UNIVERSITE DE BOURGOGNE FRANCHE-COMTE
UFR Science de la Vie, de la Terre et de l'Environnement

THESE DE DOCTORAT

Pour l'obtention du grade de

DOCTEUR DE L'UNIVERSITE DE BOURGOGNE FRANCHE-COMTE

Ecole Doctorale Environnement et Santé
Spécialité : Sciences agronomiques

Effet d'une carence en soufre combinée à un stress hydrique chez le pois protéagineux :

Etude des mécanismes moléculaires mis en jeu dans les organes puits et source au cours de la phase reproductive

Par **Charlotte HENRIET**

UMR 1347 Agroécologie, Dijon

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De quelle couleur sont les petits pois ?

Rouges parce que les petits pois sont rouges (les petits poissons rouges).

Qu'est ce qui est vert qui monte et qui descend ?

Un petit pois dans un ascenseur.

Quel est le comble pour un petit pois?

C'est d'être vert de rage de ne pas pouvoir aller en boite!

Deux petits pois discutent :

- Tu étais où hier ? On ne t'a pas vu en boîte !

Que donne un combat entre un haricot et un petit pois ?

Un Bonduelle.

Pourquoi Dijon est la ville où il y a le plus de célibataires ?

Parce que l'amour tarde (la moutarde).

Blagues Carambar, (Depuis 1965)

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Gallardo K, Besson A, Klein A, Le Signor C, Aubert G, Henriet C, Térézol M, Pateyron S, Sanchez M, Trouverie J, Avice JC, Larmure A, Salon C, Balzergue S and Burstin J (2019) Transcriptional Reprogramming of Pea Leaves at Early Reproductive Stages. Front Plant Sci. 10:1014. doi: 10.3389/fpls.2019.01014.

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C. Henriet, A. Kilandamoko, D. Aimé, J. Kreplak, T. Balliau, M. Zivy, V. Vernoud and K. Gallardo. "The interplay between sulfur nutrition and the drought response in pea: a focus on seed development and composition" International Conference on Legume Genetics and Genomics, Siófok, Hungary, 18-22 September, 2017. **Présentation orale**

C. Henriet, N. Rossin, A. Kilandamoko, M. Prudent, D. Aimé, C. Le Signor, M. Sanchez, S. Pateyron, T. Balliau, C. Rameau, J. Kreplak, G. Aubert, J. Burstin, S. Balzergues, M. Zivy, R. Thompson, K. Gallardo and V.Vernoud. "Dissection of Pea responses to water stress during seed filling and the interplay with sulfur metabolism" International Conference Advances in grain legume cultivation and use: Translating legume research into end-users reality, Novi Sad, Serbia, 26-28 September, 2017. **Présentation orale**

C. Henriet, D. Aimé, J. Kreplak, T. Balliau, M. Zivy, V. Vernoud and K. Gallardo. "How does sulfur deficiency modulate pea response to water stress? Impact on early developing and mature seeds" 11th International Plan Sulfur Workshop, Conegliano, Italy, 16-20 September, 2018. **Présentation orale**

Conférences nationales

C. Henriet, M. Zivy, V. Vernoud et K. Gallardo. « Etude du rôle de la nutrition soufrée dans la réponse du pois au stress hydrique: vers la construction de réseaux protéiques » Protéome Vert, Versailles, France, 03 Février, 2016. **Présentation orale**

C. Henriet, V. Vernoud and K. Gallardo. "The role of sulfur metabolism in the pea response to drought" 1ères Rencontres Francophones sur les Légumineuses, Dijon, France, 31 Mai–1 Juin, 2016. **Poster**

C. Henriet, A. Kilandamoko, D. Aimé, J. Kreplak, T. Balliau, M. Zivy, V. Vernoud et K. Gallardo. « Etude du rôle de la nutrition soufrée dans la réponse du pois au stress hydrique par une approche protéomique : Focus sur les graines » Protéome Vert, Versailles, France, 29 Mars, 2017. **Présentation orale**

C. Henriet, A. Kilandamoko, D. Aimé, J. Kreplak, T. Balliau, M. Zivy, V. Vernoud et K. Gallardo. "L'interaction entre la nutrition soufrée et la réponse du pois au stress hydrique" Colloque Soufre INRA, Caen, France, 12-13 Juillet, 2017. **Présentation orale**

C. Henriet, A. Kilandamoko, D. Aimé, J. Kreplak, T. Balliau, M. Zivy, V. Vernoud et K. Gallardo. « Analyse protéomique des graines de pois en embryogénèse et à maturité : Impact d'un stress hydrique combiné à une carence en soufre » Protéome Vert, Paris, France, 05 Avril, 2018. **Présentation orale**

C. Henriet, D. Aimé, T. Balliau, N. Rossin, R-F Serre, M. Térézol, J. Kreplak, M. Zivy, V. Vernoud et K. Gallardo. «Interplay between sulfur nutrition and water stress tolerance in pea : a focus on seed development and composition» Protéome Vert, Paris, France, 03-04 Juin, 2019. **Présentation orale**

Autres

C. Henriet, V. Vernoud and K. Gallardo. "The role of sulfur metabolism in the pea response to drought" Journée des doctorants de l'UMR Agroécologie, Dijon, France, 14 Mars, 2016. **Poster**

C. Henriet, V. Vernoud and K. Gallardo. "The role of sulfur metabolism in the pea response to drought" Journées du département INRA de Biologie et Amélioration des Plantes (BAP), Lyon, France, 11-12 Avril, 2016. **Poster**

C. Henriet, V. Vernoud and K. Gallardo. "The role of sulfur metabolism in the pea response to drought" Forum des Jeunes Chercheurs de l'école doctorale Environnement-Santé, Besançon, France, 16-17 Juin, 2016. **Poster**

C. Henriet, A. Kilandamoko, D. Aimé, J. Kreplak, T. Balliau, M. Zivy, V. Vernoud and K. Gallardo. "Changes in the pea seed proteome in response to drought combined with sulfur deficiency" Journée des doctorants de l'UMR Agroécologie, Dijon, France, 10 Avril, 2017. **Poster**

C. Henriet, A. Kilandamoko, D. Aimé, J. Kreplak, T. Balliau, M. Zivy, V. Vernoud and K. Gallardo. "Changes in the pea seed proteome in response to drought combined with sulfur deficiency" Forum des Jeunes Chercheurs de l'Ecole Doctorale Environnement-Santé, Dijon, France, 15-16 Juin, 2017. **Poster**

C. Henriet. « Rôle de la nutrition soufrée dans la réponse du pois au stress hydrique » Séminaire UMR 1347, Dijon, 26 Juin, 2017. **Présentation orale**

C. Henriet, D. Aimé, J. Kreplak, T. Balliau, M. Zivy, V. Vernoud and K. Gallardo. "How does sulfur deficiency modulate pea response to water stress? Impact on early developing and mature seeds" Journée des doctorants de l'UMR Agroécologie, Dijon, France, 15 Mai, 2018. **Présentation orale**

Summer school

C. Henriet, V. Vernoud and K. Gallardo. "The role of sulfur metabolism in the pea response to drought" SPS Summer School 2016: From gene expression to genomic network, Saint-Lambert, France, 17-22 Juillet, 2016. **Poster**

LISTE DES ABREVIATIONS ET SIGLES

1-DE : One-Dimensional Electrophoresis
4PMI : Plant Phenotyping Platform for Plant and Micro-organism Interactions
6PGD : 6-Phosphogluconate dehydrogenase
7S : Globulines 7S (pauvres en soufre)
7S/11S : 7S globulins to 11S globulins
11S : Globulines 11S (relativement pauvres en soufre comparé aux 7S)
ABA : Acide abscissique
ABI5 : ABA Insensitive 5 (basic leucine zipper transcription factor)
AA : Acides aminés
AAP8 : Amino acid permease 8
AER : 2-alkenal reductase
ANOVA : Analysis of variance
AOR : Alkenal/one oxidoreductase
APK : APS kinase
APR : APS réductase
APS : Adénosine 5'-phosphosulfate
As : Arsenic
ATPS1 : ATP sulfurylase 1
B : Bore
C : carbone
Ca : Calcium
CBL : Cystathionine beta-lyase
Cd : Cadnium
CDK : Cyclin-dependent kinase
CGS : Cystathionine gamma-synthase
Co : Cobalt
CO_2 : Dioxyde de carbone
Cu : Cuivre
Cys : Cystéine
CwINV : Cell wall invertase
d : days
DAP : Days After Pollination
DEG : Differentially Expressed Genes
DHPDS : Dihydrodipicolinate synthase
DNA : Deoxyribonucleic acid
DRB4 : Double-stranded RNA-binding protein 4
DTT : Dithiothreitol
dynGENIE3 : dynamical GEne Network Inference with Ensemble of trees
Fe : Fer
FSSA : Final Stage of Seed Abortion
FUS3 : FUSCA3, B3 domain factor
G1 to G4 : Group 1 of seeds to Group 4 of seeds
GmCIF1 : Glycine max cell wall invertase inhibitor
GO : Gene Ontology
GSH : Glutathion

GSSG : Glutathione disulfide
GST : Glutathione S-transferase
HNO_3 : Acide nitrique
K : Potassium
L1L: leafy cotyledon1-like
LEC1 : leafy cotyledon1
Met : Méthionine
MCM : Minichromosome maintenance
Mg : Magnesium
Mn : Manganese
Mo : Molybdène
MOS1 : MODIFIER OF snc1-1
MPa : Mégapascal
MS : Méthionine synthase
MS/MS : Tandem mass spectrometry
MSR : Methionine sulfoxide reductase
MTE2-3 : Mitochondrial pyruvate dehydrogenase
N : Nitrogen
N_2 : Diazote
NADPH : Nicotinamide adenine dinucleotide phosphate (reduced form)
nanoLC-MS/MS : Nanoflow liquid chromatography-tandem mass spectrometry
NF-Y : Nuclear Factor Y
Ni : Nickel
NO : Monoxyde d'azote
N/S : Nitrogen to Sulfur ratio
OA : Acice organique
OAS : *O*-acétylsérine
P : Phosphore
PA1 : Albumine de pois 1 (Pea Albumin 1)
PA2 : Albumine de pois 2 (Pea Albumin 2)
PAPS : Phosphoadénosine-5'-phosphosulfate
Pb : Plomb
PCR : Polymerase Chain reaction
PP2C: Protein phosphatase 2C
RIDA : Reactive Intermediate Deaminase A
RNA : Ribonucléic acid
RNA-seq : RNA sequencing
ROS : Reactive Oxygen Species
RT-qPCR : Quantitative PCR after reverse transcription
RWC : Relative Water Content
S : Soufre
S- : Carence en soufre
S-AA : acides aminés soufrés
SAM : *S*-adénosylméthionine
Se : Selenium
SDS : sodium-dodécylsulfate
SiR : sulfite réductase

SIZ1 : DNA-binding protein with MIZ/SP-RING zinc finger, PHD-finger and SAP domain
SKP1 : S-phase kinase-associated protein 1
SO_4^{2-} : Sulfate
SO_2 : Dioxide de soufre
sRNA : Small RNA
SUS2 : Sucrose synthase 2
S-WS : Double stress (carence en soufre x stress hydrique)
SMM : *S*-méthylméthionine
SNK : Student–Newman–Kheul test
SULTR : Transporteur de sulfate
SUMO : Sumoylation
TE : Eléments transposables
TEMED : Tétraméthyléthylènediamine
TILLING : Targeting Induced Local Lesions in Genomes
TRX : Thioredoxin
UPL1 : Ubiquitin-protein ligase
V : Vanadium
Var. : Variété
WS : Water stress
XIC : Extracted ion chromatograms
Zn : Zinc

SOMMAIRE

Introduction générale

Est-il possible d'assurer à toute l'humanité une alimentation suffisante, de qualité, saine et durable ? C'est cette question qui était au centre de l'exposition universelle de 2015 à Milan dont le thème était « Nourrir la planète, énergie pour la vie ». En passant d'une population mondiale de 2 milliards d'habitants en 1930, à 7 milliards en 2011, le 20ème siècle a vu la demande alimentaire grandement augmenter en quelques décennies. Pour répondre à cette demande croissante, un système de production agricole appelé agriculture intensive fut mis en place, permis par la mécanisation de celle-ci (utilisation du charbon, puis du pétrole), la création de variétés à haut rendement, et une utilisation massive de fertilisants, d'eau et de pesticides. Bien qu'ayant permis une suffisance alimentaire, ce nouveau mode d'agriculture a commencé à montrer ses limites, notamment pour la non prise en compte des conséquences environnementales et l'effet néfaste des pesticides sur la santé. Ainsi, la perte de fertilité du sol (due au large développement des cultures) compensée par l'utilisation d'intrants, la perte de la biodiversité due aux pesticides, et une augmentation de la pollution de l'eau avec une diminution de sa disponibilité, ont amené à une remise en question de ce système et à la volonté de développer une agriculture plus durable et respectueuse de l'environnement.

Parallèlement, le marché mondial des protéines végétales menace de s'effondrer, dû entre autres, à la forte demande de la Chine qui concentre d'ores et déjà 60 % des importations mondiales de soja, tirant les prix à la hausse (Les Echos, https://www.lesechos.fr/2017/06/la-chine-na-jamais-importe-autant-de-soja-157068). En effet, la convergence vers un mode de vie occidentalisé avec un régime alimentaire à base de protéines animales (viande bovine, lait), entraîne par ricochet en Asie des besoins importants de protéines végétales, importées d'Amérique (USA, Brésil, Argentine principalement), pour l'alimentation animale. A terme, les flux d'exportation en provenance d'Amérique qui bénéficient actuellement à l'Europe pourraient être détournés au profit de l'Asie. En Europe, la dépendance en protéines végétales importées est structurelle et repose sur des accords commerciaux négociés dans les années 1960. Pour 2010/2011, le niveau d'autosuffisance en France, toutes sources de protéines incluses, est estimée à 60% (Plan Protéines Végétales pour la France 2014-2020, http://agriculture.gouv.fr), ce qui souligne le poids des importations destinées à l'alimentation animale (principalement graines et tourteaux de soja).

C'est dans ces circonstances que l'agroécologie a été mise en avant afin de construire un nouveau système de production agricole permettant une agriculture durable dans le respect de l'environnement tout en répondant à la forte demande quantitative et qualitative en protéines végétales pour l'alimentation animale et humaine. Une des principales pratiques agroécologiques consiste en la réintroduction des légumineuses dans les systèmes de culture. En effet, les légumineuses produisent des graines riches en protéines, même en l'absence de fertilisation azotée grâce à leur capacité à réaliser des symbioses avec des bactéries du sol (du genre Rhizobium) fixatrices d'azote atmosphérique. Sa culture enrichit donc les sols en azote, réduisant la nécessité d'une fertilisation azotée pour les cultures suivantes. Les légumineuses sont donc des plantes d'intérêt nutritionnel pour l'alimentation animale et humaine, et d'intérêt écologique et économique par l'enrichissement des sols en azote et la diminution des besoins en engrais azotés. Preuve de leur importance au sein d'une agriculture plus durable, le développement de la production des protéines végétales est le premier axe du plan protéines végétales 2014-2020 mis en place par le gouvernement français (https://agriculture.gouv.fr/le-plan-proteines-vegetales-pour-la-france-2014-2020). De même, l'année 2016 a été déclarée année internationale des légumineuses par l'Assemblée générale des Nations Unies afin de faire prendre conscience à la population de la valeur nutritive des légumineuses et de leur contribution à une alimentation plus durable ainsi qu'à la sécurité alimentaire.

Le pois protéagineux (*Pisum sativum* L.) est la légumineuse la plus cultivée en France qui est le premier producteur européen avec 707 000 tonnes récoltées en 2017 (Source : UNIP/Arvalis/Terres Inovia/SSP, http://www.terresunivia.fr/). Le pois protéagineux produit des graines riches en protéines (environ 23%). Elles ont une forte teneur en lysine, acide aminé essentiel, mais sont pauvres en acides aminés soufrés, dont la méthionine qui est aussi un acide aminé essentiel. Les animaux et les hommes sont incapables de synthétiser les acides aminés essentiels et doivent donc obligatoirement les trouver dans leur alimentation. Les graines de pois complémentent ainsi parfaitement les apports protéiques issus des céréales qui sont à l'inverse pauvres en lysine et riches en acides aminés soufrés. Nécessitant moins d'eau que le soja, la culture du pois s'intègre parfaitement dans une agriculture agroécologique.

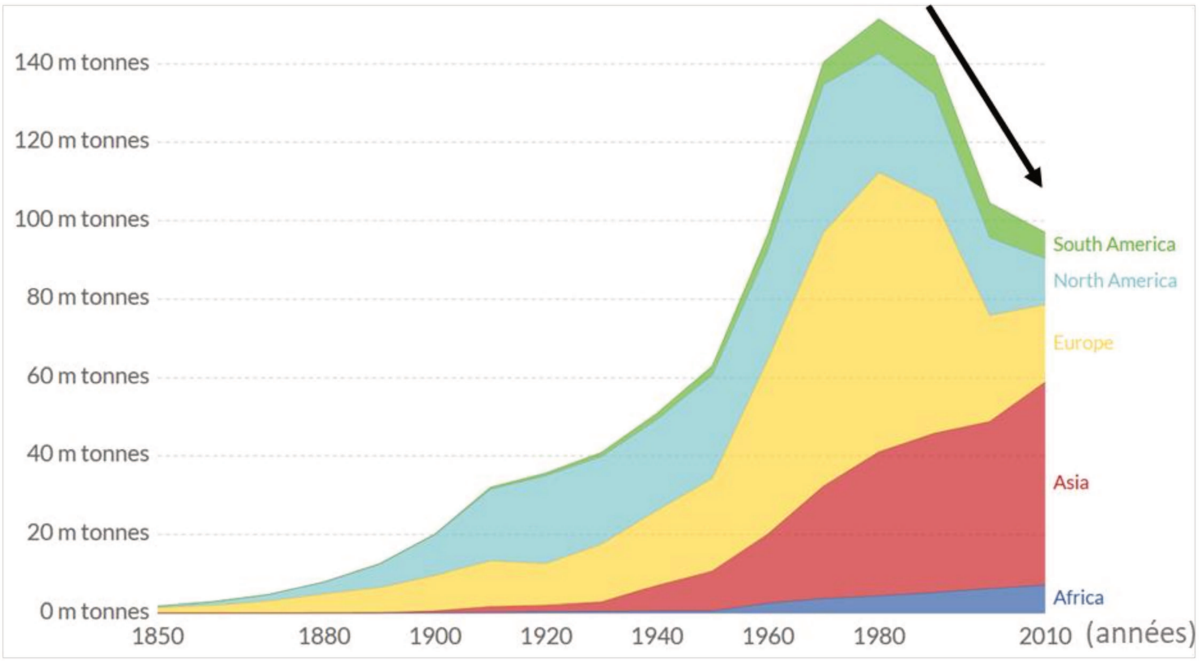

Figure 1. Evolution des émissions de dioxyde de soufre dans le monde. Les émissions de dioxide de soufre, présentées en million de tonnes, sont extraites de https://ourworldindata.org/air-pollution. De 1850 à 2000 sont les données "Clio Infra national emissions", puis sont présentées les données de Klimont et al. (2013).

Malgré ces avantages nutritionnels, économiques et écologiques, la culture du pois représente moins de 3% des surfaces agricoles en Europe (Voisin *et al.*, 2013) en raison des rendements instables entre années, d'une teneur en protéines des graines jugée trop faible comparé au soja (23% de la matière sèche chez le pois contre 40% chez le soja (Burstin *et al.*, 2011)), et d'autres facteurs de la qualité des graines (ex. digestibilité, goût). Les variations de rendements entre années sont principalement dues à des stress biotiques (champignons, insectes) et abiotiques (stress hydrique, carences nutritionnelles). Actuellement, une des contraintes environnementales majeures affectant la survie de la plante et la productivité des cultures est le stress hydrique. En effet, dans le contexte de changement climatique actuel, les risques de sécheresse sont amenés à augmenter, rendant primordial une meilleure compréhension de l'impact de ce stress sur la plante et les réponses mises en place par celle-ci dans le but d'améliorer sa tolérance. De nombreuses études sur la réponse des plantes au stress hydrique ont fait émerger un rôle de la nutrition soufrée dans la tolérance des plantes à ce stress (Rajab *et al.*, 2019; Chan *et al.*, 2013; Cao *et al.*, 2014; Gallardo *et al.*, 2014). Cependant, un appauvrissement des sols en soufre a été observé, dû à la diminution des émissions de dioxyde de soufre qui ne cessent de baisser depuis les années 80 suite à la mise en place des politiques de réduction des rejets industriels (Klimont *et al.*, 2013) (**Figure 1**). Aujourd'hui, le stress hydrique et la carence en soufre sont deux stress abiotiques qui peuvent co-exister et donc interagir. Il devient crucial de s'intéresser à l'impact d'une telle interaction sur le pois au cours de son développement, et aux conséquences sur le rendement et la qualité des graines produites.

Ainsi, les objectifs de cette thèse étaient de décrire l'impact d'une interaction entre ces deux stress abiotiques que sont le stress hydrique et la carence en soufre au cours de la phase reproductive sur le rendement et la qualité des graines de pois protéagineux, puis de caractériser au moyen d'approches omiques les mécanismes moléculaires sous-jacents en se focalisant sur les graines en cours de développement et les feuilles, sources de nutriments pour les graines.

Ce manuscrit de thèse est structuré en cinq chapitres. Le **Chapitre I** présente une synthèse bibliographique regroupant les connaissances générales utiles à la compréhension des travaux de thèse. La stratégie d'étude ainsi que les objectifs du projet y sont détaillés à la fin. Les trois chapitres suivants, **Chapitre II**, **III** et **IV**, correspondent aux résultats produits et analysés au

cours de ces travaux de thèse. Ils sont présentés sous forme d'article scientifique en anglais. Les approches utilisées ainsi que les principaux résultats sont résumés en français au début de chaque chapitre. Les Matériels et Méthodes sont détaillés dans les chapitres de résultats. Enfin, le **Chapitre V** contient une conclusion générale ainsi que des éléments de perspectives pour les travaux réalisés.

Chapitre I : Synthèse bibliographique

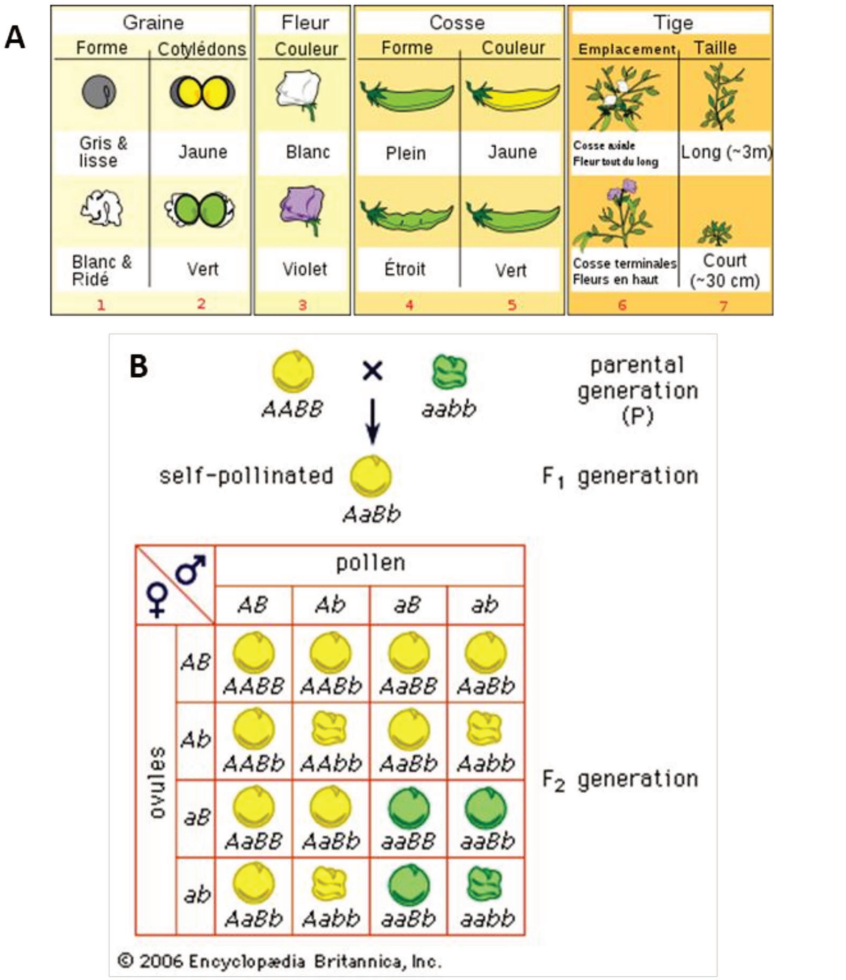

Figure 2. Illustration des travaux de Mendel (A) Tableau représentant les sept caractères phénotypiques étudiés par Mendel. **(B)** Deuxième loi de Mendel : Loi de disjonction des allèles. Croisements de pois ayant des graines rondes jaunes avec des pois ayant des graines vertes ridées. 'A' représente l'allère jaune et 'a' l'allèle vert; 'B' représente l'allèle pour une graine ronde et 'b' l'allèle pour une graine ridée. D'après Encyclopædia Britannica, Inc.

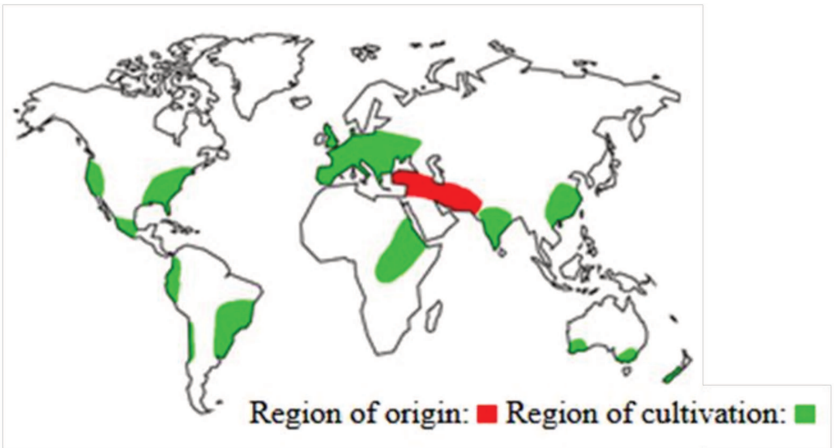

Figure 3. Origine du pois *Pisum sativum* L. Carte illustrant la region d'origine la plus probable du pois (rouge), et les regions où sa culture s'est propagée (vert).
(source : http://www1.biologie.uni-hamburg.de/b-online/schaugarten/PisumsativumL/PisumsativumL.gif)

1. Le pois : Généralités, enjeux économiques et contexte agroécologique

1.1 Le pois en science

Au milieu du XIXe siècle, les observations faites par le moine autrichien Gregor Mendel sur le pois ont permis de mettre en pratique les principes de la génétique mendélienne, fondement de la génétique moderne. Mendel a choisi les pois pour ses expériences car il pouvait les cultiver facilement, et développer des souches stables en les protégeant de la pollinisation croisée et en contrôlant leur pollinisation (Olby, 2019). Mendel utilisait plusieurs caractères pour croiser les pois tels que la couleur de la graine (verte ou jaune), la couleur des fleurs (pourpres ou blanches), la texture de leur graines (ridées ou lisses) (**Figure 2A**). Il a ensuite observé la progéniture résultante. Dans chaque cas, un trait est dominant et tous les descendants, ou génération Filiale-1 (abrégé F1), ont montré le trait dominant. Il a ensuite croisé des membres de la génération F1 ensemble et observé leur progéniture, la génération Filiale-2 (abrégée F2) (**Figure 2B**). Les plantes F2 avaient le trait dominant dans un rapport d'environ 3:1. Mendel a ainsi expliqué que chaque parent avait un «vote» lors de l'apparition de la progéniture et que le trait non dominant ou récessif n'apparaissait que lorsqu'il était hérité des deux parents. D'autres expériences ont montré que chaque trait est hérité séparément. Sans le vouloir, Mendel avait résolu un problème majeur avec la théorie de l'évolution de Charles Darwin: comment de nouveaux traits étaient préservés et non fondus dans la population, une question à laquelle Darwin lui-même n'avait pas répondu. Ses travaux, publiés en 1866 (Mendel, 1866), furent largement incompris jusqu'à ce qu'ils soient partiellement reproduits et cités autour de 1900. Le travail de Mendel fonde ainsi la génétique, une discipline qui a permis par la suite d'identifier les régions du génome du pois contrôlant divers caractères d'importance agronomique, comme le développement racinaire ou la composition protéique des graines (Bourgeois *et al.*, 2011; Bourion *et al.*, 2010). Le séquençage récent du génome du pois (Kreplak *et al.*, 2019) est un vrai tremplin pour mieux comprendre les bases moléculaire de ces caractères *via* l'identification des gènes impliqués. Le génotype choisi comme référence pour le séquençage du génome du pois est Caméor, un cultivar sélectionné en 1973 par Séminor et caractérisé par des graines riches en protéines

(24-28%). Il s'agit du cultivar utilisé pour réaliser ces travaux de thèse. D'une taille de 4,3 Gb, il est composé de au moins 44 756 gènes (Kreplak *et al.*, 2019).

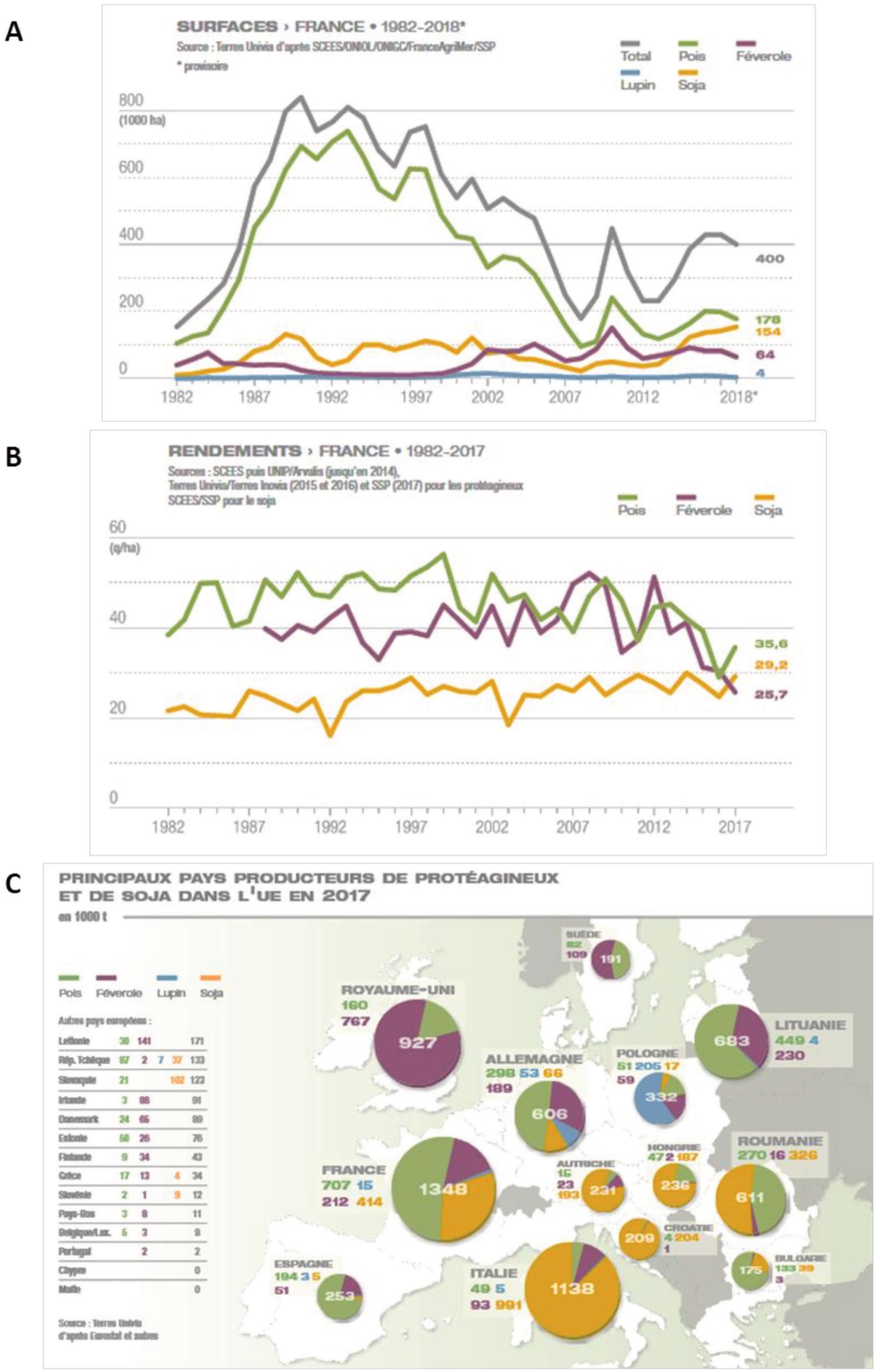

Figure 4. Production de protéagineux et de soja en France et en Europe. (A) Evolution des surfaces cultivées en France de 1987 à 2017. **(B)** Rendement des cultures en France de 1987 à 2017. **(C)** Carte représentant les principaux pays producteurs de protéagineux et de soja dans l'Union Européenne en 2017. (D'après Terre Univia)

1.2 Production et utilisation du pois protéagineux

Le pois a été domestiqué il y a environ 10 000 ans au Proche-Orient (**Figure 3**), d'où il s'est étendu à l'ouest à travers le bassin méditerranéen, et à l'est jusqu'en Chine et en Afrique de l'Est (Ambrose, 1995; Smýkal *et al.*, 2011). Les années 80 marquent le début d'un intérêt croissant pour la culture du pois (*Pisum sativum* L.) en France en passant d'une surface cultivée de 104 000 ha en 1982 à 739 926 ha en 1993 (FAOSTAT) (**Figure 4A**). Cette multiplication par sept des surfaces cultivées en 10 ans est due à la mise en place d'aide de la PAC (Politique Agricole Commune) sous la forme de prix garantis. Cependant, à la suite de nombreuses réformes à partir de 1993, d'une forte instabilité des rendements (**Figure 4B**), et d'une concurrence du soja et du pois importés, les superficies cultivées en pois sur le territoire français ont été réduites de manière significative, jusqu'à atteindre les 100 245 ha en 2018 (FAOSTAT) (**Figure 4A**). Malgré un rebond en 2010 (249 214 ha) (FAOSTAT), la surface cultivée reste stable depuis avec 180 999 ha cultivé en 2017 (**Figure 4A**). Aujourd'hui, le pois protéagineux reste la légumineuse la plus cultivée en Europe (2 707 000 tonnes en 2017), devant le soja (2 591 000 t) et la féverole (2 135 000 t) (Source: Terres Univia). La France est le premier producteur européen avec 707 000 tonnes récoltées en 2017 (**Figure 4C**), derrière la Lituanie (449 000 t) et l'Allemagne (298 000 t) (Source: Terres Univia).

Le pois en culture pure est considéré comme un excellent précédent, laissant dans le sol un reliquat d'azote de 20 à 40kg/ha (Cavailles, 2009). Il est le plus souvent suivi d'une culture exigeante en azote telle que le blé, induisant une diminution des besoins en engrais azotés de la plante. Il peut aussi être utilisé en culture d'association avec une céréale, induisant de nombreux avantages agronomiques tels que : des rendements plus réguliers, une meilleure valorisation des ressources azotées, une meilleure compétitivité face aux adventices, un effet limitant sur la verse, et une résistance accrue aux maladies (Corre-Hellou *et al.*, 2013).

Le pois protéagineux est utilisé pour la teneur élevée en protéines de ses graines, et cultivé principalement pour l'alimentation animale (Burstin *et al.*, 2011). Produite localement, cette culture participe à l'autonomie protéique des élevages. Grâce à une teneur élevée en lysine (acide aminé essentiel limitant pour la croissance), le pois associé au tourteau de colza ou aux céréales (plus pauvres en lysine mais plus riches en méthionine et cystéine) peut se substituer au tourteau de soja, souvent importé, améliorant ainsi le bilan environnemental des filières animales. Le second débouché du pois protéagineux est l'alimentation humaine, par la

consommation directe des graines décortiquées appelées « pois cassées », ou leur utilisation en industrie agroalimentaire. En effet, dans un contexte où il est nécessaire de rééquilibrer les régimes alimentaires, occidentaux notamment, vers la consommation de plus de protéines végétales par rapport aux protéines animales, les débouchés en alimentation humaine sont à la hausse, créant de nouveaux besoins.

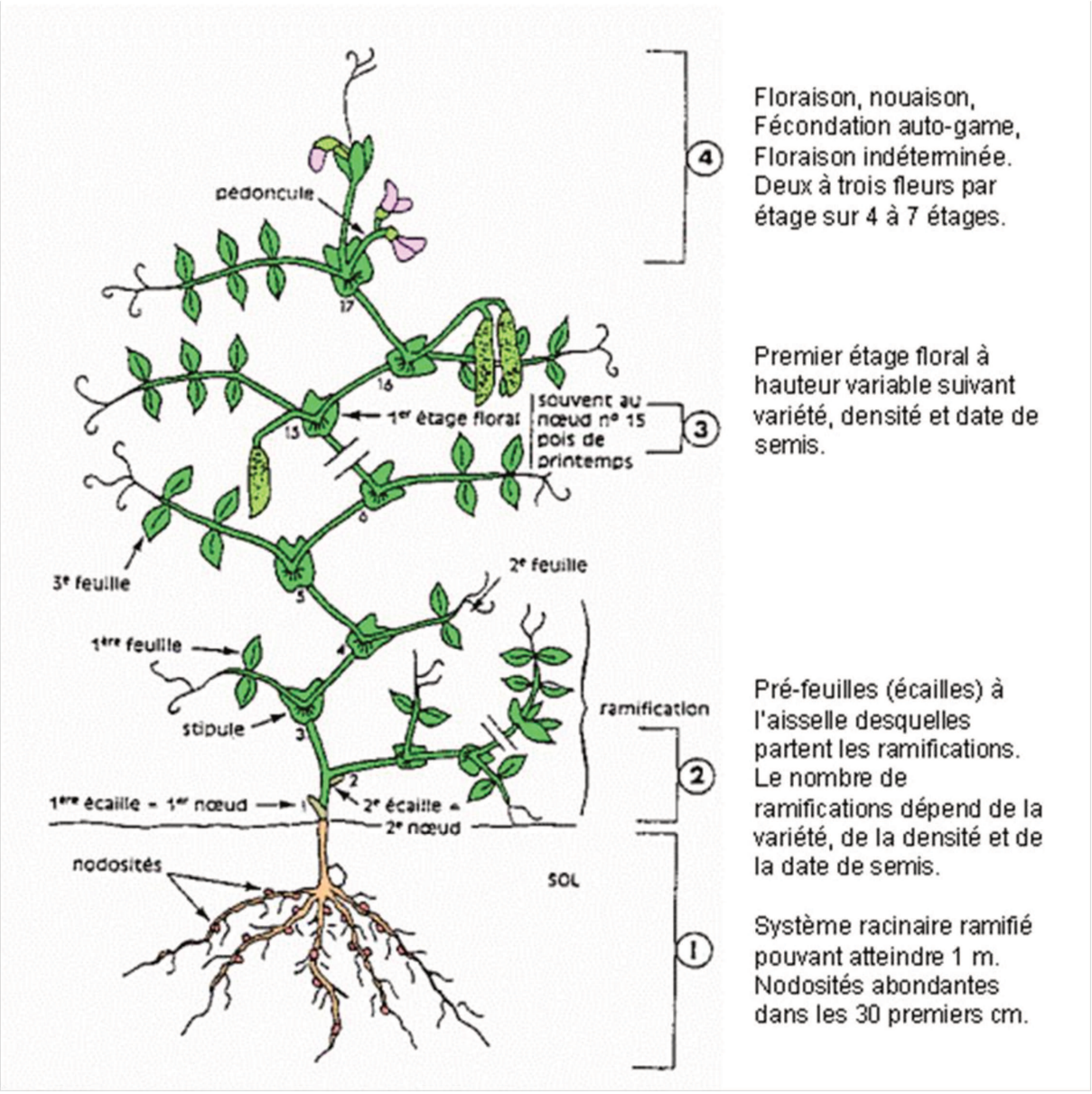

Figure 5. Architecture d'une plante de pois (D'après Boyeldieu, 1991)

1.3 Morphologie du pois

Le pois protéagineux est une plante grimpante herbacée annuelle, appartenant à la famille des Fabacées (**Figure 5**). Le système racinaire est constitué d'un pivot relativement peu développé et très ramifié dans la couche superficielle du sol (Tricot *et al.*, 1997). Les racines accueilleront les nodosités, site de fixation de l'azote par une bactérie de type Rhizobium entrée en symbiose avec la plante.

L'appareil aérien est constitué d'une tige principale et de ramifications (**Figure 5**). Cette tige est constituée de nœuds émettant des feuilles ou des ramifications à partir du troisième nœud, les deux premières feuilles primordiales étant réduites à des écailles. Les feuilles sont composées d'une à quatre paires de folioles sessiles, opposées et terminées par une vrille simple ou ramifiée, et possèdent à leur base deux grandes stipules. Chez certaines variétés, les folioles sont partiellement ou totalement transformées en vrilles. C'est le cas des variétés « afila » où toutes les folioles sont remplacées par des vrilles. Inversement chez les variétés « acacia », les vrilles sont transformées en folioles. Les premiers nœuds sont végétatifs (**Figure 5**), l'apparition du premier nœud reproducteur variant entre les variétés, de même que le nombre de fleurs par nœud. La fécondation des fleurs est principalement autogame, permettant la sélection de lignées pures et le maintien de variétés stables, mais compliquant l'obtention de nouveaux hybrides. Les gousses, fruits issus de la fécondation, portent un nombre variable de graines.

1.4 Cycle de développement du pois protéagineux

Le pois est une plante annuelle sans dormance, qui peut être semée sans nécessité de vernalisation. Il existe deux catégories de variétés de pois protéagineux : le pois de printemps et le pois d'hiver. Le pois de printemps se sème en février-mars, se récolte en juillet, en même temps que les blés. Le cycle végétatif des pois de printemps est d'environ 140 jours et peut descendre à 90 jours pour les variétés ultra-précoces. Le pois d'hiver se sème début novembre et se récolte fin juin, avant les blés. Il est surtout présent dans les régions Centre, Ile-de-France, Picardie, Barrois et Bourgogne. Les variétés d'hiver, dont le cycle végétatif est de 240 jours, permettent de gagner en précocité de récolte et en rendement.

Deux grandes phases se distinguent dans le cycle du pois : la phase végétative et la phase reproductive (**Figure 6**). Chez le génotype Caméor, utilisé pour la réalisation de cette thèse, la

phase végétative dure environ 6 semaines. Cette phase comprend la germination et la levée de la graine, suivie de la mise en place des racines, de la tige et des feuilles issues des nœuds végétatifs (**Figure 6**). Chez ce même génotype, la phase reproductive commence dès la mise en place des nœuds reproducteurs et l'apparition des premières fleurs, 6 semaines après le semis à un stade 13-15 nœuds. Les fleurs apparaissant au fur et à mesure de l'apparition des nouveaux nœuds reproducteurs (1 nouveau nœud tous les deux jours environ) et dans nos conditions de culture, Caméor produit environ 7 reproducteurs. On trouve des gousses à des stades de développement différents du bas vers le haut de la plante : lorsque les gousses sont en remplissage sur les nœuds inférieurs, des fleurs continuent d'apparaître sur les derniers nœuds formés. A maturité, les gousses deviennent déhiscentes, libérant les graines au moindre choc.

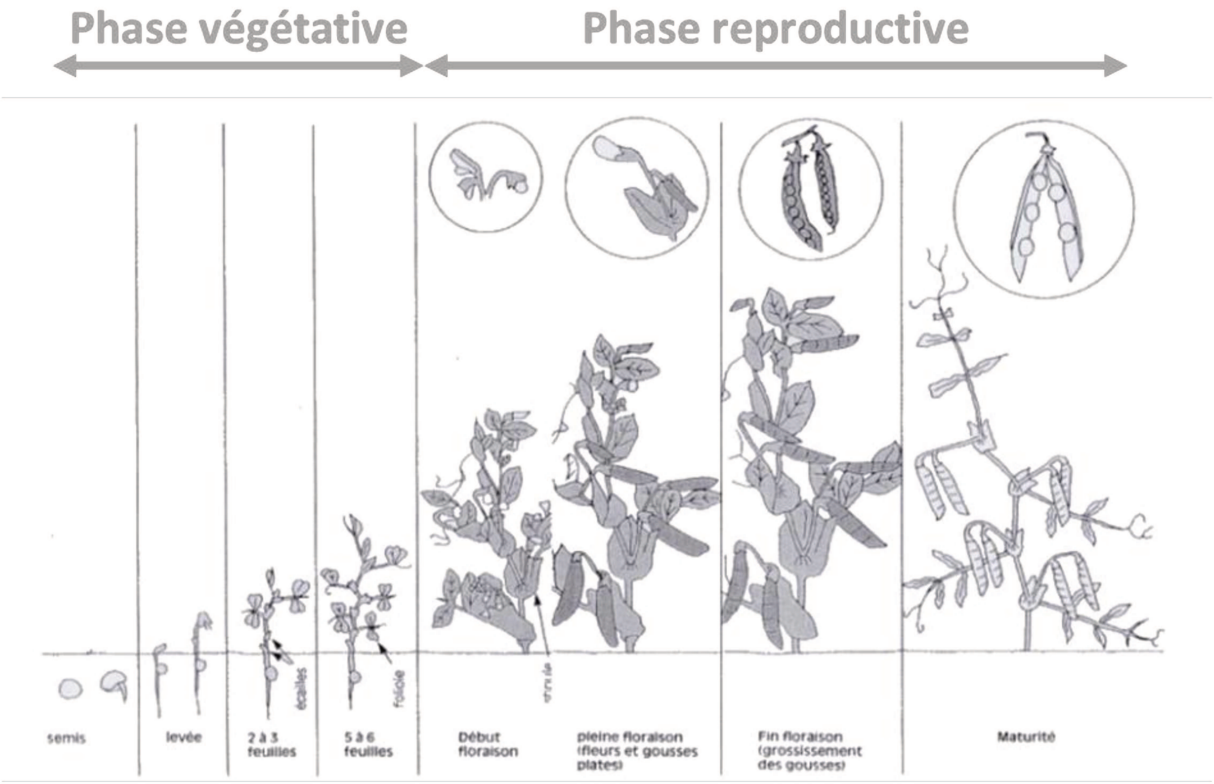

Figure 6. Schéma des principaux stades du développement du pois. (D'après Boyeldieu, 1991)

2. La graine de pois: développement et composition

2.1 Phases du développement de la graine de pois

Le développement de la graine de pois est un processus complexe divisé en trois phases successives : l'embryogénèse, le remplissage correspondant à l'accumulation des réserves (protéines et amidon) dans les cotylédons, et la maturation, phase pendant laquelle se fait l'acquisition de la tolérance à dessiccation (**Figure 7**). L'embryogénèse est caractérisée par d'intenses divisions cellulaires au sein de l'embryon et par un processus d'histo-différenciation permettant la mise en place des différents tissus de l'embryon. Le développement de l'embryon a largement été documenté chez *Arabidopsis thaliana* (Baud *et al.*, 2002; Gutierrez *et al.*, 2007) ainsi que chez *Medicago truncatula* (Wang *et al.*, 2012; Wang and Grusak, 2005) mais la description de cette phase chez le pois reste limitée (Marinos, 1970). Lors de ces stades précoces de développement, les tissus qui entourent l'embryon, l'albumen et le tégument, ont un rôle prépondérant dans l'assimilation de nutriments. Une accumulation temporaire de réserves a lieu dans ces tissus ainsi qu'un métabolisme intermédiaire intense servant à convertir et fournir des nutriments pour la croissance de l'embryon. L'embryogenèse est ainsi associée à une forte concentration en hexoses dans la graine (**Figure 8**), grâce notamment à l'activité d'invertases localisées dans le tégument qui permettent l'hydrolyse du saccharose arrivant à la graine par le phloème (Weber *et al.*, 1995). Il a été également suggéré que cette forte concentration en hexoses pourrait avoir un effet direct sur la prolifération cellulaire (Borisjuk *et al.*, 1998).

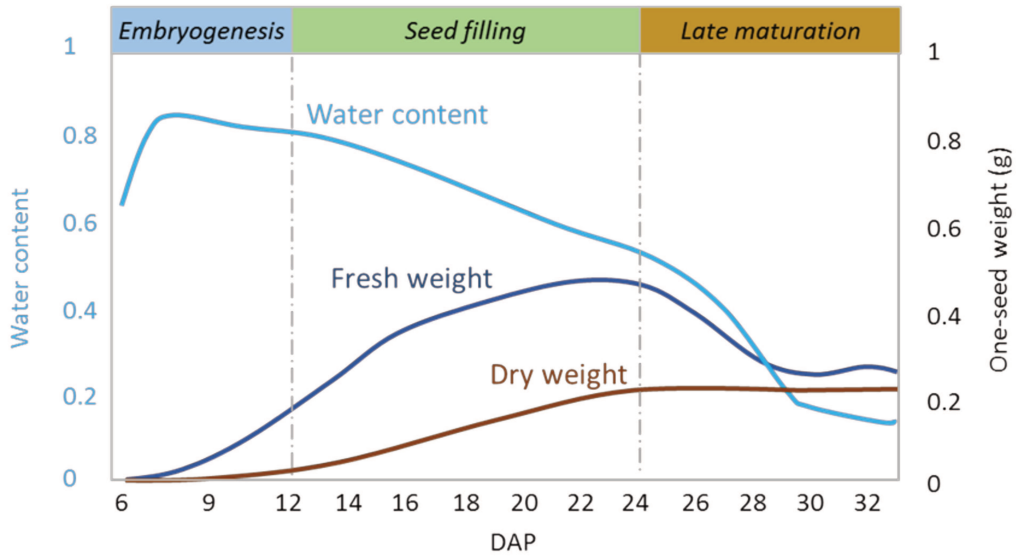

Figure 7. Schéma des principaux stades du développement du pois. DAP, days after pollination (D'après Judith Burstin, UMR Agroécologie, Dijon)

À la fin de l'embryogenèse, le nombre de cellules dans les cotylédons est établi (Smith, 1973) et l'embryon passe en mode remplissage. Chez les légumineuses, le nombre de cellules formées dans les cotylédons détermine majoritairement la capacité de l'organe de stockage à accumuler de la matière sèche lors de la phase de remplissage (Munier-Jolain and Ney, 1998). Le stade de transition entre l'embryogenèse et le remplissage est caractérisé par une diminution des divisions cellulaires et une croissance basée sur l'expansion cellulaire (Lemontey *et al.*, 2000). L'importance de la composition en sucres a été soulignée lors de cette phase de transition, notamment chez la féverole, avec une augmentation de la concentration en saccharose, nécessaire pour l'initiation du stockage des réserves (**Figure 8**) (Borisjuk *et al.*, 2004; Weber *et al.*, 1995). Ceci est corrélé à la différenciation des cellules de l'épiderme des cotylédons en cellules de transfert, et à l'expression de transporteurs de sucres (Tegeder *et al.*, 1999) et d'acides aminés (Tegeder *et al.*, 2000). Il a été démontré que l'absence de différenciation de ces cellules bloque le développement de l'embryon (Borisjuk *et al.*, 2002). En plus d'un contrôle métabolique de la transition embryogenèse-remplissage, un contrôle par les hormones, en particulier l'ABA, a également été établi (Radchuk *et al.*, 2010). L'accumulation des protéines de réserve est initiée très tôt lors de l'expansion cellulaire des cotylédons, et progresse séquentiellement durant toute la période de remplissage (**cf paragraphe 2.4**). Enfin, durant la période de maturation, la graine va subir une période de dessiccation intense associée à une réduction de ses activités métaboliques, conduisant à un état de quiescence métabolique (**Figure 8**).

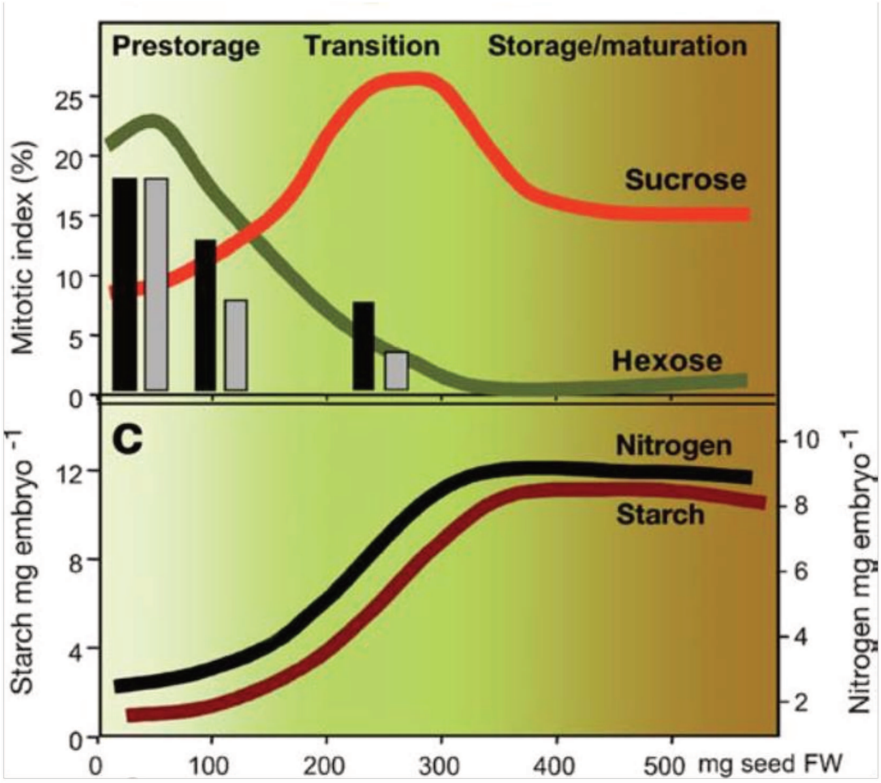

Figure 8. Caractéristiques physiologiques et biochimiques du développement de la graine de légumineuse (exemple de *Vicia Faba*). La phase d'embryogenèse ("pre-storage") est caractérisée par un indice mitotique élevé des cellules des cotylédons (en noir, region adaxiale des cotylédons, en gris, region abaxiale) ainsi que par une forte concentration en hexoses. Le passage vers la phase de maturation ("transition") est caractérisé par un changement métabolique lié à une augmentation de la concentration en saccharose, permettant l'initiation de l'accumulation des réserves carbonnées (amidon) et azotées (protéines de réserve). L'accumulation de l'azote et de l'amidon est maximale pendant la phase de remplissage. Figure issue de Weber et al., (2005).

2.2 Composition d'une graine mature de pois

A maturité, les graines de pois protéagineux sont principalement constituées d'amidon (50%), stocké sous forme de grains d'amidons, et de protéines (23% en moyenne, jusqu'à 28% pour certains cultivars), stockées dans des organites spécialisés : les corpuscules protéiques (**Figure 9**). La teneur et la composition en protéines des graines de pois varient en fonction du génotype et des conditions de culture (Perrot, 1995). Deux grandes familles de protéines de réserve se distinguent : les globulines (55 à 65%) et les albumines (20 à 30%) (**Figure 10**). Les globulines sont les protéines de réserve majeures dans la graine de pois et ont pour fonction de stocker l'azote mobilisable au moment de la germination (Guéguen *et al.*, 2016). Elles sont insolubles dans l'eau et ont été classées en deux groupes selon leur coefficient de sédimentation : les globulines 7S (vicilines et convicilines) et les globulines 11S (légumines) (**Figure 10**). Les légumines (11S) sont des hexamères de masse moléculaire entre 350 et 400 kDa. Chaque sous-unité est constituée d'un polypeptide acide α d'environ 40 kDa et d'un polypeptide basique β d'environ 20 kDa liés par un pont disulfure (Perrot, 1995). Les vicilines (7S) sont des glycoprotéines trimériques d'environ 50 kDa et ne présentent pas de pont disulfure. Les globulines présentent une forte teneur en acides aminés amides (glutamine et asparagine) et en arginine, et une déficience en acides aminés soufrés (cystéine et méthionine) et en tryptophane. Cependant, les globulines 11S sont relativement plus riches en acides aminés soufrés (1.4 à 2.3% dans leurs séquences) que les globulines 7S (0.5 à 0.9%). Les albumines sont solubles dans l'eau et présentes en quantité plus faible que les globulines. Chez le pois, les albumines majeures sont nommées PA1 et PA2 et sont plus riches en lysine et acides aminés soufrés que les globulines. L'albumine PA1 est un dimère de 11kDa composé de deux polypeptides de 6 kDa (Gatehouse *et al.*, 1985; Higgins *et al.*, 1986). L'albumine PA2 est un dimère de sous-unités de 25 kDa, très riche en feuillets β et possédant trois cystéines (Gruen *et al.*, 1987).

Les graines de pois contiennent aussi des facteurs dits « antinutritionnels », comme les inhibiteurs trypsiques et les lectines (**Figure 10**). Ils permettent une protection contre des attaques biotiques (champignons, bactéries) mais diminuent la digestibilité des graines de pois. Bien que des travaux aient permis de réduire la présence de ces composés antinutritionnels dans les graines (Page *et al.*, 2003), certains caractères restent à améliorer et à stabiliser comme l'équilibre en acides aminés, déterminé par la composition en protéines

de réserve. Les globulines étant les plus abondantes, l'étude des processus concourant à leur accumulation, notamment sous contrainte environnementale, revêt un intérêt majeur en vue d'augmenter et de stabiliser la teneur en protéines des graines de pois.

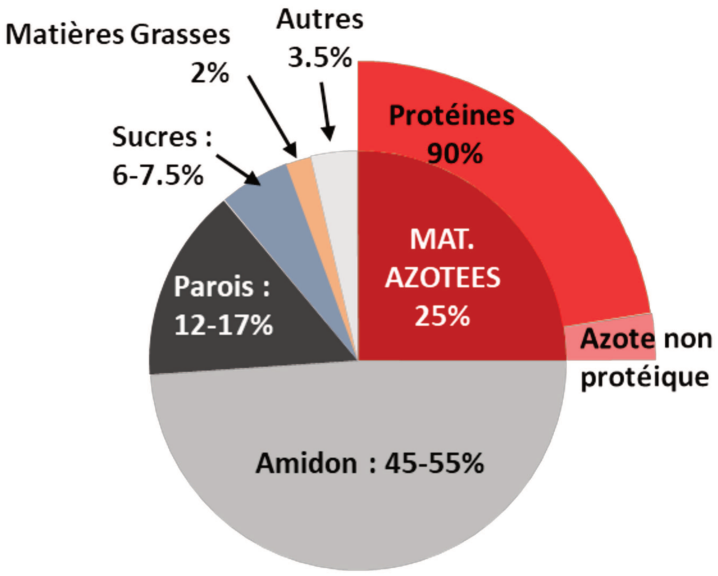

Figure 9. Composition moyenne d'une graine de pois mature, en pourcentage de la masse sèche. (d'après Gérard Duc, UMR Agroécologie, Dijon).

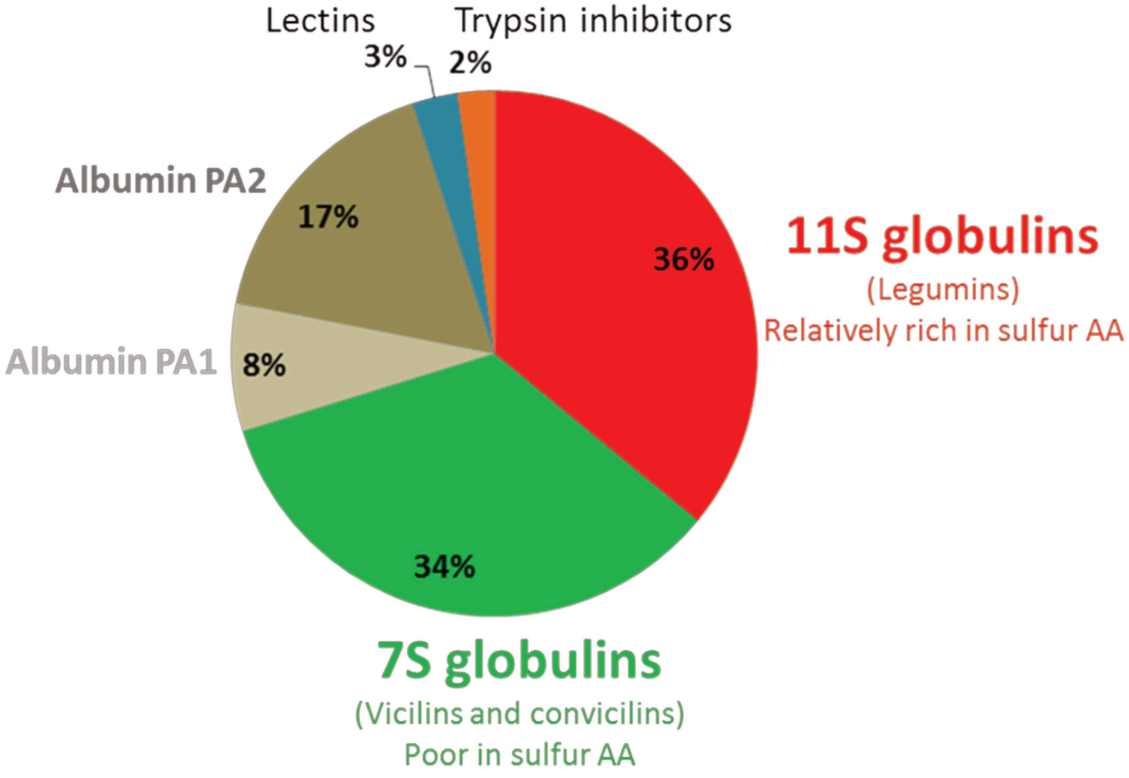

Figure 10. Composition protéique moyenne des graines de pois à maturité, en pourcentage par rapport aux protéines totales. (Adapté de Perrot et al., 1995)

2.4 La régulation de l'accumulation des protéines de réserve

L'accumulation des protéines de réserve dépend des concentrations en nutriments essentiels tels que le soufre, le carbone et l'azote. Le soufre et l'azote sont deux éléments indispensables à la biosynthèse des protéines de réserve. L'azote augmente la vitesse d'accumulation des protéines durant la phase de remplissage des graines (Shewry and Halford, 2002), et le soufre régule la synthèse des protéines riches en soufre. En effet, un appauvrissement en soufre entraine des profonds changements métaboliques tels qu'une diminution de la concentration en globulines 11S (Chandler *et al.*, 1983) et une accumulation de composés organiques et minéraux riches en azote. Par conséquent, le rapport N:S dans les tissus de la plante peut refléter la capacité de la plante à synthétiser des protéines plus ou moins riches en soufre (Brunold and Suter, 1984). L'accumulation des protéines de réserve est soumise à une régulation transcriptionelle par la disponibilité en soufre et en azote (Chandler *et al.*, 1984; Gatehouse *et al.*, 1985; Evans *et al.*, 1984). En effet il a été montré que la vitesse d'accumulation des globulines 11S et 7S était fortement corrélée au niveau d'expression des gènes codant ces globulines chez le pois (Spencer *et al.*, 1990; Higgins *et al.*, 1988; Evans *et al.*, 1985; Beach *et al.*, 1985). Ainsi, les quantités de protéines dépendent directement des niveaux de leurs transcrits. Les gènes codant les globulines 11S (légumines) et 7S (vicilines et convicilines) sont nombreux et identifiés en clusters sur les chromosomes du pois (Casey *et al.*, 2001; Le Signor *et al.*, 2017; Kreplak *et al.*, 2019). Par ailleurs, la variation d'abondance des globulines 11S et 7S a récemment été associée à des polymorphismes de séquences au niveau de gènes qui codent des facteurs de transcription spécifiques des graines ou des molécules signales responsables de l'adressage de ces dernières aux différents compartiments (Le Signor *et al.*, 2017). De même les modifications post-traductionnelles des protéines durant leur transport génèrent des modifications dans l'abondance des protéines de réserve (Chandler *et al.*, 1984).

3. Rôles des feuilles au cours de la période reproductive

Le métabolisme foliaire change profondément au cours du développement. Les jeunes feuilles se comportent comme des organes puits, importateurs d'assimilats lorsqu'elles se développent, puis deviennent des organes sources lorsqu'elles atteignent leur maturité et qu'elles produisent plus de photoassimilats que nécessaire à leur propre métabolisme (Turgeon, 1989). Cette conversion du statut de puits à celui de source marque une transition fondamentale dans la physiologie de la feuille et de nombreuses études ont été consacrées à la compréhension de ses caractéristiques biochimiques et structurelles (Meng *et al.*, 2001; Jeong *et al.*, 2004; Turgeon, 1989).

Le développement des graines et la composition en protéines dépendent des ressources mobilisées par la plante à partir des nutriments absorbés au niveau des racines et remobilisés à partir des feuilles. Les processus de remobilisation consistent au transport *via* le phloème des réserves nutritives solubles et assimilables qui dépendent de la relation source-puits. Dans une récente étude à laquelle j'ai contribué par mes compétences en traitement de données omiques, les mécanismes moléculaires associés aux statuts puits ou source des feuilles de pois ont été explorés (Gallardo *et al.*, 2019). Parmi les résultats obtenus, il est à souligner d'importants changements dans le transcriptome des feuilles des nœuds reproducteurs au début du développement des graines (de la floraison à 14 jours après floraison). Parmi les gènes up-régulés dans ces feuilles sont des transporteurs de sulfate susceptibles de soutenir le métabolisme du soufre dans les feuilles de la partie reproductive.

La période de remplissage et de maturation des graines de pois nécessite des grandes quantités d'azote, or l'azote absorbé est insuffisant pour combler les besoins des graines (Salon *et al.*, 2001). Un processus de remobilisation progressif de l'azote préalablement accumulé dans les organes sources (feuilles, tiges, gousses) vers les organes puits se met alors en place. Chez le pois (génotype Caméor), l'azote remobilisé représente 71% de l'azote total accumulé dans les graines (Schiltz *et al.*, 2005). Les protéines photosynthétiques des feuilles et des tiges représentent la principale source d'azote remobilisé (Schiltz *et al.*, 2004). L'azote issu de la dégradation de ces protéines est converti en acides aminés transportables, particulièrement des amides comme la glutamine et l'asparagine qui sont les principaux acides aminés transportés par le phloème vers les graines (Moison *et al.*, 2018; Masclaux-Daubresse

et al., 2006). Chez le pois, lors du remplissage des graines, des gènes susceptibles de contribuer à la remobilisation des nutriments des feuilles vers les graines ont été identifiés, dont certains sont impliqués dans l'autophagie ou le transport d'azote (Gallardo *et al.*, 2019). Quant au soufre, il est majoritairement remobilisé vers les graines sous forme de glutathion (GSH), de sulfate et de S-méthylméthionine (SMM) (Tan *et al.*, 2010; Hawkesford, 2003; Wongkaew *et al.*, 2018).

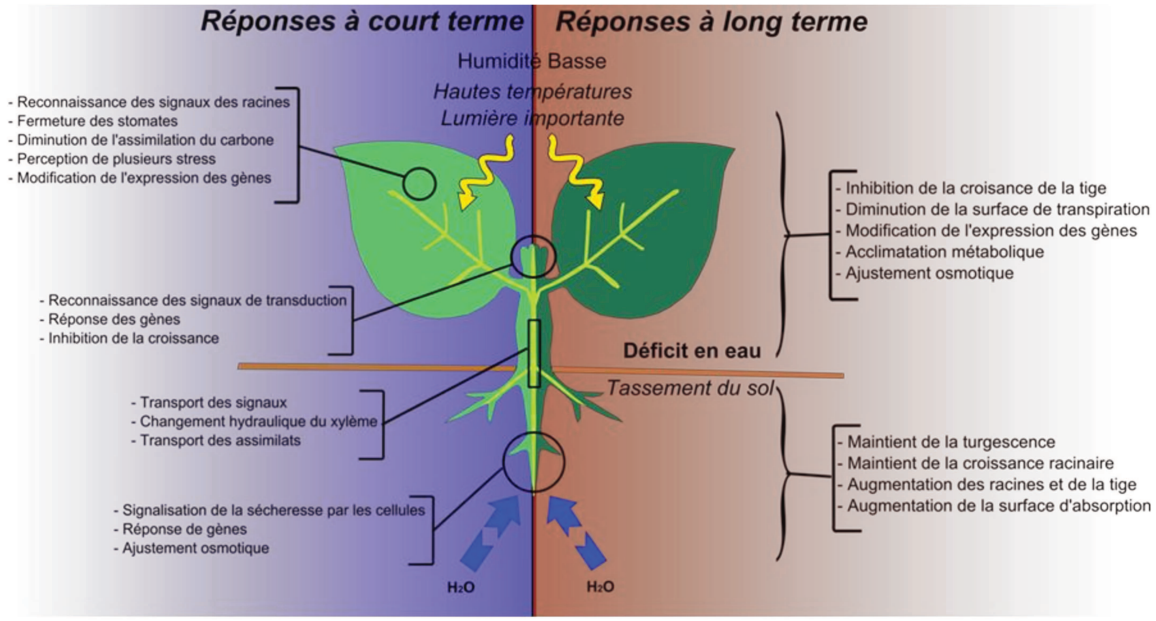

Figure 11. Réponse des plantes à la sécheresse. Les mécanismes physiologiques et biochimiques associés à la réponse à court terme (gauche) et long terme (droite) des plantes à la sécheresse sont listés. (Adapté de Chaves et al., 2003)

4. Stress hydrique et nutrition soufrée

4.1 Pourquoi étudier des stress combinés

Au champs, les plantes sont exposées simultanément à un certain nombre de stress abiotiques et biotiques et il est reconnu que des stress combinés sont une menace plus probable pour les plantes que les stress individuels (Rizhsky *et al.*, 2004; Mittler, 2006; Kissoudis *et al.*, 2014). Ainsi, il serait plus intéressant aujourd'hui de se concentrer sur la compréhension des réponses des plantes dans des conditions de stress multiples afin de développer des génotypes mieux adaptés aux aléas climatiques en champs. La stratégie d'adaptation de la plante à une combinaison de stress comprend à la fois une réponse « partagée » et une réponse « unique ». Les réponses partagées désignent les réponses moléculaires et physiologiques communes aux différents stress et les réponses uniques étant spécifiques aux stress individuels ou à des stress combinés donnés (Rizhsky *et al.*, 2002; Rizhsky *et al.*, 2004; Prasch and Sonnewald, 2013; Sewelam *et al.*, 2014).

4.2 Le stress hydrique

La croissance et la productivité des plantes sont affectées négativement par un déficit hydrique qui est défini par l'état physiologique d'une plante quand la quantité d'eau évaporée par transpiration au niveau des feuilles excède celle prélevée dans le sol par les racines. Les effets les plus précoces sont une réduction de l'élongation cellulaire et la fermeture des stomates médiée par l'acide abscissique (ABA) qui entraine une baisse de la conductance stomatique. Cela permet une réduction des pertes hydriques et un maintien, dans une certaine mesure, du potentiel hydrique de la plante (Jongdee *et al.*, 2002). Ce maintien passe par un ajustement du potentiel osmotique permettant à la plante de maintenir une certaine turgescence cellulaire et donc sa teneur en eau (Anjum *et al.*, 2011) (**Figure 11**). Cet ajustement est effectué *via* l'accumulation d'osmoprotectants sous forme d'osmolytes (polyamines, choline-O-sulfate), de sucres (trehalose, mannitol) et de proline (Chan *et al.*, 2013). Si la fermeture des stomates réduit les pertes d'eau, elle entraine une diminution de l'activité photosynthétique qui peut affecter la croissance des plantes et la production de biomasse (Jewell *et al.*, 2010; Chaves *et al.*, 2003; Fahad *et al.*, 2017). La répartition de la biomasse au sein de la plante peut être également affectée, comme l'allocation du carbone vers les racines au dépend des feuilles et de la tige (DaCosta and Huang, 2006). Le déficit

hydrique est un problème important pour les légumineuses, entrainant une inhibition de l'activité fixatrice d'azote (Streeter, 2003), un raccourcissement de la racine primaire et une augmentation des racines latérales. Le stress hydrique peut aussi modifier la composition des graines. Par exemple, chez le soja, *Glycine max* L., un stress hydrique lors du remplissage des graines augmente la quantité de protéines dans les graines matures mais diminue la quantité d'huile (Dornbos and Mullen, 1992). Le pois protéagineux est extrêmement sensible aux épisodes de stress hydrique subis lors de la phase reproductive associée à la production de graines (source : Terres Inovia, **Figure 12**), soulignant l'importance de s'intéresser aux bases moléculaires de la réponse du pois au stress hydrique lors de cette phase.

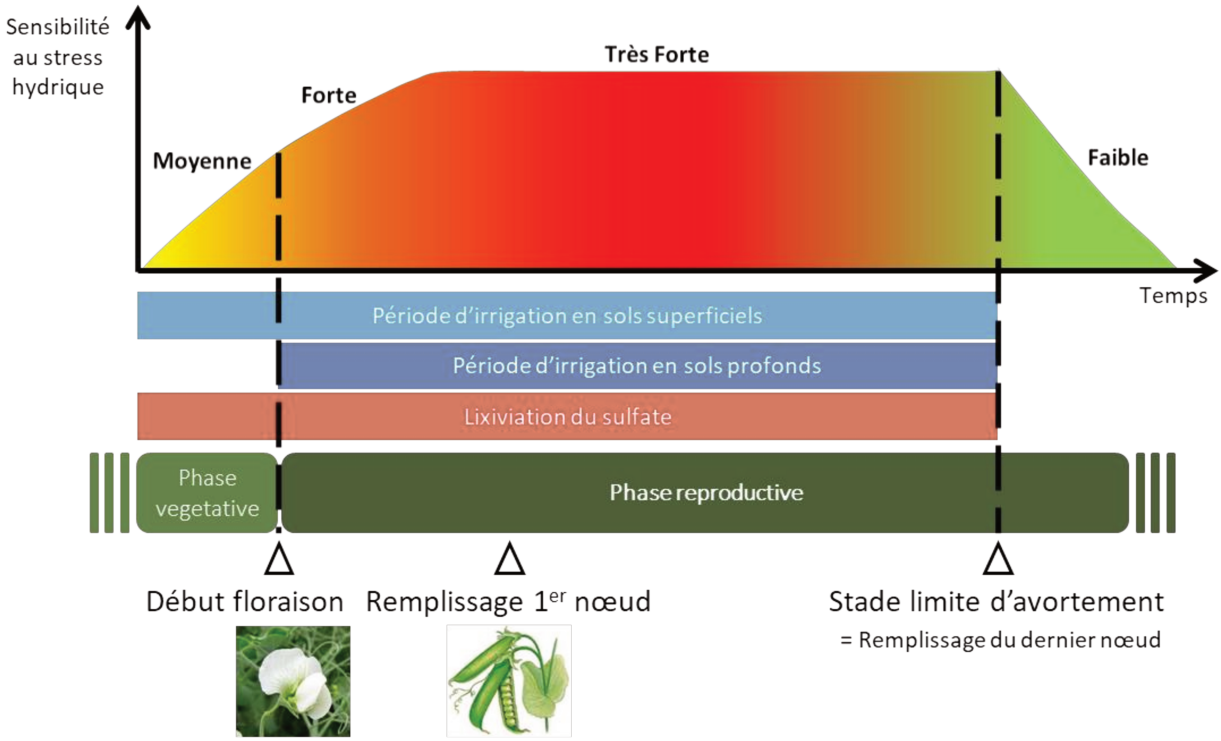

Figure 12. Les phases de développement sensibles au stress hydrique chez le pois.
(Adapté de http://www.terresinovia.fr)

4.3 La nutrition soufrée

La nutrition soufrée connait aujourd'hui un vrai regain d'intérêt, y compris de la communauté scientifique. Le soufre est quatrième dans la liste des macronutriments majeurs nécessaires à la plante après l'azote, le phosphore et le potassium, jouant notamment un rôle dans la production d'acides aminés (qualité de la graine) et d'autres composés soufrés (tolérance et résistance aux stress).

4.3.a Les besoins en soufre des plantes cultivées

La quantité de soufre nécessaire pour produire une tonne de graines varie en fonction des cultures. Environ 8 kg de soufre (de 5 à 13) sont nécessaires pour produire une tonne de graines de légumineuses, contre 12 kg et 3-4 kg de soufre pour les crucifères et céréales, respectivement (Jamal *et al.*, 2010). En effet, les cultures ont des besoins en soufre différents selon si elles accumulent ou non des composés soufrés abondants comme les glucosinolates chez les crucifères et/ou des quantités importantes de protéines dans leurs graines dont la synthèse nécessite des quantités non négligeables de soufre (le cas des légumineuses).

4.3.b Absorption et transport du soufre

Le soufre est majoritairement absorbé au niveau racinaire sous forme de sulfate (SO_4^{2-}). Le sulfate est absorbé et transporté par des systèmes de transport actifs dépendants du pH et couplés à une pompe à protons (H^+-ATPase). Chez *Arabidopsis thaliana*, la famille des transporteurs de sulfate (SULTR) est constituée de 14 membres classés en quatre groupes selon leur similarité de séquence (SULTR 1 à 4) (Hawkesford, 2003). Les transporteurs du groupe 1 (SULTR1) sont des transporteurs de haute affinité facilitant l'absorption du sulfate par les racines ou sa translocation des organes sources aux organes puits (Takahashi *et al.*, 2000). Le groupe 2 (SULTR2) est constitué des transporteurs de faible affinité et sont exprimés dans les tissues vasculaires permettant la translocation du sulfate des racines vers l'ensemble de la plante (Takahashi *et al.*, 2011; Smith *et al.*, 1995; Smith *et al.*, 2000; Yoshimoto *et al.*, 2002; Buchner *et al.*, 2004), et notamment vers les graines en développement (Awazuhara *et al.*, 2005). Les transporteurs du groupe 3 (SULTR3) sont exprimés dans différents organes (feuilles, fleurs, graines) et SULTR3;1 est localisé à la membrane chloroplastique. Les transporteurs du groupe 3 ont été montré comme étant impliqués dans la translocation du sulfate dans la graine (Zuber *et al.*, 2010). Les transporteurs du groupe 4 (SULTR4) sont localisés à la membrane tonoplastique. Le sulfate absorbé au niveau racinaire ou alloué dans les organes végétatifs est soit stocké dans les vacuoles, soit réduit et assimilé pour donner des métabolites qui peuvent eux-mêmes être transportés dans la plante. C'est le cas du GSH et de la SMM qui contribuent au chargement de la graine en soufre (Tan *et al.*, 2010; Gigolashvili and Kopriva, 2014). Les mécanismes de transport de ces métabolites vers les graines restent à élucider.

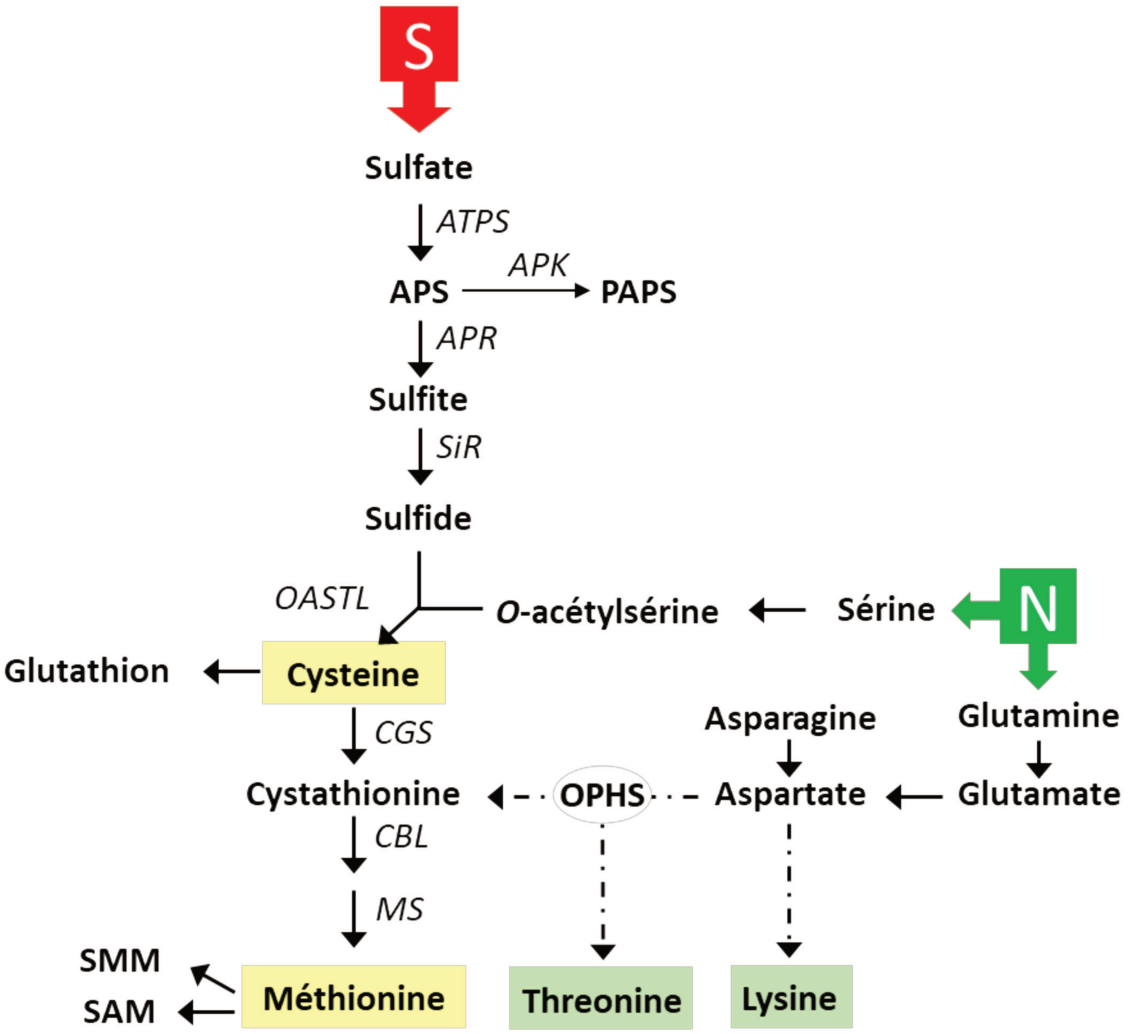

Figure 13. Interaction entre les métabolismes du soufre et de l'azote pour la synthèse des acides aminés soufrés. La réduction du sulfate conduit à la synthèse de sulfide qui est utilisé, avec l'O-acetylsérine, pour la synthèse de cystéine. La cystéine est ensuite utilisée pour la synthèse de méthionine en plusieurs réactions. La première réaction est la synthèse de cystathionine, qui requière la *O*-phosphohomosérine (OPHS) dérivée du métabolisme de l'aspartate. ATPS, ATP sulfurylase; APR, adénosine 5'-phosphosulfate réductase; SiR, sulfite réductase; OASTL, *O*-acetylérine-thiol-lyase; CGS, cystathionine gamma-synthase; CBL, cystathionine beta lyase; MS, méthionine synthase. APS, adénosine 5'-phosphosulfate; PAPS, 3'-phosphoadénosine-5'-phosphosulfate; SAM, *S*-adénosylméthionine; SMM, *S*-méthylméthionine.

4.3.c Assimilation du soufre et lien avec le métabolisme de l'azote

Le métabolisme du sulfate fait intervenir des premières étapes de réduction (**Figure 13**). En présence d'ATP, le sulfate est converti en adénosine 5'-phosphosulfate (APS) par l'ATP sulfurylase. L'APS peut être phosphorylé par l'APS kinase (APK) et former du 3'-phosphoadénosine-5'-phosphosulfate (PAPS), un précurseur pour la synthèse de métabolites secondaires, dont plusieurs jouent un rôle dans les processus de défense (Anjum *et al.*, 2015). L'APS peut aussi être réduit en sulfite (SO_3^{2-}) par l'APS réductase (APR), puis en sulfide (S^{2-}) par la sulfite réductase (SiR). L'ion sulfide est ensuite couplé à l'*O*-acétylsérine (OAS) dans une réaction conduisant à la synthèse de cystéine. L'*O*-acétylsérine est dérivée de la sérine, montrant le lien étroit entre les métabolismes du soufre et de l'azote. La cystéine est ensuite utilisée pour la synthèse de méthionine, un autre acide aminé soufré également connecté au métabolisme de l'azote car son précurseur est l'aspartate. La synthèse de méthionine est réalisée en trois étapes successives catalysées par la cystathionine gamma-synthase (CGS), la cystathionine beta-lyase (CBL), et la méthionine synthase (MS) (Ravanel *et al.*, 1998). La méthionine peut ensuite être incorporée dans les protéines, stockée sous forme de SMM, et/ou utilisée pour la synthèse de *S*-adénosylméthionine (SAM) (**Figure 13**). En plus de son rôle de donneur de groupe méthyle, la SAM est le précurseur de la synthèse de polyamines, de biotine et d'éthylène jouant un rôle crucial dans le développement et la croissance des plantes (Ravanel *et al.*, 1998).

4.4 Le rôle de la nutrition soufrée dans la réponse au déficit hydrique

De nombreux transporteurs de sulfate sont fortement régulés par le déficit hydrique (Gallardo *et al.*, 2014; Ahmad *et al.*, 2016), ce qui permettrait à la plante de réguler l'allocation du sulfate aux différents organes sous contrainte hydrique. Il a également été montré chez le maïs et le peuplier que la concentration en sulfate augmente dans le xylème en réponse à un déficit hydrique (Malcheska *et al.*, 2017; Ernst *et al.*, 2010), suggérant que le sulfate joue un rôle de molécule signal permettant la fermeture des stomates (**Figure 14**). Le sulfate permet aussi la biosynthèse de cystéine qui est requise pour la synthèse d'ABA conduisant à la fermeture des stomates et donc à la réduction de la perte d'eau (Cao *et al.*, 2014; Malcheska *et al.*, 2017). En effet, la cystéine est un donneur de soufre pour la sulfuration du cofacteur molybdène nécessaire à la synthèse d'ABA. Le métabolisme du soufre est à l'origine de la production d'autres métabolites soufrés jouant des rôles osmoprotectants (composés dérivés du PAPS),

antioxydants (GSH) ou réparateurs (ex. méthylations) (Chan *et al.*, 2013) (**Figure 14**). Plusieurs enzymes du métabolisme soufré sont régulées par un déficit hydrique au niveau de leur transcription et de leur activité enzymatique (Ahmad *et al.*, 2016; Larrainzar *et al.*, 2014). Aussi, il a été montré qu'un cultivar de colza ayant une meilleure efficience d'absorption et d'utilisation du soufre résiste mieux à un stress hydrique (Lee *et al.*, 2016), appuyant l'importance de la nutrition soufrée dans la tolérance des plantes à la sécheresse.

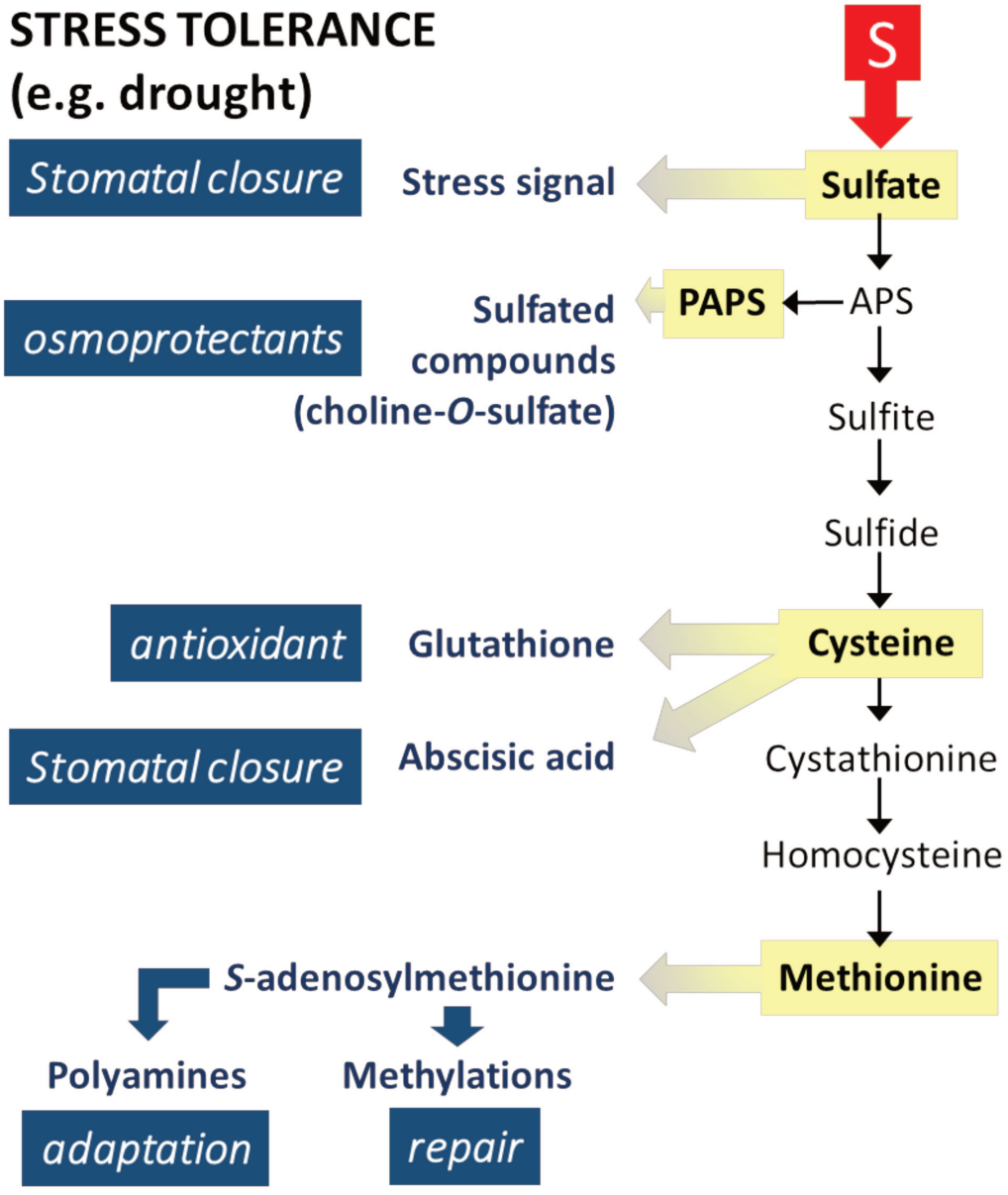

Figure 14. Interaction entre la nutrition soufrée et la réponse des plantes au stress hydrique. Le sulfate joue un rôle central dans la réponse des plantes au stress hydrique en agissant comme molécule signal pour la fermeture des stomates ou en permettant la synthèse de métabolites impliqués dans les processus antioxydants, osmoprotectants, de réparation, d'adaptation, ou de fermeture des stomates. APS, adénosine 5'-phosphosulfate; PAPS, 3'-phosphoadénosine-5'-phosphosulfate.

4.5 Carence en soufre

Un appauvrissement des sols en soufre est observé depuis plusieurs décennies, devenant un facteur limitant pour la production végétale dans de nombreux pays. Cette carence en soufre des sols est due à la diminution des émissions de dioxyde de soufre qui ne cessent de baisser depuis les années 80 (**Figure 1**), suite à la mise en place des politiques de réduction des rejets industriels, et aux changements des pratiques de fertilisation dans les parcelles agricoles (Lewandowska and Sirko, 2008). Ainsi, des symptômes de carence en soufre ont commencé à apparaître aux champs sous la forme de jaunissement des feuilles (Buchner *et al.*, 2004). La **Figure 15** illustre le jaunissement des feuilles de pois et de colza en réponse à une carence en soufre (Courbet *et al.*, 2019; Henriet *et al.*, 2019), soulignant l'importance de la nutrition soufrée pour le maintien du métabolisme des feuilles. Chez la légumineuse modèle *Medicago truncatula*, il a été démontré qu'une carence en soufre à un stade végétatif, *i.e.* apparition des ramifications tertiaires, affecte la production de fourrages et de graines et réduit l'allocation de carbone vers les graines. Il est à noter que ces graines sont caractérisées par une faible vigueur germinative et une faible teneur en saccharose et en oligosaccharides de la famille du raffinose nécessaires à l'allongement de l'axe embryonnaire lors de l'imbibition (Zuber *et al.*, 2013). Des effets similaires sur la germination ont été obtenus chez le colza (D'Hooghe *et al.*, 2014), montrant l'importance de la nutrition soufrée de la plante mère, qu'elle soit crucifère ou légumineuse, pour le maintien de la qualité physiologique des semences. De plus, une carence en soufre diminue la concentration en protéines de réserve relativement riches en soufre (globulines 11S chez les légumineuses), effet compensé par une accumulation des protéines relativement pauvres en soufre (globulines 7S) permettant de maintenir la teneur en protéines des graines (Chandler *et al.*, 1983; Zuber *et al.*, 2013). La régulation de l'expression des gènes codant les globulines dépend de la nutrition soufrée et azotée, et implique l'O-acetylsérine et la méthionine qui agissent comme molécules signal (Tabe *et al.*, 2010; Chandler *et al.*, 1984). De plus, une insuffisance de soufre dans la plante réduit l'efficacité d'absorption de l'azote (Fazili *et al.*, 2008). L'expression de nombreux gènes impliqués dans le transport (SULTR1;1, SULTR2;1, SULTR3;5, SULTR4;1 et SULTR4;2) et le métabolisme du soufre (ATP-sulfurylase, sulfotransférases) augmente en réponse à une carence en soufre (Takahashi *et al.*, 2000; Parmar *et al.*, 2007; Buchner *et al.*, 2004). L'augmentation de l'expression de ces gènes en réponse à une carence en soufre suggère la

mise en place de mécanismes adaptatifs visant à activer le métabolisme du soufre disponible dans les tissus (Zuber *et al.*, 2013), notamment le soufre mis en réserve au sein des vacuoles et dont l'efflux est permis grâce aux transporteurs du groupe 4. Enfin, les gènes codant pour la nitrate réductase et la glutamine synthétase impliquées dans l'assimilation de l'azote sont au contraire régulés négativement en réponse à une carence en soufre chez *Nicotiana tabacum*, soulignant le lien étroit entre la nutrition soufrée et azotée (Migge *et al.*, 2000).

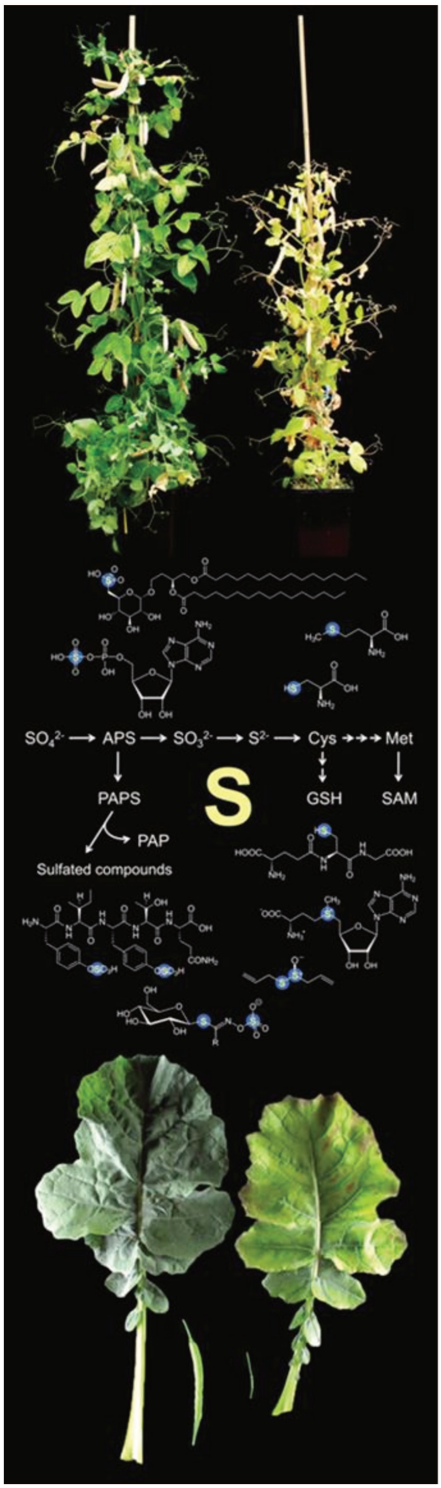

Figure 15. Effet d'une carence en soufre – bandeau de la couverture du volume 70 (issue 16) de J. Exp. Bot. Cette illustration montre l'effet d'une carence en soufre (droite) chez le pois protéagineux (haut) et le colza (bas). Ces illustrations font référence aux publications Henriet et al., (2019) pour le pois et Courbet et al., (2019) pour le colza.

5. Objectifs de la thèse et démarches

Ce projet de thèse a été initié au sein du pôle de Génétique et Adaptation des Plantes à des Systèmes de culture Innovants (GEAPSI) de l'UMR Agroécologie, dans l'équipe Remplissage des graines et stress abiotiques (FILling & Abiotic Stresses, FILEAS). Ces travaux s'inscrivent dans un contexte de changement climatique où un stress hydrique et une carence en soufre, deux stress abiotiques, vont être amenés à co-exister et donc interagir. Comme indiqué dans la synthèse bibliographique, ces stress étudiés de façon individuelle sont connus pour impacter négativement les plantes d'intérêt agronomique, réduisant rendement et qualité des graines produites. Or, aucune étude ne s'est intéressée à l'interaction entre ces deux stress et les conséquences qui en découlent. L'objectif principal de cette thèse était de développer des connaissances et une meilleure compréhension de l'impact d'une carence en soufre combinée à un stress hydrique modéré lors de la phase reproductive, et des réponses mises en place par la plante dans le but d'améliorer sa tolérance. La thèse est centrée sur le pois protéagineux, *Pisum sativum* L., dont la culture produit des graines riches en protéines, enrichit les sols en azote pour les cultures suivantes, et limite l'utilisation d'engrais azotés, réduisant significativement les pertes de nitrate par lessivage et les émissions de gaz à effet de serre. Le cultivar utilisée est Caméor qui produit des graines riches en protéines. Le séquençage récent de son génome (Kreplak *et al.*, 2019), la disponibilité d'un transcriptome (Alves-Carvalho *et al.*, 2015) ainsi qu'une collection de mutants TILLING (Targeting Induced Local Lesions in Genomes, Dalmais *et al.*, 2008) pour ce génotype offrent des perspectives d'études moléculaires approfondies.

Afin d'obtenir une vision la plus complète possible de l'impact de l'interaction entre la carence en soufre et le stress hydrique chez le pois, l'étude a été réalisée à différents niveaux en partant d'une vision globale de son impact par le phénotypage des plantes entières, jusqu'à la caractérisation des mécanismes moléculaires sous-jacents par l'étude de l'expression des gènes, et de l'accumulation des protéines, métabolites et d'éléments. L'étude est basée sur le couple graines-feuilles pour leur relation organe puits - organe source, le premier organe ayant un intérêt nutritionnel et donc économique, le second étant le « moteur » de la plante et acteur majeur pour la production des graines.

Pour réaliser une telle étude, une seule expérimentation en serre de grande ampleur a été mise en place sur une durée de 5 mois. Au total, 450 plantes du génotype Caméor de pois ont été cultivées et placées sur la plateforme de phénotypage à haut de débit de Dijon (4PMI) pour appliquer et suivre le stress hydrique. Au cours des stress, 228 plantes ont été prélevées pour échantillonner les feuilles et graines afin de réaliser des analyses moléculaires, et pour réaliser un phénotypage de la plante entière *via* la mesure de plus de 50 caractères. Au final, 103 plantes ont été amenées à maturité pour une analyse du rendement et de la qualité des graines produites ainsi qu'un phénotypage détaillé de la plante entière.

Le matériel ainsi produit a permis de répondre à trois objectifs principaux, dont les résultats sont représentés dans ce manuscrit en trois chapitres sous la forme d'un article scientifique publié (**Chapitre II et Annexe 1**) et de deux articles en préparation (**Chapitre III et IV**).

Le premier objectif visait à étudier l'impact du stress hydrique et d'une carence en soufre appliqués séparément ou combinés sur le rendement et la qualité des graines matures de pois. (*i*) Les composantes de rendement et de qualité des graines de pois ont été mesurées, notamment la composition protéique par une analyse des profils protéiques des graines matures, obtenus par électrophorèse monodimensionnelle. (*ii*) Les composantes écophysiologiques associées aux variations de rendement et de composition des graines ont été déterminées par la mesure de la répartition du carbone, de l'azote et du soufre entre les différents organes de la plante, y compris les graines, à maturité. *(iii*) Les mécanismes moléculaires mis en jeu à un stade clé du développement de la graine, correspondant au stade de transition vers le remplissage, ont été mis en évidence par une approche transcriptomique par RNA-Seq afin d'identifier des gènes potentiellement impliquées dans le contrôle des variations de composition de la graine. (**Chapitre II et Annexe 1**)

Le second objectif visait à identifier les mécanismes moléculaires mis en jeu dans les graines (organes puits de nutriments) au cours des stades précoces de leur développement, et leur modulation en réponse à une carence en soufre et/ou un stress hydrique. (*i*) Un atlas des protéines de la graine à trois stades clés de développement (embryogenèse, transition embryogenèse-remplissage, et synthèse des protéines de réserve) a été développé par protéomique quantitative de type shotgun. (*ii*) Leur cinétique d'accumulation à ces différents stades de développement a été étudiée et les protéines régulées en réponse aux stress ont

été identifiées. *(iii)* Les mécanismes moléculaires gouvernant la réponse des graines aux stress ont été étudiés par une approche « réseau » à partir des données protéomiques. (**Chapitre III**)

Le troisième objectif consistait à identifier les mécanismes moléculaires mis en jeu dans les feuilles issues des premiers nœuds reproducteur (organes sources de nutriments) au cours des stades précoces de la phase reproductive, et leur modulation en réponse à une carence en soufre et/ou un stress hydrique. Afin d'étudier dans leur globalité les régulations métaboliques et transcriptionnelles, une approche multi-omique combinant transcriptomique, protéomique, métabolomique et ionomique a été utilisée. (**Chapitre IV**)

En parallèle de ce travail, j'ai participé à une étude comparative du transcriptome des feuilles entre le pois et *Medicago truncatula* au cours du processus de remobilisation de l'azote vers les graines. L'article correspondant, dont je suis co-auteur, est disponible en **Annexe 2**.

<u>Chapitre II :</u> Modulation du rendement et de la composition protéique des graines de pois en réponse à une carence en soufre combinée à un stress hydrique

<u>Chapitre II :</u> Modulation du rendement et de la composition protéique des graines de pois en réponse à une carence en soufre combinée à un stress hydrique

Approches

Analyse de la composition protéique des graines matures de pois récoltées dans 4 conditions : non limitante en soufre et en eau, carencée en soufre, stress hydrique modéré, combinaison des deux stress.

Analyse de la teneur, de la quantité et de la répartition du carbone, de l'azote et du soufre dans les différents organes des plantes de pois récoltées à maturité.

Etude transcriptomique (RNA-seq) des graines de pois au stade de transition entre l'embryogenèse et le remplissage, et étude de l'effet des stress simples et combinés sur le transcriptome de ces graines.

Principaux résultats

- La combinaison d'une carence en soufre et d'un stress hydrique modéré affecte de manière synergique le rendement ainsi que les composantes de rendement (taille d'une graine et nombre de graines).

- L'abondance relative des globulines 11S relativement riches en soufre dans les graines est beaucoup moins affectée par le stress combiné que par la carence en soufre seule : le stress hydrique atténue l'effet négatif de la carence en soufre sur la composition protéique des graines.

- Cet effet est probablement dû à la forte diminution de la taille des graines en réponse au stress combiné qui réduit leur demande en azote mais pas en soufre, permettant ainsi l'ajustement du ratio globulines 7S /globulines 11S.

- L'analyse du transcriptome de graines immatures de pois prélevées à la fin de la période de stress combiné (stade de transition entre l'embryogenèse et l'accumulation des protéines de réserve) montre la mise en place de processus de régulation spécifiques au double stress impliquant un jeu de facteurs de transcription et de SUMO-ligases.

<u>Ce travail a été publié dans la revue Journal of Experimental Botany.</u>

<u>Henriet C</u>, Aimé D, Térézol M, Kilandamoko A,Rossin N, Combes-Soia L, Labas V, Serre RF, Prudent M, Kreplak J, Vernoud V and Gallardo K (2019) Combining water stress with sulfur deficiency in pea impedes reproductive development but reduces imbalance in seed protein composition. J Exp Bot. 70: 4287-4304. doi: 10.1093/jxb/erz114.

Water stress combined with sulfur deficiency in pea affects yield components but mitigates the effect of deficiency on seed globulin composition

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ABSTRACT

Water stress and sulfur (S) deficiency are two constraints increasingly faced by crops due to climate change and low-input agricultural practices. To investigate their interaction in the grain legume pea (*Pisum sativum*), sulfate was depleted at the mid-vegetative stage and a moderate 9-d water stress period was imposed during the early reproductive phase. The combination of the stresses impeded reproductive processes in a synergistic manner, reducing seed weight and seed number, and inducing seed abortion, which highlighted the paramount importance of sulfur for maintaining seed yield components under water stress. On the other hand, the moderate water stress mitigated the negative effect of sulfur deficiency on the accumulation of S-rich globulins (11S) in seeds, probably due to a lower seed sink strength for nitrogen, enabling a readjustment of the ratio of S-poor (7S) to 11S globulins. Transcriptome analysis of developing seeds at the end of the combined stress period indicated that similar biological processes were regulated in response to sulfur deficiency and to the combined stress, but that the extent of the transcriptional regulation was greater under sulfur deficiency. Seeds from plants subjected to the combined stresses showed a specific up-

regulation of a set of transcription factor and SUMO ligase genes, indicating the establishment of unique regulatory processes when sulfur deficiency is combined with water stress.

KEYWORDS

Abiotic stresses, drought, nutrient partitioning, *Pisum sativum*, sulfur, seed quality, seed transcriptomics, storage proteins

INTRODUCTION

Legumes are able to accumulate large amounts of proteins in their seeds even in the absence of nitrogen (N) fertilizer thanks to their ability to interact symbiotically with soil-borne Rhizobiaceae that fix atmospheric dinitrogen. In pea (*Pisum sativum*), one of the most cultivated pulse crops, seeds contain between 18–34% protein on a dry-weight basis (Burstin *et al.*, 2011). As in other legumes, pea seed proteins are poor in the sulfur (S) amino acids methionine and cysteine (S-AA). However, being rich in lysine, they complement the protein intake from cereals, which are in contrast poor in lysine (Burstin *et al.*, 2011). About 70% of seed proteins in legumes are seed-storage proteins consisting of 7S (vicilins, convicilins) and 11S (legumins) globulins (Boulter and Croy, 1997). These abundant proteins differ in their nutritional properties. Notably, 11S globulins contain higher levels of S-AA than 7S globulins (1.5% and 0.6%, respectively, in *Medicago truncatula*) (Zuber *et al.*, 2013). Hence, variations in the 7S/11S ratio (which has a mean value of 1 in the pea cultivar 'Caméor'; Bourgeois *et al.*, 2009) could influence the nutritional quality of legume seeds by affecting the dietary intake of methionine and cysteine. Variations in this ratio could also influence the functionality of protein isolates such as their solubility, foaming, and emulsifying capacities (Dagorn-Scaviner *et al.*, 1986; Rangel *et al.*, 2003), or their textural properties (Mujoo *et al.*, 2003).

Variations in environmental conditions influence the final seed yield and the content and composition of proteins in pea (Karjalainen and Kortet, 1987; Bourgeois *et al.*, 2009). One of the environmental constraints affecting crop productivity is water stress (WS), which is predicted to occur more frequently and severely with climate change. In pea, water stress is a major yield-limiting factor (Fougereux *et al.*, 1997), and its impact depends on the intensity

of the stress and its duration, and on the phenological stage at which it occurs (Ney *et al.*, 1994; Guilioni *et al.*, 2003; Prudent *et al.*, 2015). Previous studies on water stress have suggested that S nutrition could play a key role in the plant response (Chan *et al.*, 2013, and references therein). Under water stress, sulfate concentration increases in the xylem sap (Ernst *et al.*, 2010; Malcheska *et al.*, 2017) and enhances stomatal closure in the shoot by promoting the expression of genes involved in abscisic acid (ABA) synthesis (Malcheska *et al.*, 2017). Moreover, a rapeseed cultivar with improved efficiency of S uptake and utilization is more resistant to water stress (Lee *et al.*, 2016), supporting the importance of S in tolerance to water stress.

Due to controls on emissions, areas with S-deficient soils are increasing (Mcgrath *et al.*, 2003) and molecular indicators are being developed to diagnose S deficiency (Etienne *et al.*, 2018). In legumes, the S-starvation response can be reduced through mycorrhizal colonization under high phosphate levels (Sieh *et al.*, 2013). S deficiency reduces photosynthesis through a negative effect on both chlorophyll content and the rate of photosynthesis per unit chlorophyll (Terry, 1976), and affects nitrogen fixation and assimilation (Scherer and Lange, 1996; Zhao *et al.*, 1999; Varin *et al.*, 2010). S deficiency also affects the quality of legume seeds, such as germination capacity and the accumulation of 11S globulins, which is compensated by an increased accumulation of 7S globulins (Blagrove *et al.*, 1976; Chandler *et al.*, 1983; Chandler *et al.*, 1984; Spencer *et al.*, 1990; Zuber *et al.*, 2013). Variations in legume seed globulins have been shown to be primarily controlled at the transcriptional level, with the changes in transcript abundance closely reflecting the pattern of synthesis of the corresponding globulins (Chandler *et al.*, 1984; Gallardo *et al.*, 2007).

Since water stress may occur simultaneously with S deficiency in cropping systems with low fertilizer input, the impact of deficiency combined with a water-stress period is clearly very important but has not previously been studied. Recent studies have highlighted the fact that the plant response to a combination of two abiotic stresses is unique and that the outcome of the interaction cannot be extrapolated from the effects of the individual stresses (Pandey *et al.*, 2015; Zhang and Sonnewald, 2017). Plants adapt their responses to combined stress factors, displaying unique and/or common responses compared to single stresses. The interaction between two stresses can be either additive (adding the impacts of two hypothetical stressors) or synergistic (a response that is greater than the additive response)

and, in some cases, mitigation strategies have been revealed (Pandey *et al.*, 2015). In this study, we provide a first overview of the combined effects of water stress and S deficiency on pea productivity, nutrient partitioning between plant parts, and seed quality traits. Moderate water stress was applied at the beginning of the reproductive phase, a period during which this stress frequently occurs in the field. Clear differences in the plant and seed characteristics in response to the combined stress compared to the individual stresses were observed, highlighting synergistic effects on reproductive processes and mitigating effects on seed globulin composition, which could be explained by changes in seed sink strength for N. By studying the transcriptome of developing seeds, specific molecular signatures for S deficiency and for the combined stress were identified, some of which could play key roles in the transcriptional regulation of globulin accumulation.

MATERIALS AND METHODS

Plant growth conditions

Seeds of *Pisum sativum* L. ('Caméor' genotype) were pre-germinated for 5 d in a Fitoclima S600 germinator (Aralab, Rio de Mouro, Portugal) at 20 °C in the dark. The germinated seeds were sown individually in 2-l pots containing a mixture of perlite/sand (3/1, v/v) at a day/night temperature of 19/15 °C, with a 16-h photoperiod with artificial lighting (250 µmol m–2 s–1). The seedlings were irrigated with a nitrate- and S-rich nutrient solution (S+) as previously described (Zuber *et al.*, 2013; 2 mM Ca(NO3)2 as the only modification) for 3 weeks (to the 5-/6-node stage). Half of the plants were then subjected to S deficiency (S–) by using the same solution lacking MgSO4.7H2O but containing 1.16 mM MgCl2, after rinsing the substrate twice with deionized water and then twice with the S– solution. After 8 d of S deficiency, plants at the 8-node stage (on the primary branch: emerging secondary branches were removed throughout development) were transferred to an automated Plant Phenotyping Platform for Plant and Micro-organism Interactions (4PMI, Dijon, France). Plants were automatically weighed and watered four times a day in order to maintain a soil relative water content corresponding to the maximum (100%) water-holding capacity of the substrate. At flowering of the second/third flowering node, irrigation was stopped for half of the plants until the soil water content reached 50% of the maximum water-holding capacity of the substrate. Once

this target value was reached it was maintained for 9 d. Plants were then re-watered normally with their appropriate solution (S+ or S–) until maturity (**Figure 16A**).

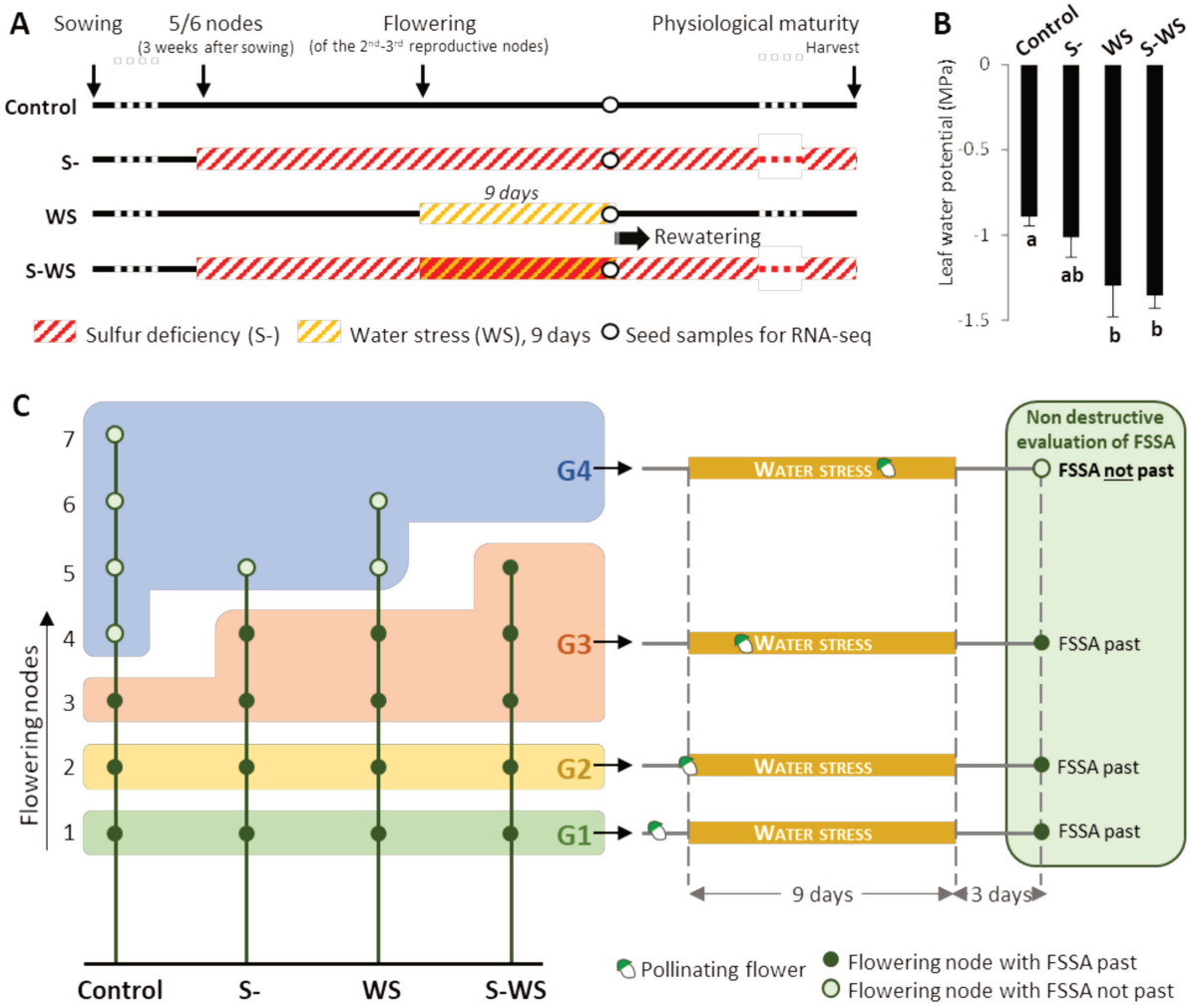


Figure 16. Experimental design for studying the interaction between water stress and S deficiency in pea (cv. 'Caméor'). (A) Developmental stages at which stresses were imposed. Control, plants were well watered under non-limiting S conditions; S–, plants were deprived of S from the mid-vegetative stage until harvest; WS, plants were subjected to water stress from flowering of the 2nd or 3rd reproductive node for 9 d, then re-watered for recovery; S–WS, plants were subjected to a combination of the two stresses. Developing seeds were collected at the end of the water-stress period for transcriptome analyses, and other plants were harvested at maturity for phenotyping and analyses of seed composition. **(B)** Leaf water potential measured at the end of the water-stress period. Data are means (±SE), *n*=4 plants. Different letters indicate significant differences as determined by ANOVA followed by a SNK test (*P*<0.1). **(C)** Schematic representation of how mature seeds were collected. For each plant, seeds from each individual flowering node (dots on the left diagram) were collected at maturity and pooled into four groups (G1–G4). G1 seeds corresponded to flowering nodes that flowered before the beginning of the water stress period; G2 seeds corresponded to the flower that opened on the day that water stress was imposed; G3 and G4 seeds corresponded to nodes that flowered during the water-stress period. G1–G3 seeds had passed the final stage of seed abortion (FSSA) 3 d after the end of the water-stress period, while G4 seeds had not. Note that no G4 seeds developed on the double-stressed plants.

Physiological measurements and collection of seed samples

The midday leaf water potential was measured at the end of the water-stress period on the last fully expanded leaf of four plants per treatment in a C52 sample chamber coupled to a HR-33T Dew Point Microvoltmeter (Wescor Inc.). The chlorophyll content of the leaf from the first reproductive node of 7–10 plants per treatment was estimated throughout the reproductive period using a SPAD-502 chlorophyll meter (Minolta).

For each plant, the flower that opened on the day that water stress was applied was tagged, thus allowing us to separate seeds at harvest that corresponded to flowers that opened before water stress, on the day it was applied, and during the stress period. A pea pod sizer developed by Arvalis-Institut du Végétal was used to non-destructively estimate whether or not the seeds of the plants had reached the final stage of seed abortion (FSSA, i.e. the stage at which seeds no longer abort: pod thickness >0.7 cm) (Munier-Jolain *et al.*, 2010). FSSA measurements were carried out 3 d after the end of the water-stress period, which corresponded to 12 d after pollination (DAP) for the tagged pod. Mature seeds were harvested in four groups (G1–G4) according to the date of anthesis of the corresponding flower, with G3 and G4 being further defined according to the FSSA measurements (**Figure 16C**). For germination tests, 20 mature seeds (G1–G4 pooled) from each of three replicate plants per treatment were germinated in the dark at 20 °C as described by Benamar *et al.*, (2003). A seed was considered as germinated when the radicle protruded through the seed coat.

For transcriptomics, developing seeds were harvested from tagged pods at 9 DAP, corresponding to 9 d after the beginning of the WS period (**Figure 16A**). The experiment was carried out in a randomized complete block design with either three (S– and WS treatments) or four (control and S–WS treatments) biological replicates per treatment, each replicate consisting of two plants pooled together.

Measurements of S, N, and C contents in mature seeds and plant parts

S, N, and carbon (C) contents were determined on dried (80 °C for 48 h), ground tissues from four plants per treatment. Samples included seeds (separated into four groups as described before), pod walls, roots, vegetative parts (leaves and stems developed before flowering, i.e. below the first reproductive node), and reproductive parts (leaves and stems of the reproductive nodes). N and C contents were determined from 5 mg of ground tissue using the

Dumas procedure on a Flash 2000 Elemental Analyzer (ThermoFisher Scientific) with two technical replicates per sample. S content was determined from 20 mg of ground tissue mixed with 5 mg of tungsten trioxide on an elemental PYRO cube analyser (Elementar), with two technical replicates per sample. Using these data, the quantity of elements in each plant part was calculated by multiplying the element concentrations by the total biomass of each part. Nutrient partitioning between plant parts was calculated as a percentage relative to the total quantity of each element in the entire plant.

Extraction, separation, and relative quantification of seed proteins using 1-D electrophoresis gels

Total soluble proteins were extracted as previously described (Gallardo *et al.*, 2007) from four biological replicates of mature seeds using 500 µl of urea/thiourea buffer for 10 mg of seed powder. Protein concentration was determined according to Bradford, (1976) using a Bio-Rad Protein Assay. For each seed sample, 10 µg proteins were separated by one-dimensional electrophoresis (1-DE) in a SDS polyacrylamide gel using the XCell4 SurelockTM Midi-Cell system (Life Technology) [Resolution gel: 4.1 mM Tris-HCl pH 8.8, 40% (v/v) acrylamide/bisacrylamide (30%/0.8%), 1% (g/v) SDS, 0.05% (g/v) ammonium persulfate, 0.05% (v/v) TEMED; Concentration gel: 0.6 mM Tris-HCl pH 6.8, 13% (v/v) acrylamide/bisacrylamide (30%/0.8%), 1% SDS, 0.05% ammonium persulfate, 0.05% TEMED; Electrophoresis buffer: 50 mM Trizma base, 380 mM glycine, 0.1% (g/v) SDS]. After staining with Coomassie Blue R250 (Bio-Rad), gels were scanned using an Odyssey Infrared Imaging System (LI-COR) with an intensity of 7.5 and a resolution of 84 µm. Protein band detection and quantification were performed using Phoretix 1D (v11.2, TotalLab Limited). In each well, the quantitative data were normalized by dividing the volume of each protein band by the total band volume, and the molecular weight (kDa) of each band was calculated using a low-range protein ladder (Bio-Rad).

Identification and annotation of mature seed proteins

Protein bands were excised from the gel and protein digestion was carried out according to Labas *et al.*, (2015). The resultant peptides were extracted in 5% formic acid (10 min sonication, supernatant removed and saved), followed by incubation in 100% acetonitrile/1% formic acid (1:1, 10 min), and a final incubation with 100% acetonitrile (5 min, supernatant

was again removed and saved). These two peptide extractions were pooled and dried using a SPD1010 speedvac system (Thermosavant, Thermofisher Scientific). The resultant peptide mixtures were analysed by nanoflow liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) for protein identification. Peptide separation and data acquisition were performed according to Labas *et al.*, (2015) on a LTQ Orbitrap Velos mass spectrometer (ThermoFisher Scientific) coupled to an Ultimate® 3000 RSLC Ultra High Pressure Liquid Chromatographer (Dionex) controlled by Chromeleon Software (v6.8 SR11; Dionex). MS/MS ion searches were performed using Mascot (v2.3.2, Matrix Science) via Proteome Discoverer 2.1 software (ThermoFisher Scientific) against the pea gene atlas (Alves-Carvalho *et al.*, 2015). The search parameters included trypsin as a protease with two allowed missed cleavages and carbamidomethylcysteine, methionine oxidation, and acetylation of N-term protein as variable modifications. The tolerance of the ions was set to 5 ppm for parent and 0.8 Da for daughter-fragment ion matches. Peptides and proteins identified by Mascot were validated using Scaffold (v4.8.3, Proteome Software): protein identifications were accepted when they contained at least two identified peptides and when the probability was at least 95% and 99% as specified by the PeptideProphet and ProteinProphet algorithms, respectively (Keller *et al.*, 2002). To annotate each protein band, we used the quantitative values obtained by mass spectrometry. These values corresponded to the Normalized Weighted Spectra counts calculated by Scaffold 4.8.3 (**Supplementary Table S1**) that reflected the abundance of each protein identified within a band. We assigned a protein name to a band only when the Normalized Weighted Spectra count indicated a high abundance for this protein (i.e. a major protein).

RNA sequencing, read mapping, and differential analyses

Total RNAs were extracted from 9-DAP seeds using an RNeasy Plant Mini Kit (QIAGEN), treated with an RNase-Free DNase Set (QIAGEN), and then purified by lithium chloride precipitation. Quantification of RNA was performed using a spectrophotometer NanoDropTM 2000 (ThermoFisher Scientific) and RNA quality was assessed on a 2100 Bioanalyzer (Agilent Technologies). The same RNA pools were used for RNA sequencing (RNA-seq) and reverse-transcription quantitative PCR (RT-qPCR). RNA-seq was performed on an Illumina HiSeq3000 to generate 150-nucleotide-long paired-end reads. RNA-seq libraries were prepared using an Illumina TruSeq Stranded mRNA sample prep kit according to the manufacturer's instructions.

Briefly, mRNAs were selected using poly-T beads, fragmented to generate double-stranded cDNA, and ligated to adapters. Eleven cycles of PCR were applied to amplify the libraries. Library quality was assessed using a Fragment Analyzer (Agilent Technologies) and libraries were quantified by qPCR using a Kapa Library Quantification Kit (Roche). RNA-seq experiments were performed on an Illumina HiSeq3000 using a paired-end read length of 2×150 pb with Illumina HiSeq3000 sequencing kits. The sequence quality of the raw data was assessed using the FastQC v0.11.2 software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and raw data were corrected using the k-mer-based error correction software Rcorrector (-k 31) (MacManes, 2015, Preprint; Song and Florea, 2015). The corrected data were trimmed for low-quality and adapter sequences using Trimmomatic v0.32 (Bolger *et al.*, 2014) with the following parameters: ILLUMINACLIP:TruSeq3PE2:2:40:15, LEADING/TRAILING:2, SLIDINGWINDOW:4:15, and MINELN:25. Trimmed reads with less than 25 bp and unpaired reads were discarded. The corrected and trimmed reads were aligned against the Pea Reference Genome sequence v1 (https://urgi.versailles.inra.fr/Species/Pisum/Pea-Genome-project) using the HISAT2 v2.0.5 alignment program (Kim *et al.*, 2015) with the specific parameter --dta for output compatibility with StringTie 1.2.2 (Pertea *et al.*, 2015). For each library, more than 90% of the reads were uniquely mapped to the genome (**Table 1**).

Table 1. Summary of RNA-seq mapping results

Samples Name	Condition	Biological repeat	Paired-end reads number*	Total reads number	Reads mapped		Reads mapped once		Multiple reads mapped		Reads unmapped	
					Counts	%	Counts	%	Counts	%	Counts	%
93_HISAT.log	Control	1	29472882	58945764	57790583	98.04	53594519	90.92	4196064	7.12	1155181	1.96
94_HISAT.log	Control	2	23544089	47088178	46138319	97.98	42833163	90.96	3305156	7.02	949859	2.02
95_HISAT.log	Control	3	19409507	38819014	38037868	97.99	35417371	91.24	2620497	6.75	781146	2.01
96_HISAT.log	Control	4	27239503	54479006	53297681	97.83	49422708	90.72	3874973	7.11	1181325	2.17
97_HISAT.log	WS	1	23791904	47583808	46553001	97.83	43348835	91.10	3204166	6.73	1030807	2.17
98_HISAT.log	WS	2	24714093	49428186	48429363	97.98	45062951	91.17	3366412	6.81	998823	2.02
99_HISAT.log	WS	3	26927181	53854362	52741109	97.93	49009845	91.00	3731264	6.93	1113253	2.07
100_HISAT.log	S-	1	26480476	52960952	51878974	97.96	48256174	91.12	3622800	6.84	1081978	2.04
102_HISAT.log	S-	2	32946979	65893958	64413039	97.75	60043746	91.12	4369293	6.63	1480919	2.25
103_HISAT.log	S-	3	17954537	35909074	34919289	97.24	32624989	90.85	2294300	6.39	989785	2.76
104_HISAT.log	S-WS	1	14679990	29359980	28607498	97.44	26729750	91.04	1877748	6.40	752482	2.56
105_HISAT.log	S-WS	2	17460709	34921418	34052863	97.51	31937127	91.45	2115736	6.06	868555	2.49
106_HISAT.log	S-WS	3	24825088	49650176	48496745	97.68	44973351	90.58	3523394	7.10	1153431	2.32
107_HISAT.log	S-WS	4	23656577	47313154	46134451	97.51	43117634	91.13	3016817	6.38	1178703	2.49

* after adapters trimming and low quality reads filtering

A protocol was developed based on Pertea *et al.*, (2016) to annotate new transcripts. New transcript assembly was performed using StringTie 1.2.2 with default parameters and with a file containing reference gene models as a guide. For each library, a StringTie prediction was made based on HISAT2 mapping files. Assemblies from the different samples were then merged using the StringTie merge function. The Gffcompare v0.9.9e software

(https://github.com/gpertea/gffcompare) was used to classify the predicted transcripts compared to the reference, and only new transcripts were kept (class code U, i.e. the predicted transcript is intergenic in comparison to known reference transcripts). To remove potential transposable elements (TEs) from our set of new assembled transcripts, transcript and TE coordinates were compared using the Bedtools intersect v2.26.0 software (Quinlan and Hall, 2010) with the option --wao. Transcripts with more than 50% coverage with a TE were removed from our data set. Long non-coding transcripts were identified using the FEElnc v0.1.0 software (Wucher *et al.*, 2017) with default parameters. When a coding protein gene had at least one of its alternative transcripts identified as a long non-coding RNA (lncRNA), all alternative sequences were mapped against the protein databases of Medicago truncatula v4 (http://www.medicagogenome.org/) and Arabidopsis thaliana TAIR10 (https://www.arabidopsis.org/). When a match (e-value<0.001) was obtained for at least one of the transcripts, the gene and its alternative transcripts were considered as coding RNAs. Coding transcripts were translated into proteins using the load, longestorf, and translate commands from the JCVI toolbox (Tang *et al.*, 2015). The entire dataset was mapped against the *Medicago truncatula* proteome (v4) and the Arabidopsis thaliana proteome (TAIR10) using Blastp with an e-value of 0.001. For each species, the best hit for both coverage and identity was selected. Protein domain characterization and assignment of gene ontology (GO) terms were performed using InterProScan5 (Jones *et al.*, 2014). Mapped reads were assigned to the pea reference genome v1 enriched with the new transcripts using FeatureCounts v1.5.0-p3 (Liao *et al.*, 2014). Only paired reads that mapped once and that were correctly mapped on the same chromosome were counted (-p -B -C). Reads were assigned to their overlapping meta-features. Counts of genes were pre-filtered to keep only rows that had at least 10 reads total. Pairwise differential gene expression between control and stress treatments was obtained using the DESeq2 R package (1.12.3 version) (Love *et al.*, 2014). Genes were considered as differentially expressed when the false discovery rate (FDR)-adjusted P-value was ≤0.05. GO term enrichment analysis was conducted using the TopGO R package (2.34.0 version; Alexa and Rahnenfuhrer, 2018), with the Elim method and Fisher's exact test (P<0.001).

RT-qPCR

Samples of 5 µg of DNAse-treated RNA were reverse-transcribed with an iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's protocol in a final volume of 20 µl. Quantitative real-time PCR was carried out on a LightCycler 480 apparatus (Roche) as described by Noguero *et al.*, (2015) with GoTaq qPCR Master Mix (Promega), using 3 µl of 50× diluted cDNA and 0.2 µM of each primer in a final volume of 10 µl. Reactions were performed in duplicates from each biological replicate. Expression levels relative to the housekeeping reference genes Actin (Psat5g063760) and Histone (Psat6g056720) were calculated using the ΔΔCT method (Schmittgen and Livak, 2008). Primers used for the reference genes and deregulated genes are given in **Supplementary Table S2**.

Statistical analyses

Statistical analyses of phenotypic and physiological data, 1-DE gel-derived protein data, and CNS content, quantities, and partitioning were conducted using Statistica Version 7.0 (StatSoft). ANOVAs followed by Student–Newman–Kheul (SNK) post hoc tests were used to determine significant differences ($P<0.05$, except for leaf water potential where $P<0.1$).

RESULTS

Sulfur deficiency combined with water stress dramatically affects seed yield components

To investigate the interplay between S nutrition and the response to water stress, *Pisum sativum* plants (cv. 'Caméor') deprived of S from the mid-vegetative stage (to minimize vacuolar storage of sulfate) were subjected to a moderate water stress (WS) for 9 d during the early reproductive phase, i.e. at flowering of the second or third reproductive nodes (S–WS treatment, **Figure 16A**). Control plants (well-watered, in non-limiting S conditions) and plants subjected to individual stresses (WS or S–) were examined for comparison. Leaf water potential measurements confirmed that the water stress was moderate (–1.3 MPa, **Figure 16B**). The effect of individual and combined stresses was evaluated at maturity by measuring plant, pod, and seed characteristics (**Table 2**). Water stress did not significantly affect seed yield, although there was a slight decrease in individual seed weight and in the number of

reproductive nodes. In contrast, S deficiency alone or combined with water stress significantly decreased yield by 38% and 65%, respectively. The stronger impact of the combined stress could be explained by a marked decrease of both individual seed weight (–34%) and seed number per plant (–48%). In addition, the combined stress increased seed abortion (+58% compared to the control), leading to fewer seeds per pod. These effects were not observed in response to the individual stresses, thus demonstrating the dramatic effect of S starvation combined with moderate water stress on seed production. Furthermore, pea plants subjected to the same S deficiency but experiencing a 3-d longer period of water stress (12 d) did not survive: the leaves dried out even though the water stress was moderate (**Figure 17A**).

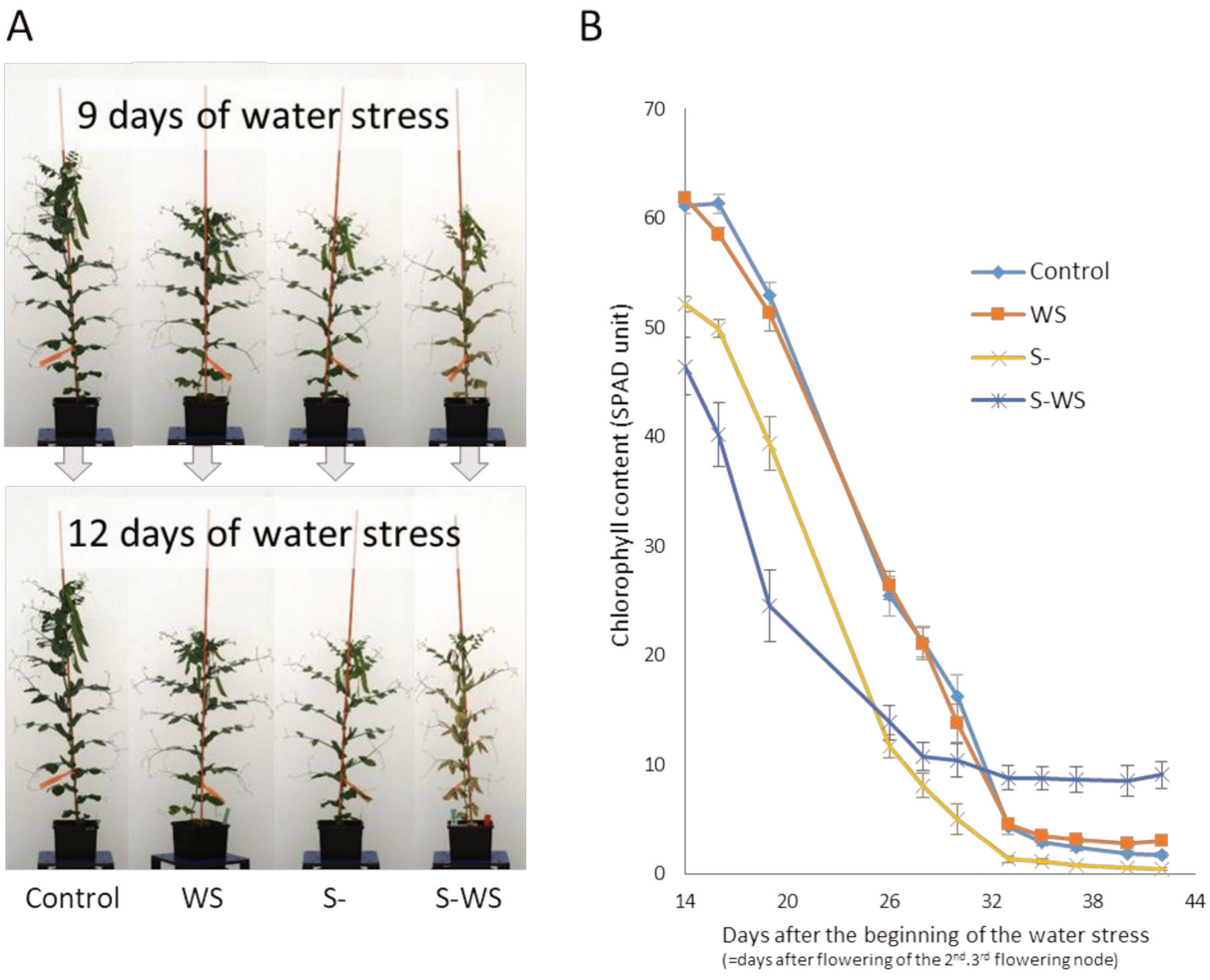


Figure 17. Effect of water stress combined with sulfur deficiency on pea plant phenotype and estimated leaf chlorophyll content. A, Pictures representative of plants after 9 and 12 days of water stress from the beginning of flowering. The same plants were photographed at three-day interval. **B,** Mean chlorophyll content (SPAD unit) in leaves of the first reproductive node during seed maturation. Values are mean ± SE ($7 \leq n \leq 10$). S-, S-deficiency; WS, water stress; S-WS, combined stress.

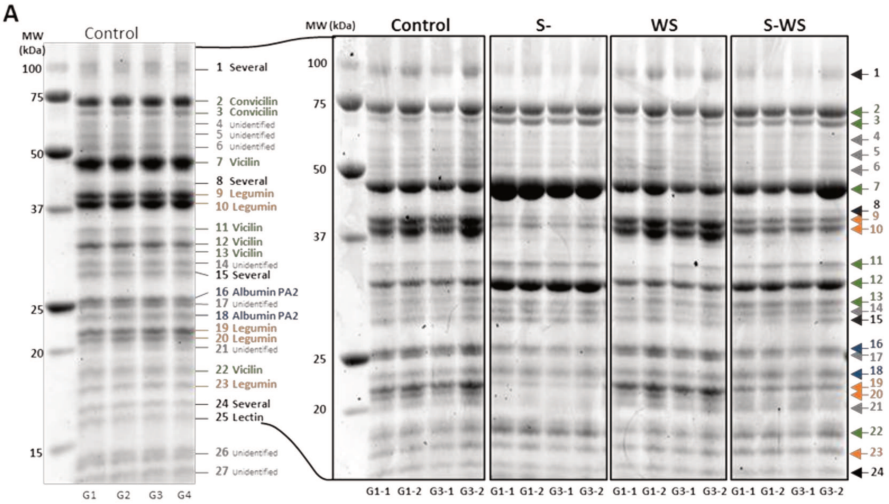
To determine whether the stresses affected early- and late-produced seeds differently, mature seeds were collected in four different groups (G1–G4) depending on (i) the time of pollination of the corresponding flower (before, at the time water stress was imposed, or during the water-stress period) and (ii) whether or not seeds had reached the FSSA 3 d after the end of the water-stress period (see Methods; **Figure 16C**). G1 seeds corresponded to flowering nodes that flowered before the beginning of the water stress period. G2 seeds corresponded to the flower that opened on the day that water stress was imposed. Both G3 and G4 seeds corresponded to flowering nodes that flowered during the water stress period but they could be distinguished according to the FSSA measurements: G3 seeds had reached FSSA 3 d after the end of the water-stress period, while G4 seeds were still in embryogenesis at this time. Plants subjected to the individual or combined stresses produced more nodes carrying G3 seeds and fewer nodes carrying G4 seeds than control plants (**Table 2**), indicating accelerated seed production. Interestingly, in the combined stress treatment, seeds from all reproductive nodes had reached FSSA (i.e. there were no G4 seeds), while seeds from the last reproductive nodes of plants grown in control or single-stress conditions were still in embryogenesis. In addition, all groups of seeds grown under the combination of water stress and S deficiency displayed a significant decrease in individual seed weight compared to the control treatment. Hence, the combined stress negatively affected individual seed weight whatever the developmental stage of the seeds exposed to water stress.

Table 2. Effect of water stress combined or not with S-deficiency on phenotypic characteristics at harvest in pea.

Phenotypic characteristics at maturity	Well-watered								Water-stressed							
	Sulfur supply			Sulfur deficiency					Sulfur supply				Sulfur deficiency			
	Control			S-					WS				S- WS			
	Mean	SE	SNK test	Mean	SE	SNK test	Ratio to Control		Mean	SE	SNK test	Ratio to Control	Mean	SE	SNK test	Ratio to Control
Plant characteristics at harvest																
Seed yield per plant (g)	12.83	0.650	a	7.96	0.471	b	**0.62** ↘	**-38%**	11.63	0.463	a	0.91	4.45	0.547	c	**0.35** ↘ **-65%**
Total plant biomass (g)	23.03	0.798	a	14.67	0.831	b	**0.64** ↘	**-36%**	21.32	0.967	a	0.93	10.46	0.617	c	**0.45** ↘ **-55%**
Harvest index	0.56	0.018	a	0.54	0.011	a	0.97		0.55	0.007	a	0.98	0.42	0.026	b	**0.74** ↘ **-26%**
Number of vegetative nodes	12.86	0.404	a	12.57	0.481	a	0.98		12.60	0.163	a	0.98	12.80	0.200	a	1.00
Number of reproductive nodes	8.86	0.340	a	6.43	0.202	c	**0.73** ↘	**-27%**	7.30	0.213	b	**0.82** ↘ **-18%**	5.70	0.153	d	**0.64** ↘ **-36%**
Number of nodes with pods	6.71	0.244	a	4.86	0.189	c	**0.72** ↘	**-28%**	5.70	0.474	b	**0.85** ↘ **-15%**	4.80	0.211	c	**0.71** ↘ **-29%**
Pod and seed characteristics at harvest (G1 to G4 seeds pooled)																
Seed number per plant	48.57	2.349	a	33.71	1.742	b	**0.69** ↘	**-31%**	48.20	1.718	a	0.99	25.50	2.535	c	**0.53** ↘ **-48%**
Pod number per plant	9.86	0.404	a	7.29	0.421	b	**0.74** ↘	**-26%**	9.40	0.452	a	0.95	7.00	0.298	b	**0.71** ↘ **-29%**
One-seed weight (g)	0.26	0.003	a	0.24	0.005	b	**0.89** ↘	**-11%**	0.24	0.005	b	**0.91** ↘ **-9%**	0.17	0.008	c	**0.66** ↘ **-34%**
Seed number per pod	5.66	0.494	a	4.85	0.216	a	0.86		5.56	0.242	a	0.98	3.24	0.354	b	**0.57** ↘ **-43%**
Number of aborted seeds per pod	2.62	0.521	a	2.24	0.276	a	0.85		2.08	0.245	a	0.79	4.15	0.426	b	**1.58** ↗ **+58%**
Gmax (%)	100	0	a	73.33	26.67	a	0.73		100	0	a	1.00	71.67	14.81	a	0.72
Number of reproductive nodes																
G1	0.71	0.244	a	1.43	0.488	a	2.00		1.10	0.158	a	1.54	1.30	0.242	a	1.82
G2	1.00	0.000	a	1.00	0.000	a	1.00		1.00	0.000	a	1.00	1.00	0.000	a	1.00
G3	1.00	0.408	b	1.43	0.636	ab	1.43		1.60	0.422	ab	1.60	2.30	0.412	a	**2.30** ↗ **+130%**
G4	4.00	0.289	a	0.86	0.535	c	**0.21** ↘	**-79%**	2.00	0.577	b	**0.50** ↘ **-50%**	n/a	n/a	n/a	n/a
Seed number per group																
G1	10.7	1.667	ab	13.5	2.255	a	1.27		10.5	1.443	a	0.98	4.75	0.250	b	0.45
G2	6.75	0.854	a	5.50	0.500	a	0.81		7.00	0.408	a	1.04	2.00	0.408	b	**0.30** ↘ **-70%**
G3	16.5	2.327	ab	11.8	3.924	b	0.71		24.3	2.016	a	1.47	20.3	2.839	ab	1.23
G4	19.8	3.425	a	7.00	n/a	a	0.35		8.67	1.856	a	0.44	n/a	n/a	n/a	n/a
One-seed weight (g)																
G1	0.25	0.009	a	0.24	0.006	a	0.96		0.21	0.012	a	0.85	0.13	0.017	b	**0.54** ↘ **-46%**
G2	0.27	0.009	a	0.24	0.002	a	0.90		0.23	0.013	a	0.85	0.11	0.018	b	**0.42** ↘ **-578%**
G3	0.27	0.002	a	0.24	0.008	a	0.89		0.24	0.012	a	0.90	0.19	0.011	b	**0.69** ↘ **-31%**
G4	0.28	0.005	a	0.23	n/a	b	**0.84** ↘	**-16%**	0.25	0.010	ab	0.90	n/a	n/a	n/a	n/a

The harvest index corresponds to the ratio of seed yield to total dry biomass, and Gmax to the maximum percentage of germinated seeds after 96 hours. The ratios of values for stressed plants versus control plants are indicated. For each trait, different letters indicate significant differences ($P<0.05$, ANOVA followed by a SNK test, $7<n<10$ for all traits except for seed number and weight per group from G1 to G4 where $n=4$). For Gmax determination, G1 to G4 seeds were pooled and 20 seeds per plant (3 plants per condition) were used. The colored bold values indicate whether the ratio decreased (dark blue scale) or increased (red) significantly. When the differences were statistically significant, the percentage of increase or decrease relative to the control is indicated. SE, standard error. Note that no G4 seeds developed on the double-stressed plants. n/a, not applicable.

A



B

Type of regulation	Band number	MW (kDa)	Annotation		Ratio to Control (Relative protein abundance) S- G1	S- G2	S- G3	S- G4	WS G1	WS G2	WS G3	WS G4	S- WS G1	S- WS G2	S- WS G3	S-AA %	Lys %	Trp %
↗ S-WS & S-	3	64	Convicilin	7S	**1.89**	**2.13**	**2.67**	**2.54**	0.83	0.89	1.06	1.06	**1.89**	**1.82**	**2.40**	0.70	7.71	0.53
	7	48	Vicilin	7S	**1.50**	**1.73**	**1.63**	**1.77**	0.83	0.85	0.88	0.82	0.95	0.98	**1.39**	0.86	7.56	0
	11	34	Vicilin	7S	**1.71**	1.58	**2.21**	1.63	0.76	0.84	0.82	0.59	1.37	1.29	**1.98**	0.86	7.56	0
	12	32	Vicilin	7S	**2.49**	**2.27**	**2.43**	**2.50**	1.13	1.10	1.03	0.96	1.76	**1.54**	**1.78**	0.47	6.99	0
	22	19	Several proteins		**2.57**	**2.39**	**2.44**	**2.86**	1.14	1.09	1.21	0.89	1.38	1.29	**2.07**			
	24	17	Several proteins		1.16	**1.36**	1.26	**1.80**	1.09	1.03	0.95	1.01	**1.76**	**1.44**	1.31			
	25	16	Lectin		**4.02**	**3.31**	**3.27**	**3.20**	1.45	1.01	1.06	1.23	**1.88**	**2.24**	**2.22**			
	26	14	unidentified		**2.19**	**2.06**	**2.09**	**2.10**	1.10	0.92	1.12	0.90	**1.39**	**1.57**	**1.60**			
↗ S-WS	14	30	unidentified		1.38	0.79	0.72	0.56	1.50	0.99	0.70	0.89	**2.16**	1.31	1.00			
	18	25	Albumin PA2	2S	1.40	1.00	0.89	0.75	1.27	1.06	0.86	1.07	**1.84**	1.33	1.18	3.03	6.93	0.43
	23	18	Legumin	11S	1.20	1.08	1.19	1.03	1.17	1.09	1.14	1.16	**1.45**	**1.50**	**1.47**	1.40	4.56	0.70
↘ S-WS & S-	6	51	unidentified		**0.33**	**0.39**	**0.37**	**0.22**	0.98	1.09	1.44	1.03	**0.34**	**0.45**	0.73			
	8	43	Several proteins		**0.14**	**0.13**	**0.18**	0.20	1.17	1.21	1.11	1.42	1.28	1.22	**0.47**			
	9	40	Legumin	11S	**0.14**	**0.15**	**0.18**	**0.19**	0.98	0.98	1.02	1.15	**0.67**	**0.68**	**0.43**	2.31	4.24	0.58
	10	38	Legumin	11S	**0.22**	**0.27**	**0.31**	**0.27**	0.96	0.98	0.89	0.99	**0.60**	**0.69**	**0.47**	2.31	4.24	0.58
	16	26	Albumin PA2	2S	**0.56**	**0.52**	**0.52**	0.66	1.07	0.81	0.99	1.04	**0.45**	**0.37**	**0.52**	3.03	6.93	0.43
	19	23	Legumin	11S	**0.23**	**0.17**	**0.20**	**0.11**	1.23	1.31	1.04	1.14	0.81	**0.78**	**0.41**	2.31	4.24	0.58
	20	22	Legumin	11S	**0.16**	**0.05**	**0.09**	**0.08**	1.03	0.75	0.80	1.19	0.60	**0.56**	**0.26**	2.31	4.24	0.58
	27	13	unidentified		**0.32**	**0.44**	**0.51**	**0.25**	1.21	1.29	1.30	1.16	0.45	0.87	0.46			
↘ S-	17	26	unidentified		0.50	0.70	**0.70**	0.61	0.90	1.37	1.18	0.73	0.81	0.85	0.91			
↗ S-WS & ↘ S-	21	21	unidentified		**0.34**	**0.27**	**0.41**	0.23	1.18	1.18	1.29	1.16	1.41	**1.77**	1.06			
Non-regulated by stresses	1	97	Several proteins		1.10	0.56	0.54	0.51	0.99	1.13	1.09	0.89	0.61	0.38	0.48			
	2	70	Convicilin	7S	0.97	0.96	1.01	0.97	0.92	0.95	1.00	0.97	0.98	1.01	0.99	0.48	7.92	0.65
	4	59	unidentified		0.51	0.62	1.37	0.79	0.80	1.03	2.84	1.26	1.10	0.90	2.23			
	5	55	unidentified		0.32	0.42	0.51	0.36	0.63	0.93	2.25	0.73	0.47	0.51	1.54			
	13	31	Vicilin	7S	2.22	1.43	1.47	1.44	1.48	1.18	1.21	1.51	1.80	1.66	1.34	0.47	6.99	0
	15	29	Several proteins		1.24	1.23	1.25	1.27	1.02	1.00	0.93	0.85	1.28	1.19	1.23			
				2S	0.89	**0.72**	**0.69**	0.71	1.15	0.91	0.93	1.05	0.99	0.78	0.83			
				7S	**1.57**	**1.63**	**1.66**	**1.69**	0.91	0.93	0.96	0.91	**1.17**	**1.16**	**1.42**			
				11S	**0.23**	**0.24**	**0.28**	**0.24**	1.03	1.01	0.96	1.09	**0.70**	**0.73**	**0.48**			
			All globulins:	11S + 7S	0.94	0.97	0.99	0.99	0.97	0.97	0.96	1.00	0.95	0.96	0.96			
				7S/11S	**7.81**	**7.57**	**6.25**	**7.06**	0.89	0.92	1.01	0.84	1.69	1.66	3.01			

Figure 18. Effects of water stress combined with S deficiency on protein composition of mature pea seeds from seed groups G1–G4. (A) Protein profiles of mature seeds from plants with or without stresses. Representative one-dimensional electrophoresis (1-DE) protein patterns for mature control seeds from G1–G4 are shown on the left. The 27 individual bands that were detected and quantified in all the seed groups are shown. Protein annotation refers to major proteins in each band, according to the Normalized Weighted Spectra counts (Supplementary Table S1). Six protein bands corresponded to 7S globulins (vicilins and convicilin, in green) and five to 11S globulins (legumins, in orange). One 1-DE gel illustrating qualitative changes in the accumulation of the 7S and 11S globulins between the different treatments is shown on the right for two biological replicates of G1 (G1-1, G1-2) and G3 (G3-1, G3-2). **(B)** Quantitative variations in response to stresses. Significant changes in the relative abundance of the protein bands are shown for each group of seeds compared to the control. The ratio of values for stressed plants versus control plants is indicated for each group. Note that no G4 seeds could be harvested for S–WS plants. The 7S/11S ratio corresponds to the relative quantity of 7S divided by that of 11S. The colors indicate whether the ratio decreased (grey scale) or increased (red scale) when values were significantly different to that of control seeds, as determined by ANOVA followed by a SNK test ($P<0.05$, $n=4$ plants). The percentage of amino acids in the sequence of storage-proteins is indicated (S-AA, sulfur amino acids, methionine and cysteine). S–, S deficiency alone; WS, water stress alone; S–WS, combined stresses.

Seed protein composition is less affected by combined stress than by S deficiency alone

To examine the influence of the individual and combined stresses on seed protein composition, total proteins of mature seeds of each group (G1–G4) in the different treatments were separated by 1-DE. A total of 27 individual bands were detected and quantified (**Figure 18A**), and 19 could be annotated following nanoLC-MS/MS analyses (**Supplementary Table S1**). The relative abundance of each protein band for each group of seeds is given in **Annex 3** and expressed as a ratio to the control in **Figure 18B**. These data showed that the short and moderate water stress alone did not significantly modify seed protein composition. In contrast, S deficiency drastically affected the accumulation of major proteins (i.e. storage proteins), as seen in **Figure 18A**. As expected, the major proteins whose relative abundance increased in response to S deficiency corresponded to 7S globulins (vicilins and convicilins, 0.47–0.86% S-AA in their sequence), and the major proteins whose relative abundance decreased corresponded to 11S globulins (legumins, 2.31% S-AA) and 2S albumins (3.03% S-AA). This resulted in a significantly higher 7S/11S ratio in G1–G4 seeds from S– plants compared to the control (by 6.25- to 7.81-fold). Interestingly, fewer differences in the accumulation of 7S and 11S globulins were observed when S deficiency was combined with water stress, especially for G1 seeds (**Figure 18**), and the 7S/11S ratio was not significantly affected by the combined stress (**Figure 18B, Annex 3**). These data clearly demonstrated that the storage protein composition of all groups of seeds was much less affected by the combined stress than by S deficiency alone. The possible impact of these changes on seed germination capacity was investigated using three biological replicates of seeds pooled from all the groups G1–G4. Although the results were not statistically significant, the maximum percentage of germinated seeds after 96 h of imbibition (Gmax, **Table 2**) was lower for S– and S–WS plants compared to control seeds, which achieved 100% germination after 68 h.

Nitrogen/Sulfur balance in seeds subjected to S deficiency and/or water stress

The mitigating effect on seed globulins of the combined stress compared to S deficiency alone prompted us to measure the S, N, and C concentrations in the same seed samples. The absolute quantity of each element per seed was calculated as well as the N/S and C/N ratios (**Annexes 4 and 5, summarized in Table 3**). In mature pea seeds, N is predominantly stored in

the form of proteins (N content multiplied by 5.4 represents the protein content according to (Mariotti *et al.*, 2008) while C is mainly stored as starch (Bastianelli *et al.*, 1998). Hence, the variations observed in this study for seed N and C contents reflected modifications in protein and starch contents in the seeds. The data indicated that the moderate water stress did not result in major changes in the SNC contents (%) and quantities (mg seed–1). In contrast, S deficiency alone or combined with water stress significantly decreased the S content and quantity per seed for all groups, and increased the N content in seeds in groups G1–G3. Interestingly, the N and C quantities per seed did not vary in response to S deficiency alone, whereas they decreased significantly in response to the combined stress (**Table 3**).

Table 3. SNC content, quantity, N/S and C/N ratios in mature seeds from G1 to G4.

SNC measurements in seeds	Ratio to Control										
	S-				WS				S-WS		
	G1	G2	G3	G4	G1	G2	G3	G4	G1	G2	G3
Content (%)											
Sulfur	**0.31**	**0.30**	**0.31**	**0.30**	1.05	**0.92**	0.99	1.08	**0.58**	**0.50**	**0.40**
Nitrogen	**1.21**	**1.16**	**1.12**	1.07	1.04	0.96	1.00	1.03	**1.32**	**1.23**	**1.14**
Carbon	1.00	0.99	**0.99**	**0.99**	1.01	1.00	1.00	1.00	0.99	1.00	**0.98**
Absolute quantity in one seed (mg)											
Sulfur	**0.29**	**0.27**	**0.28**	**0.25**	0.89	**0.78**	0.89	0.97	**0.31**	**0.22**	**0.28**
Nitrogen	1.17	1.05	0.99	0.89	0.89	0.82	0.90	0.92	**0.70**	**0.51**	**0.79**
Carbon	0.96	0.90	0.88	0.82	0.86	0.85	0.90	0.90	**0.53**	**0.42**	**0.68**
N/S	**4.04**	**3.91**	**3.61**	**3.65**	0.99	1.05	1.00	0.96	**2.30**	**2.49**	**2.86**
C/N	**0.83**	**0.85**	**0.88**	0.92	0.97	1.04	1.00	0.98	**0.75**	**0.81**	**0.86**

The ratios of values obtained for stressed plants versus control plants are indicated for each group of seeds from G1 to G4. Bold and color values were significantly different compared to that of control plants ($P<0.05$, ANOVA, SNK test, n=4 plants). The colors indicate whether the ratio decreased (grey scale) or increased (dark red scale). N/S and C/N ratios correspond to the absolute quantity of nitrogen (N) or carbon (C) divided by that of sulfur (S) or N. S-, Sulfur-deficiency alone; WS, water stress alone; S-WS, combined stress.

Therefore, it is likely that the seeds of plants subjected to the combined stress had a reduced N and C demand compared to S– plants, which was consistent with their lower weight (–11% in S– versus –34% in S–WS, **Table 2**). Consequently, the seed N/S ratio only increased by 2.5-fold in response to the combined stress, whereas it increased by 4-fold in response to S deficiency (**Table 3**). Significantly, the seed N/S ratio was strongly correlated with the 7S/11S

ratio (R=0.9, P<0.001, **Figure 19**), suggesting that the N/S ratio can be used as an indicator of globulin composition in pea seeds.

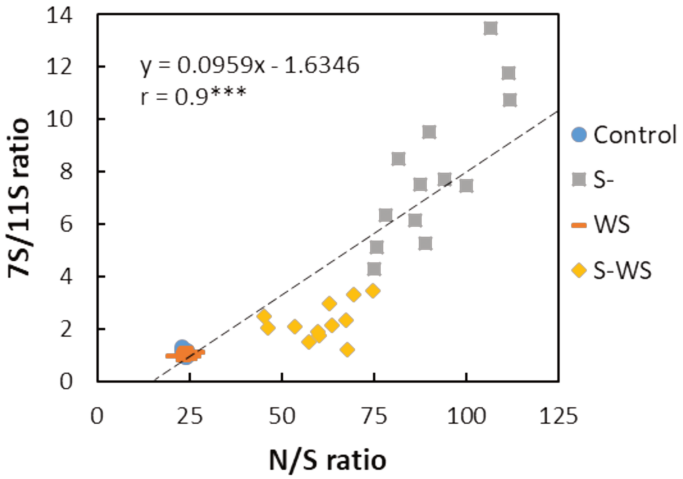


Figure 19. Correlation between the N/S and 7S/11S ratios in mature pea seeds from G1 to G4. The N/S ratio was calculated from N and S quantities per seed and the 7S/11S ratio was calculated from the relative quantity of 7S and 11S globulins. ***, *P*<0,001 (Pearson Correlation, n=4 plants per condition). S-, S-deficiency ; WS, water stress ; S-WS, combined stress.

Differences in nutrient allocation between tissues of stressed plants

To link the seed characteristics with nutrient content and partitioning in different plant parts, SNC contents were measured in pod walls, roots, and reproductive and vegetative plant parts (**Figure 20A**), and the absolute quantity (**Figure 20B–D**) and partitioning (**Figure 20E–G**) of each element was calculated. There was a sharp decrease in S content and total S quantity in all compartments of the plants deprived of S (**Figure 20A, B**) and the partitioning data indicated a reduced proportion of S in roots of these plants (**Figure 20E**), suggesting a major utilization of the root S reserves. In contrast, the proportion of S in pod walls increased significantly, suggesting a higher transfer of S into the pod walls and/or defects in S remobilization from them to the seeds (**Figure 20E**). Specifically, when S deficiency was combined with water stress, the proportion of N and C accumulated in the seed compartment decreased while it increased in pod walls, roots, and vegetative plant parts (**Figure 20F, G**). These results reflected a decreased allocation of N and C to seeds in response to the combined stress. In support of this, higher chlorophyll contents were observed in leaves of the combined-stressed plants by the end of the reproductive phase (**Figure 17B**), suggesting later senescence.

A

Nutrient content and ratio in the different compartment	Well-watered							Water-stressed						
	Sulfur supply			Sulfur deficiency				Sulfur supply				Sulfur deficiency		
	Control			S-				WS				S-WS		
	Mean	SE	SNK test	Mean	SE	SNK test	Ratio to Control	Mean	SE	SNK test	Ratio to Control	Mean	SE	SNK test	Ratio to Control
Sulfur Content (%)															
Seeds	0.19	0.001	a	0.05	0.003	c	0.29 ↘	0.19	0.003	a	0.99	0.08	0.002	b	0.42 ↘
Pod walls	0.07	0.002	a	0.04	0.003	c	0.54 ↘	0.06	0.008	ab	0.88	0.05	0.003	bc	0.66 ↘
Reproductive plant parts	0.23	0.023	a	0.05	0.006	c	0.23 ↘	0.18	0.035	a	0.80	0.07	0.001	c	0.30 ↘
Vegetative plant parts	0.29	0.030	a	0.06	0.002	b	0.20 ↘	0.26	0.026	a	0.91	0.05	0.004	b	0.18 ↘
Roots	0.57	0.066	a	0.07	0.003	b	0.13 ↘	0.64	0.034	a	1.13	0.07	0.002	b	0.12 ↘
Nitrogen Content (%)															
Seeds	4.54	0.036	b	4.96	0.081	a	1.09 ↗	4.48	0.073	b	0.99	5.20	0.128	a	1.15 ↗
Pod walls	0.38	0.012	a	0.38	0.049	a	1.02	0.35	0.008	a	0.92	0.88	0.158	b	2.33 ↗
Reproductive plant parts	1.33	0.052	a	1.02	0.070	a	0.77	1.19	0.076	a	0.90	1.21	0.088	a	0.91
Vegetative plant parts	1.60	0.030	a	1.34	0.122	a	0.84	1.48	0.054	a	0.93	1.75	0.190	a	1.10
Roots	3.28	0.035	a	3.08	0.133	a	0.94	2.91	0.173	a	0.89	3.20	0.187	a	0.98
Carbon Content (%)															
Seeds	45.56	0.059	a	45.09	0.094	b	0.99 ↘	45.60	0.040	a	1.00	44.92	0.115	b	0.99 ↘
Pod walls	43.83	0.066	a	44.26	0.203	a	1.01	42.89	0.632	a	0.98	44.16	0.081	a	1.01
Reproductive plant parts	40.27	0.098	a	41.08	0.133	a	1.02	40.66	0.504	a	1.01	41.22	0.446	a	1.02
Vegetative plant parts	42.43	0.063	a	42.50	0.051	a	1.00	42.71	0.106	a	1.01	42.64	0.203	a	1.00
Roots	41.08	0.502	a	37.66	0.211	b	0.92 ↘	38.49	0.426	b	0.94 ↘	38.69	0.844	b	0.94 ↘
N/S															
Seeds	23.96	0.107	c	91.50	6.357	a	3.82 ↗	24.00	0.417	c	1.00	65.67	2.549	b	2.74 ↗
Pod walls	5.15	0.226	b	9.88	1.255	b	1.92	5.67	0.802	b	1.10	19.01	4.376	a	3.69 ↗
Reproductive plant parts	6.00	0.529	b	20.56	3.223	a	3.43 ↗	7.07	0.939	b	1.18	17.97	1.093	a	3.00 ↗
Vegetative plant parts	5.69	0.554	c	23.29	2.453	b	4.09 ↗	5.73	0.332	c	1.01	33.70	4.206	a	5.92 ↗
Roots	5.98	0.637	b	41.40	2.763	a	6.92 ↗	4.62	0.463	b	0.77	45.32	3.635	a	7.58 ↗
C/N															
Seeds	10.03	0.068	a	9.09	0.167	b	0.91 ↘	10.19	0.172	a	1.02	8.65	0.201	b	0.86 ↘
Pod walls	116.78	3.771	a	121.51	15.853	a	1.04	124.34	3.690	a	1.06	58.33	14.867	b	0.50 ↘
Reproductive plant parts	30.49	1.111	a	40.93	2.799	b	1.34 ↗	34.66	2.243	ab	1.14	34.67	2.292	ab	1.14
Vegetative plant parts	26.59	0.493	a	32.44	2.684	a	1.22	28.95	1.059	a	1.09	25.37	3.270	a	0.95
Roots	12.55	0.239	a	12.29	0.453	a	0.98	13.36	0.801	a	1.06	12.20	0.619	a	0.97

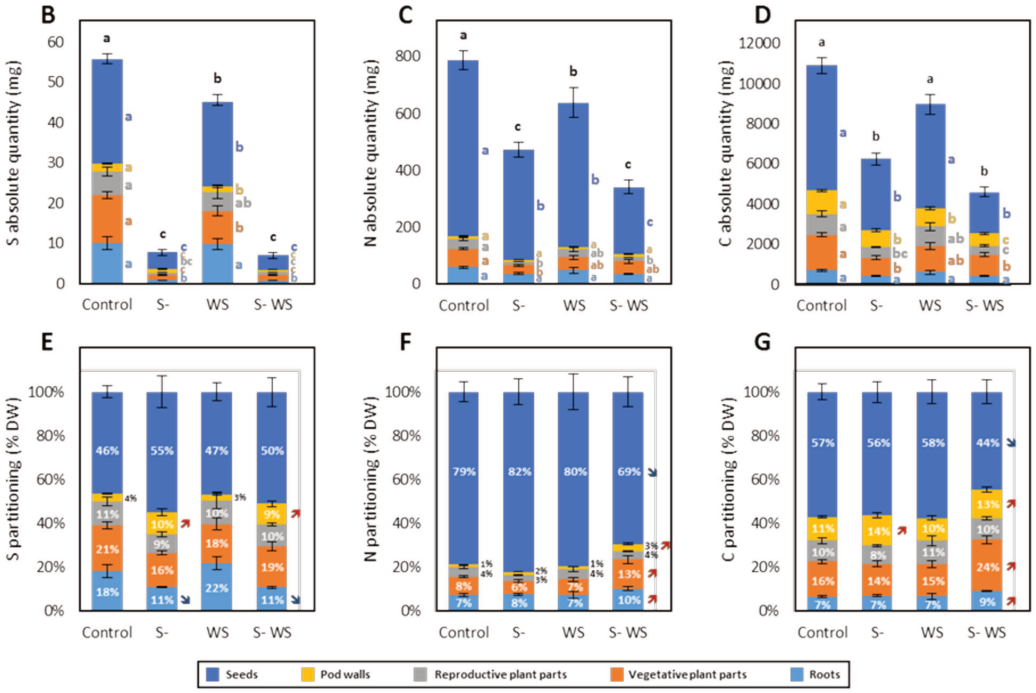


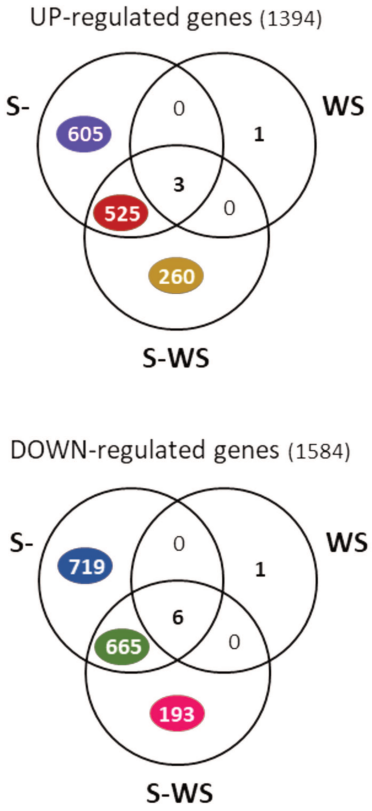
Figure 20. Effects of water stress combined with S deficiency on the accumulation and distribution of N, C, and S in different pea plant compartments at maturity. (A) Sulfur (S), nitrogen (N), and carbon (C) contents (%), and N/S and C/N ratios, in each compartment. **(B–D)** Absolute quantity (mg) of S, N, and C in each compartment. **(E–G)** Proportions of N, C, and S in the dry biomass (% dry weight) of each compartment. Data are means (±SE). Bold and colored values (A), different letters (B–D), or arrows (E–G) indicate significant differences as determined by ANOVA followed by a SNK test (P<0.05, n=4 plants per condition). S–, Sulfur deficiency alone; WS, water stress alone; S–WS, combined stresses. Data are shown for seeds pooled from G1–G4.

Transcriptome changes in developing seeds subjected to drought and/or S deficiency

To gain insights into the molecular processes occurring in developing seeds at the end of the combined stress period, a transcriptome analysis was carried out for G2 seeds that experienced the combined stress throughout their early development (0–9 DAP, **Figure 16**). Samples collected from the different treatments at 9 DAP were subjected to paired-end RNA-seq. Between 15–33 million paired-end reads were generated per sample after adapter trimming and filtering of low-quality reads, and 91% were uniquely mapped to the pea reference genome (**Table 1**). Of the 28 100 genes expressed in the seed samples, 2976 were differentially expressed in response to the stresses (P-adjust<0.05). Annotation, sequences, and expression values of these differentially expressed genes (DEGs) are given in **Supplementary Table S2**. The reliability of the Illumina RNA-seq data was validated for 11 genes using quantitative RT-PCR. A strong correlation between the RNA-seq and RT-qPCR data was obtained (R^2=0.97), thus validating our differential analysis (**Annex 6**). In total, 1394 and 1584 genes exhibited significantly higher and lower expression, respectively, in stress conditions compared to the control (**Fig. 21A**), and about 40% of the transcriptomic changes (1199 out of 2976 DEGs) were shared between the S– and S–WS treatments. Only two genes (out of 2976) had opposite regulation in response to the different stresses: a ferritin gene (Psat7g247120) was down-regulated in response to S deficiency but up-regulated in response to the combined stress, while a gene encoding an unknown protein (Psat5g068840) was up- and down-regulated in S– and S–WS conditions, respectively (**Supplementary Table S2**). Interestingly, the number of DEGs was significantly higher in S– (2523 genes) compared to S–WS (1652 genes), indicating important transcriptional modifications in response to S deficiency alone (**Figure 21A**). In contrast, moderate water stress did not induce a strong reprogramming of the transcriptome in developing seeds since only 11 genes were differentially expressed in the WS treatment (four genes induced and seven repressed; **Figure 21A, Supplementary Table S2**). Among the genes down-regulated by water stress were two legumins (Psat0s1923g0200 and Psat6tg055080), which were also down-regulated in the S– and S–WS treatments. In total, eight globulins were among the DEGs: their expression decreased in response to S deficiency with or without water stress (**Annex 7**). Further examination of the RNA-seq data revealed 21 globulin transcripts expressed at low levels

(**Annex 7**), indicating that, under our conditions, the G2 seeds at 9 DAP were at a transition stage towards storage protein accumulation.

A



B

	GO Term	Description	Annotated	Significant	*P*-value
UP-REGULATED GENES		Response to S- only (605 genes)			
	GO:0055114	Oxidation-reduction process	1913	70	1.3E-06
	GO:0043086	Negative regulation of catalytic activity	144	12	3.1E-05
		Response to S- and S-WS only (525 genes)			
	GO:0043086	Negative regulation of catalytic activity	144	17	1.1E-10
	GO:0006508	Proteolysis	885	37	4.9E-08
	GO:0055114	Oxidation-reduction process	1913	60	4.1E-07
	GO:0009086	Methionine biosynthetic process	10	4	1.1E-05
	GO:0006791	Sulfur utilization	7	3	1.3E-04
	GO:0019419	Sulfate reduction	2	2	2.4E-04
	GO:0008272	Sulfate transport	23	4	4.1E-04
		Response to S-WS only (260 genes)			
	GO:0016925	Protein sumoylation	3	2	1.4E-04
	GO:0006355	Regulation of transcription, DNA-templated	1452	21	8.0E-04
DOWN-REGULATED GENES		Response to S- only (719 genes)			
	GO:0006412	Translation	717	63	6.1E-21
	GO:0006879	Cellular iron ion homeostasis	6	4	3.6E-06
	GO:0006826	Iron ion transport	13	4	1.5E-04
	GO:0009765	Photosynthesis, light harvesting	24	5	1.6E-04
	GO:0015979	Photosynthesis	145	15	3.6E-04
	GO:0042254	Ribosome biogenesis	91	8	9.9E-04
		Response to S- and S-WS only (665 genes)			
	GO:0006412	Translation	717	79	1.1E-24
	GO:0009765	Photosynthesis, light harvesting	24	12	1.2E-13
	GO:0042180	Cellular ketone metabolic process	562	37	1.1E-07
	GO:0044106	Cellular amine metabolic process	394	27	2.8E-06
	GO:0015979	Photosynthesis	145	25	8.7E-06
	GO:0009309	Amine biosynthetic process	183	16	1.7E-05
	GO:0006526	Arginine biosynthetic process	17	5	4.8E-05
	GO:0006414	Translational elongation	54	8	5.8E-05
	GO:0009082	Branched-chain amino acid biosynthesis	20	5	1.1E-04
	GO:0042026	Protein refolding	12	4	1.7E-04
	GO:0015995	Chlorophyll biosynthetic process	26	5	4.2E-04
	GO:0005978	Glycogen biosynthetic process	17	4	7.3E-04
		Response to S-WS only (193 genes)			
	GO:0006801	Superoxide metabolic process	23	3	4.2E-04

Figure 21. Effect of water stress combined with S deficiency on the transcriptome of pea seeds from group 2 (G2) at 9 d post-pollination. (A) Venn diagrams showing the numbers of up- and down-regulated genes in response to stresses compared to the control. **(B)** GO term enrichment analysis for the differentially expressed genes in response to stresses. Terms in the 'Biological Process' category are shown for genes up- and down-regulated in response to the different stresses. For each term, the number of genes present in the genome ('annotated'), the number of genes present in the gene lists ('significant'), and the associated *P*-value (Fisher's test, threshold of $P<0.001$) are given. S–, S deficiency alone; WS, water stress alone; S–WS, combined stress.

Biological processes regulated in developing seeds in response to stresses

A GO enrichment analysis (Elim method/Fisher's test, P<0.001) of the DEGs indicated that several biological processes were similarly enriched in the list of genes responding specifically to S– and in the list of genes responding to both S– and S–WS (**Figure 21B, Supplementary Table S3**). These were related to 'Translation' and 'Photosynthesis' (down-regulated genes), and to 'Oxidation reduction processes' and 'Negative regulation of catalytic processes' (up-regulated genes). This indicated that similar biological processes were regulated in response to S deficiency alone and to the combined stress, but that these processes were regulated to a greater extent under S deficiency alone.

GO terms specifically enriched in the set of genes up-regulated in response to S– and S–WS only (525 genes) were related to proteolysis, and S metabolism and transport (**Fig. 21B**). These data and our observations that mature seeds produced under S– or S–WS conditions had different N/S ratios prompted us to examine the transcriptional regulation of genes for S and N transport and metabolism in response to the stresses. The expression of genes for 20 of the 26 enzymes in **Figure 22** varied in response to S deficiency and/or to the combined stress, and the fold-change in expression was in most cases higher in response to S deficiency. Most of the genes involved in nitrate reduction and assimilation, such as nitrate reductase and glutamine synthetase, and in S metabolism, such as adenosine 5´-phosphosulfate (APS) reductase and methionine synthase, were up-regulated in response to S deficiency with or without water stress. One exception was the first enzyme of sulfate reduction (ATP sulfurylase 1, ATPS1), the expression of which was down-regulated under both conditions, probably because of the low amounts of sulfate in S–WS and S– seeds. In connection with this, the expression of a sulfite oxidase, which synthesizes sulfate from sulfite, increased in these seeds, reflecting a need to provide sulfate from other S sources. Genes encoding sulfate, nitrate, and amino acid transporters were differentially expressed in response to the stresses (**Figure 22**). The most up-regulated was the homolog of AAP8 (Psat1g164680), which in Arabidopsis plays a role in supplying the developing embryo with amino acids (Schmidt *et al.*, 2007).

Interestingly, the GO terms 'Protein Sumoylation' and 'Regulation of Transcription' were over-represented in the list of genes up-regulated in response to the combined stress (260 genes, **Figure 21B**). This suggested the establishment of translational and transcriptional regulation

processes in response to the combined stress. A list of the genes encoding transcription factors (TFs) and SUMO-ligases specifically induced in the S–WS treatment is given **in Supplementary Table S4**. The closest Arabidopsis homologs of the pea TFs are mainly involved in embryogenesis, seed development/filling, and stress responses, and four of them have been described as being involved in abscisic acid (ABA)-related processes, including ABA Insensitive 5 (ABI5).

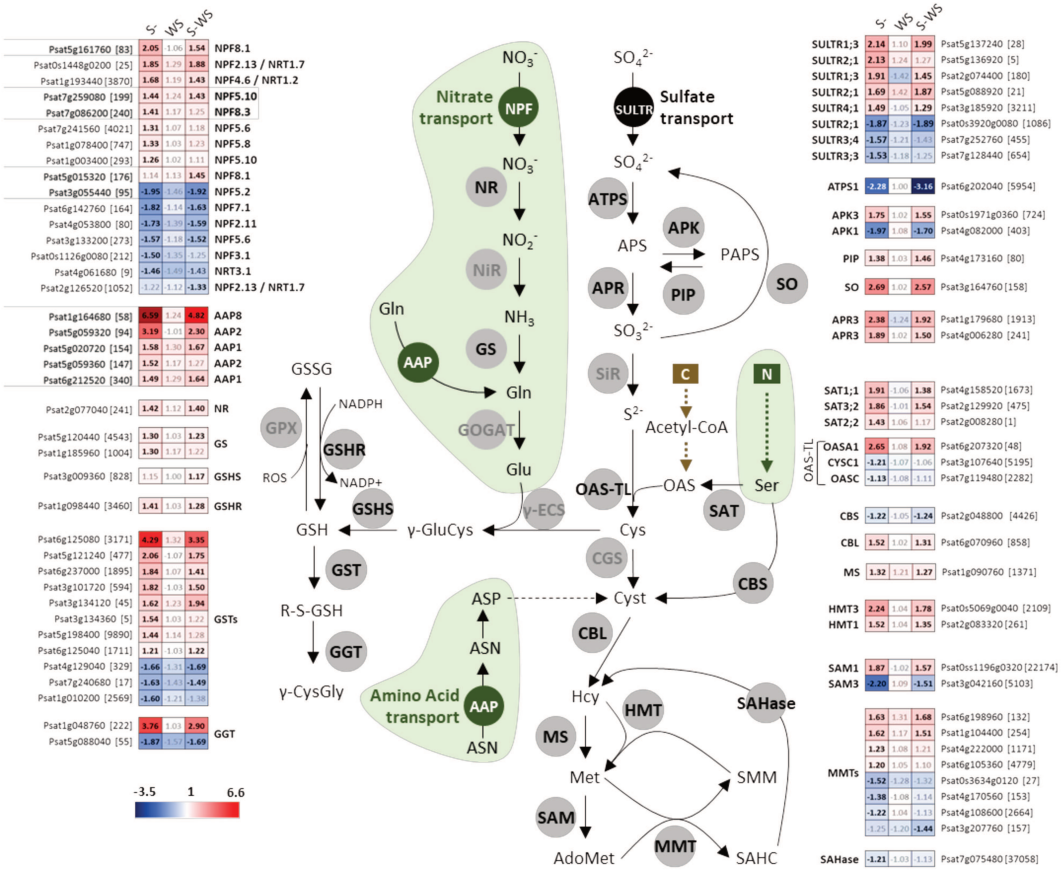


Figure 22. Effects of water stress combined with S deficiency on the expression of genes related to S and N transport and metabolism in pea. For each gene, the fold-change in expression under stress conditions compared to the control is given. The values are color-coded from low expression in blue to high expression in red, and values and enzymes in bold indicate significant differences between stress and control conditions. S–, S deficiency alone; WS, water stress alone; S–WS, combined stresses. Gene annotation refers to the closest *Arabidopsis thaliana* homolog (Supplementary Table S3). For each gene, the mean expression level under control conditions (expressed in counts) is given in square brackets. Enzymes: APK, APS kinase; APR, APS reductase; ATPS, ATP sulfurylase; CBL, cystathionine β-lyase; CBS, cystathionine β-synthase; CGS, cystathionine γ-synthase; γ-ECS, γ-glutamylcysteine synthase; GGT, γ-glutamyl transferase; GOGAT, glutamate synthase; GS, glutamine synthetase; GSHS, glutathione synthetase; GSHR, glutathione reductase; GPX, glutathione peroxydase; GST, glutathione S-transferase; HMT; homocysteine-*S*-methyltransferase; MMT, S-adenosylmethionine methyltransferase; MS, methionine synthase; NiR, nitrite reductase; NR, nitrate reductase; OAS-TL, OAS thiol-lyase; PIP, 3´(2´),5´-bisphosphate nucleotidase; SAHase, *S*-adenosylhomocysteine hydrolase; SAM, *S*-adenosylmethionine synthetase; SAT, serine acetyltransferase; SiR, sulfite reductase; SO, sulfite oxidase. Metabolites: AAP, amino acid permease; AdoMet, *S*-adenosylmethionine; APS, adenosine 5´-phosphosulfate; ASN, asparagine; ASP, aspartate; Cys, cysteine; Cyst, cystathionine; Gln, glutamine; Glu, glutamate; γ-GluCys, γ-glutamylcysteine; γ-CysGly, γ-cysteinylglycine; GSH, glutathione; GSSG, glutathione disulfide; Hcy, homocysteine; Met, methionine; OAS, O-acetylserine; PAPS, 3´-phoshoadenosine 5´-phosphosulfate; SAHC, *S*-adenosylhomocysteine; SMM, S-methylmethionine. Transporters: AAP, amino acid permeases; NPF, nitrate peptide transporter family; SULTR, sulfate transporters (green shading indicates N transport and metabolism).

DISCUSSION

This study investigated the effects of two major constraints for crop growth and yield that are expected to occur increasingly in the near future in the context of climate change and low fertilizer input, namely water stress and S deficiency. The combined stress was applied during the flowering period, a key phase during which seed yield and quality are both established (Ney *et al.*, 1994). By comparing nutrient contents and partitioning with changes in seed yield components and protein composition, we have formulated an integrative view of the effects of the interaction between water stress and S deficiency in pea (**Figure 23**). Moreover, a transcriptome analysis of developing pea seeds (9 DAP) revealed the molecular processes occurring at the end of the combined stress period. Our results indicated synergistic and mitigating effects of these two combined abiotic stresses on seed yield components and seed composition, respectively, and revealed genes involved in the early response of pea seeds to these stresses.

Sulfur nutrition helps to maintain yield in pea plants exposed to a moderate water stress episode

Our results showed that short-term and moderate water stress during flowering in pea did not significantly affect seed yield and total plant biomass, although the individual seed weight and the number of reproductive nodes were slightly reduced (**Table 2, Figure 23**). A similar weak response was also observed by Ney *et al.*, (1994), who reported that a short-term drought in pea of about 6 d (with a leaf water potential between –1.1 MPa and –1.4 MPa) did not lead to major developmental changes except for the number of flowering nodes. In contrast, S deficiency negatively affected yield components and seed composition (**Figure 23**), indicating that the amount of S accumulated during the first 3 weeks of plant growth was not sufficient to maintain seed production and quality. The combination of water stress and S deficiency had a synergistic effect on the reduction of seed yield (**Table 2, Figure 23**), leading to a decrease of the harvest index, which suggests failure in the allocation of assimilated photosynthates to seeds (Sinclair, 1998). In addition, S-deficient plants experiencing a water-stress period that was 3 d longer did not survive (**Figure 17**), which demonstrated the critical need to maintain S nutrition in order for pea to maintain yield when facing water stress, even if the stress is moderate.

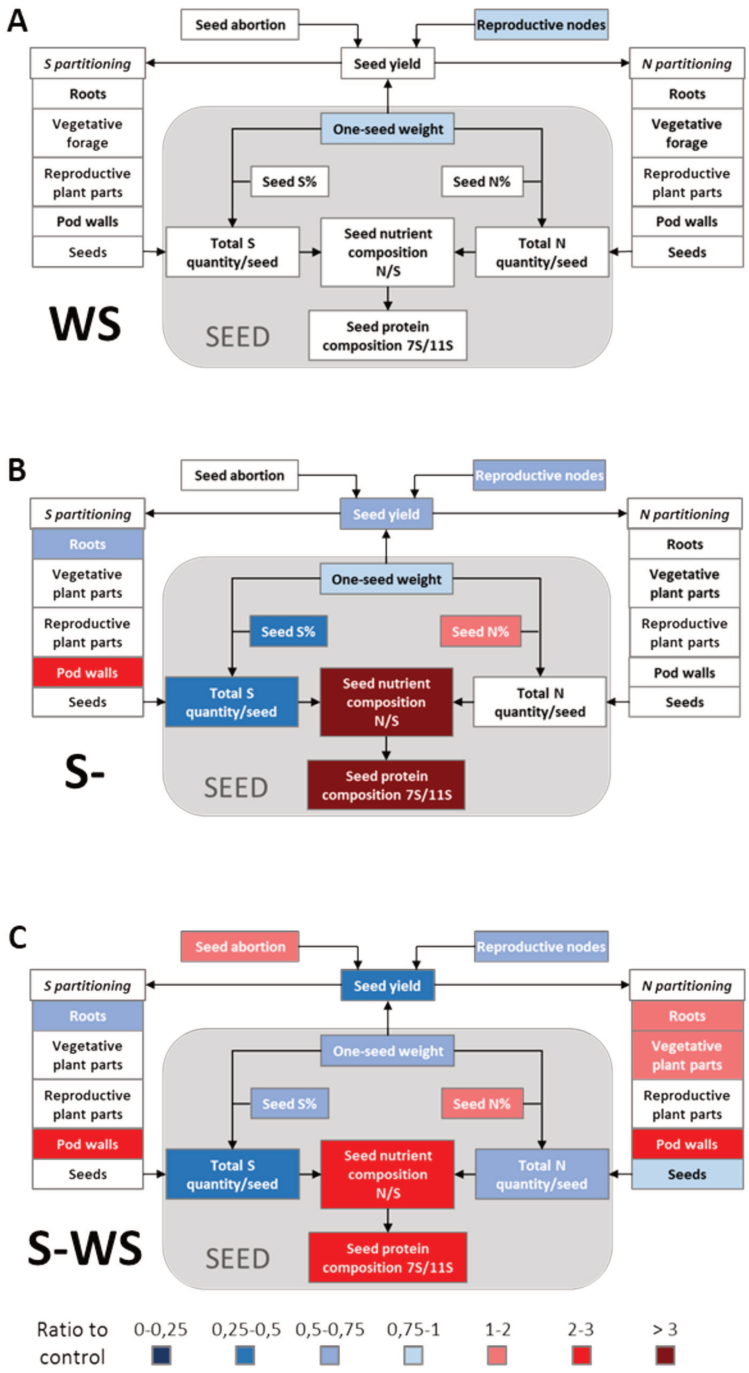


Figure 23. Summary of the effects of water stress with or without S deficiency on yield components, nutrient partitioning, and seed nutrient and protein composition at harvest in pea. **(A)** Summary for water-stress conditions. **(B)** Summary for sulfur-deficient conditions. **(C)** Summary for combined stress condition. The colors correspond to the ratios of values for stressed plants versus control plants, and indicate whether the ratio was significantly decreased (blue) or increased (red). S–, S deficiency; WS, water stress; S–WS, combined stresses.

The strong response of S-deprived pea plants to water stress may be explained by a reduced adaptation to water stress. This could be in part attributed to the role of S in the production of metabolites that play roles in repair (e.g. the methyl donor S-adenosylmethionine), osmoprotection or antioxidation (Chan *et al.*, 2013; Anjum *et al.*, 2015). In particular, detoxification of increased levels of reactive oxygen species (ROS) produced during the drought period (Cruz de Carvalho, 2008) may necessitate an increased production of the ROS-scavenger molecule glutathione, the level of which drastically decreases in plants grown under S deficiency (Nikiforova *et al.*, 2003; Ostaszewska-Bugajska *et al.*, 2015). Alternatively, the response of double-stressed plants may be attributed to the role of sulfate and sulfide in signaling stomatal closure (Ernst *et al.*, 2010; Malcheska *et al.*, 2017; Rajab *et al.*, 2019), possibly by promoting ABA biosynthesis or gating open the anion channel of guard cells (Cao *et al.*, 2014; Malcheska *et al.*, 2017). Plants grown under combined stress conditions could thus be more susceptible to drought due to defects in stomatal closure. In addition, studies in maize have shown that drought can affect the long-distance mobility of sulfate (Ahmad *et al.*, 2016). Such an impairment of the root-to-shoot transport of sulfate might exacerbate the negative effect of S deficiency on the production in double-stressed leaves of sulfate-derived metabolites that are essential for the survival of the plant.

One of the main findings of our study was that the combined stress impeded reproductive development of pea. Double-stressed plants produced fewer reproductive nodes, fewer and smaller seeds, and FSSA was reached earlier in these plants (**Figure 16, Table 2**), reflecting adaptive mechanisms aimed at accelerating seed production to ensure the survival of the species, as previously observed in response to drought (Desclaux and Roumet, 1996) or S deficiency (Hoefgen and Nikiforova, 2008). This was associated with a reduced leaf chlorophyll content during the early reproductive phase (**Figure 17**), an indication of senescence, which is a highly regulated process providing nutrients to newly formed organs, including developing seeds (Guiboileau *et al.*, 2010). The reduced level of leaf chlorophyll could be attributed to a lower production of sulfate-derived molecules and in particular sulfide, which has been shown to prevent autophagy and senescence in Arabidopsis (Álvarez *et al.*, 2012; Dong *et al.*, 2017). By contrast, chlorophyll content remained higher in leaves of the double-stressed plants by the end of the reproductive period (**Figure 17**), presumably because the seed compartment no longer needed to be supplied with nutrients. This was consistent with the nutrient

partitioning between plant organs, especially for C and N, the contents of which remained high in the roots and in the vegetative parts of the plant, and increased in the pod walls, a sign that nutrients could no longer be translocated to seeds (**Figure 20**).

Water stress mitigates the effects of S deficiency on the developing seed transcriptome and rebalances the protein composition in mature seeds

S deficiency decreased the relative abundance of S-rich globulins (11S) in pea seeds (**Figure 18**), as previously described for this species (Chandler *et al.*, 1984; Evans *et al.*, 1985), and for other crops (e.g. wheat, Bonnot *et al.*, 2017; rapeseed, D'Hooghe *et al.*, 2014). This has been shown to be primarily regulated at the transcriptional level (Chandler *et al.*, 1983) and it is compensated by an increase in the S-poor 7S globulins (Chandler *et al.*, 1984, **Figure 18**). Seed N content, which is proportional to seed protein content by a multiplication factor of 5.4 in pea (Mariotti *et al.*, 2008), was significantly higher in seeds from S-deficient plants (**Table 3**). This was also the case for plants subjected to the combined stresses, and hence the conditions used in this study revealed adaptive processes that kept the seed protein content high through the accumulation of 7S globulins. Interestingly, the seed protein composition was less affected by the combined stresses, reflecting a mitigating effect of water stress (**Figures 18, 23**). Similar results have been obtained in oilseed rape, where heat stress during seed filling has been shown to mitigate the negative effect of S deficiency on the S-poor/S-rich globulin ratio (Brunel-Muguet *et al.*, 2015). In our experiments, the S quantity per seed was similarly low in the S-deficient and combined-stress plants, while the N and C quantities per seed specifically decreased in response to the combined stress (**Annex 4, Figure 20, Table 3**). Because of the reduced weight of the seeds from the combined-stress plants, we hypothesized that these seeds required less N and C to be filled, enabling a better balance between N and S compared to seeds of plants deprived of S. Accordingly, the N/S ratio was less affected in seeds of the double-stressed plants compared to S deficiency alone (**Table 3, Annex 4**). Hence, our data demonstrated a lower seed sink strength for N and C but similar seed S uptake, thus rebalancing the seed protein composition (**Figure 23**). We assumed that this would reduce the sensing of S deficiency and mitigate its effect on the developing seed transcriptome. Accordingly, fewer genes were regulated in response to the combined stresses than under S deficiency alone. Moreover, similar biological processes were regulated in response to S

deficiency alone and to the combined stresses, but the extent of the transcriptional regulation was greater under S deficiency than under the combined stresses (**Figure 21**).

In order to identify candidate genes for rebalancing the N/S ratio in response to the double-stresses, we examined the expression of genes related to S and N metabolism and transport in developing seeds that were at a transition stage towards storage-protein accumulation (9 DAP, **Annex 7**). This revealed a tight regulation of these genes by S deficiency with or without water stress (**Figure 22**). In fact, the genes of almost all enzymes of S metabolism, from APS reductase to methionine synthase, were up-regulated in response to these stresses. Three up-regulated genes encoded isoforms of serine acetyltransferase, which catalyses the synthesis of O-acetylserine, the precursor of cysteine, from serine and acetyl-CoA, and thus represents a key enzyme connecting S metabolism with the N and C metabolisms. O-acetylserine has been proposed to act as a signal in the transduction pathways sensing S and N availability (Kim *et al.*, 1999): its accumulation in the siliques of Arabidopsis increases in response to low-S and high-N conditions, and exogenous application of O-acetylserine to immature soybean cotyledons regulates seed storage-protein accumulation in a similar way to S deficiency (i.e. decrease of S-poor globulins; Kim *et al.*, 1999). In our present study, the similar regulation of genes related to N and S metabolism (including serine acetyltransferase) in S– and S–WS seeds strongly suggested that the rebalancing of globulin composition in the double-stressed seeds did not involve specific regulation of these pathways, at least within the seed.

The rebalancing of the seed globulin composition in the double-stressed seeds could be controlled at the transcriptional level through the recognition by specific TFs of motifs in storage-protein promoters (Fujiwara and Beachy, 1994). Interestingly, one of the two GO terms with a significantly high occurrence in the list of genes specifically up-regulated in response to the combined stresses in seeds at 9 DAP was 'Regulation of transcription' (**Figure 21B**). This list of regulators included six TFs with a known role during seed development (**Supplementary Tables S3, S4**), such as ABI5 that is necessary for accumulation of 47-kDa vicilin in mature pea seeds (Le Signor *et al.*, 2017). Studies in common bean (*Phaseolus vulgaris*) suggest that ABI5 interacts with the 7S globulin promoter through a G-box motif (W-K Ng and Hall, 2008), which is essential for beta-phaseolin (7S) accumulation (Pandurangan *et al.*, 2016). The up-regulation of ABI5 in response to the combined stresses was intriguing since vicilins did not accumulate in seeds of the double-stressed plants to as high an extent as they

did under S deficiency alone (**Figure 18**). Hence, the activity of ABI5 might be tightly regulated in these seeds to avoid an over-accumulation of vicilins at the expense of legumins. Interestingly, the second GO term enriched in the list of genes up-regulated in response to the combined stress was 'Protein sumoylation' (**Figure 21B**), and the two genes carrying these GO terms were homologous to AT5G60410 (**Supplementary Tables S3, S4**), a small ubiquitin-related modifier (SUMO) E3 ligase named SIZ1, which has been shown in Arabidopsis to negatively regulate ABI5 activity through sumoylation (Miura *et al.*, 2009). Owing to the co-regulation of SIZ1 and ABI5 in early developing seeds in response to the combined stresses, it is possible that SIZ1 plays a role in controlling the activity of ABI5 under these conditions. In future work, it will be interesting to test whether sumoylation of ABI5 occurs in seeds through SIZ1 to prevent a high accumulation of vicilins under S deficiency in order to maintain the N/S ratio as much as possible when the sink strength for N is reduced, as observed for seeds of the double-stressed plants.

CONCLUSIONS

This study revealed the importance of S for stabilizing seed yield in pea plants facing short and moderate episodes of water stress, and showed that the adaptive responses of S-deprived plants to water stress are much more complicated than a simple additive response. The combined stresses induced pleiotropic effects that were aimed at accelerating seed production, specifically leading to seed abortion, while rebalancing the seed globulin composition, probably as the result of a lower seed sink strength for N. Moreover, combined stress mitigated the impact of S deficiency on the transcriptome of seeds at 9 DAP. The transcriptomics data clearly advance our knowledge of the molecular responses of developing pea seeds to S deficiency occurring either with or without water stress. Candidate genes for fine-tuning the regulation of globulin synthesis under stressed conditions were identified, and our future work will investigate their relevance for potential applications (e.g. for stabilizing the accumulation of S-rich globulins) by reverse genetics using TILLING (Targeting Induced Local Lesions in Genomes) mutants in pea (Dalmais *et al.*, 2008).

DATA DEPOSITION

The MS proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Vizcaíno *et al.*, 2014) with the dataset identifier PXD011029. The raw RNA-seq data have been deposited to the NCBI SRA database (http://www.ncbi.nlm.nih.gov/bioproject/) under accession number PRJNA517587.

SUPPLEMENTARY INFORMATION

Supplementary data are available at JXB online.

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<u>Chapitre III :</u> Analyse du protéome des graines de pois de l'embryogenèse au début du remplissage : impact d'une carence en soufre combinée à un stress hydrique

<u>Chapitre III :</u> Analyse du protéome des graines de pois de l'embryogenèse au début du remplissage : impact d'une carence en soufre combinée à un stress hydrique

Approches

Analyse du protéome de graines de pois récoltées à trois stades de développement (5, 9 et 12 jours après pollinisation) dans 4 conditions : non limitante en soufre et en eau, carencée en soufre, stress hydrique modéré, combinaison des deux stress.

Utilisation d'une approche réseau pour mettre en évidence les connections entre les différentes protéines, notamment celles régulées par les stress simples et combinés.

Principaux résultats

- 3184 protéines ont été identifiées et quantifiées dans les graines de pois en développement. L'étude de leur cinétique d'accumulation, de l'embryogenèse jusqu'au début du remplissage donne un premier aperçu de la dynamique du protéome des jeunes graines de pois.

- La réponse de ces graines aux stress simples ou combinés met en jeu un faible nombre de protéines impliquées dans l'élimination des espèces réactives de l'oxygène ou dans le maintien de l'équilibre redox.

- La construction d'un réseau protéique a mis en lumière des connections entre les protéines identifiées par protéomique. Les protéines régulées par les stress ont été repérées, donnant un premier aperçu du réseau protéique susceptible de maintenir l'homéostasie redox jusqu'à la phase de remplissage et d'éviter les dommages cellulaires en condition de stress.

Shotgun proteomics provides insights into the antioxidant network underlying the response of developing pea seeds to sulfur deficiency

Henriet *et al*., in preparation

ABTRACT

Pea (*Pisum sativum* L.) is a key legume crop producing seeds rich in proteins that contribute to satisfy the growing demand for plant proteins. The aim of this study was to unveil the proteome of developing pea seeds at three key stages covering embryogenesis, transition to seed filling, and beginning of storage protein synthesis, and then to investigate how this proteome was influenced by S deficiency and water stress, applied separately or combined. A total of 3184 different proteins were quantified by shotgun proteomics and 2473 were specifically or preferentially accumulated at particular stages, thus providing a unique view of the proteome dynamic of pea seeds at early reproductive stages. Furthermore, the data revealed 27 proteins whose abundance varied in response to stresses, and most of the up-regulated proteins are well known for their antioxidant function, indicating that the seed response to these stresses relies on a small number of proteins involved in detoxification processes. In particular, two NADPH-dependent oxidoreductases acting as scavengers of reactive carbonyl species were specifically induced in response to S deficiency and might contribute to maintain early growth of the embryo under this condition. Other redox-sensitive proteins (glutathione S-transferase, methionine sulfoxide reductase and two thioredoxins) were induced under both S-deficient and combined stress conditions, which is indicative of a need to adjust the redox state of the seed. Inference of a protein network identified the stress-regulated antioxidant proteins as sharing connections between them and with several other proteins. By dissecting these connections, we provide first insights into the complex antioxidant network operating in early developing seeds that would allow for the maintenance of redox homeostasis up to the filling phase and avoid cellular damages in response to stresses.

KEYWORDS

Abiotic stresses, drought, sulfur, *Pisum sativum*, seed development, seed proteomics, network

INTRODUCTION

Grain legumes such as pea (*Pisum sativum* L.) can contribute to satisfying the growing demand for plant proteins for human consumption and animal feed. They accumulate large amounts of proteins in their seeds (~23% in pea, Burstin *et al.*, 2011) even in the absence of nitrogen (N) fertilizer due to the ability of their root system to enter into symbiosis with soil bacteria that are able to fix atmospheric N. This makes legumes attractive as a source of plant proteins in order to profit from their nutritional and health benefits while reducing environmental impacts. Pea is of increasing interest for the food industry, notably because of the low allergenicity of its proteins and gluten-free characteristics (Barac *et al.*, 2010). However, the larger development of this culture is hampered by the instability of seed yield components (e.g., seed number, one-seed weight) and seed quality (e.g. protein content and composition) across years (Bourgeois *et al.*, 2009; Bénézit *et al.*, 2017). Among factors responsible for yield variations are abiotic stresses occurring during the reproductive period, such as drought, a major yield-limiting factor in pea (Martin and Jamieson, 1996), which is predicted to occur more often in the present context of global warming. A lack of sulfur (S) is also an increasing constraint faced by crops due to environmental policies aiming at reducing SO_2 emissions, which have led to a decrease of S deposition into the soil, causing S deficiency symptoms in crop plants (Gill *et al.*, 2012). The importance of S-derived metabolites, like glutathione or sulfate itself, in helping plants to combat abiotic stresses has stimulated researches on the interaction between S nutrition and abiotic stresses, such as drought (Chan *et al.*, 2013; Batool *et al.*, 2018; Henriet *et al.*, 2019).

In a recent study investigating the effect of S deficiency combined with water stress at early reproductive stages in pea, we showed that the plant response to the combination of these stresses is much more complicated than a simple additive effect (Henriet *et al.*, 2019). In particular, the combined stress strongly impacted the individual seed weight. In pea, the individual seed weight at maturity perfectly correlated with the number of cells in the cotyledons (Davies, 1975), which is established during seed embryogenesis. This phase of seed

development is characterized by a high hexose content which promotes embryo cell division. In fact, the mitotic activity in developing pea seeds has been proposed to be controlled by hormones, environmental factors, and nutrient supply (Weber *et al.*, 2005; Borisjuk *et al.*, 2002). After embryogenesis, the embryo switches from mitotic growth to growth driven by cell expansion (Weber *et al.*, 2005). In pea (cv. Caméor) this transition occurs around 10 days after pollination (DAP) (Schiltz *et al.*, 2004) and is associated with the initiation of a nutrient uptake system based on the development of embryonic transfer cells and of the expression of storage protein genes (Weber *et al.*, 2005; Borisjuk *et al.*, 2002). Storage proteins accumulate in the cotyledons during the filling phase (Herman and Larkins, 1999). The most abundant storage proteins in pea seeds are the 7S (vicilins, convicilins) and 11S (legumins) globulins, the latter containing higher levels of S-amino acids (methionine and cysteine). Thus, globulin composition, as determined by the 7S/11S ratio, largely determines the seed nutritional value. This ratio increased in response to S deficiency, which significantly decreased the relative abundance of legumins by reducing the amount of legumin transcripts (Chandler *et al.*, 1983; Chandler *et al.*, 1984). However, we recently showed that a moderate water stress could mitigate the negative effect of S deficiency on seed protein composition, probably due to less N loaded in the double-stressed seeds enabling a readjustment of the seed N/S ratio (well correlated with the seed 7S/11S ratio, Henriet *et al.*, 2019). Elucidating the complex process of pea seed development and its response to abiotic stresses will help future breeding programs aiming at improving and stabilizing seed quality traits in pea under fluctuating environmental conditions.

Seed development has been extensively studied in several species including legumes by transcriptomics, thus increasing our understanding of transcripts associated with specific developmental stages (Benedito *et al.*, 2008; Gallardo *et al.*, 2007). However, the level of a given transcript does not necessarily reflects the abundance of the corresponding protein. For instance, in *Medicago truncatula*, transcript and protein profiles were divergent across seed development for 50% of the comparisons made, which is indicative of extensive post-transcriptional events (Gallardo *et al.*, 2007). The recent release of a reference genome in pea (Kreplak *et al.*, 2019) makes now possible to apply quantitative shotgun proteomics to study seed metabolism in this grain legume crop. A further advance is to be expected from the development of protein networks, which has been successfully used to reveal central actors

in the grain proteome response to S and N nutrition in wheat (Bonnot *et al.*, 2017). Here, we have used shotgun proteomics to provide a first atlas of the protein complement of developing pea seeds, targeting three key stages of seed development: embryogenesis, transition stage to seed filling, and beginning of storage protein synthesis. We also investigated the effect of S deficiency combined or not with water stress on the seed proteome and inferred a seed protein network in which key antioxidants interact to allow for the maintenance of redox homeostasis up to the seed filling phase.

MATERIALS AND METHODS

Stress imposition and pea seed collection

Pea plants (Pisum sativum L., "Caméor" genotype) were grown as described in Henriet *et al.*, (2019). Briefly, plants were subjected to S deficiency (S-) after three weeks of growth (mid-vegetative stage). To impose water stress (WS), irrigation was stopped at flowering until soil water content reached 50% of the maximum water-holding capacity (100%) of the substrate. After 9 days (d), plants were re-watered normally. Control plants, grown in parallel, were well supplied with S and water throughout development. Pollinating flowers at the beginning of water stress imposition were tagged and seeds from these targeted pods were harvested at 5, 9 and 12 days after pollination (DAP), corresponding to 5d and 9d after the beginning of water stress and 3 d after re-watering (**Figure 24A**). For each condition and time, three (9DAP seeds in S- and WS conditions) to four (all the other time and condition) biological replicates of seeds were collected, each replicate consisting of a pool of seeds from two plants (46 seed samples in total). The number and weight of seeds per pod were recorded for one-seed weight determination. Fresh seeds were frozen in liquid nitrogen and stored at -80°C until protein extraction. Significant differences in one-seed weight between the different seed samples were identified by one-way ANOVA followed by a Student-Newman-Kheul (SNK) post-hoc test ($P<0.05$) using the R software (version 3.5.1) (R Core Team, 2018).

Protein extraction, reduction and alkylation

Fresh seeds stored at -80°C were ground in liquid nitrogen using mortar and pestle. Total proteins were extracted using 110 mg of seed powder using the procedure enabling the simultaneous precipitation and denaturation of proteins with Trichloroacetic acid and β-

mercaptoethanol in cold acetone, as described by (Méchin *et al.*, 2007). After lyophilization, the proteins were solubilized in 20 µL of ZUT buffer (6 M urea, 2 M urea, 10 mM DTT, 30 mM Tris-HCl pH 8.8, 0.1% zwitterionic acid labile surfactant) and adjusted to a final concentration of 2 µg proteins/µL. Ten µL (20 µg proteins) of each sample were incubated for 30 min at room temperature. Then, proteins were alkylated by addition of 2 µL of 330 mM iodoacetamide, 50 mM ammonium bicarbonate, followed by incubation in darkness for 1 h at room temperature. Ninety µL of 50 mM ammonium bicarbonate were added to each sample to dilute them ten times. Digestion was performed overnight at 37°C by adding 800ng of trypsin (4ul at 0,2 µg.µl^{-1}). Digestion was stop by adding 6µl of 18.6% trifluoroacetic acid in water.

Shotgun proteomics of developing pea seeds

Desalting and LC-MS/MS analyses were then performed as described by Hervé *et al.*, (2016) using a nlc425 device (Sciex, Villebon-sur-Yvette) coupled to a Q-exactive mass spectrometer (Thermo Fisher Scientific) with a glass needle (non-coated capillary silica tips, 360/20-10, New Objective In), thus forming a nanoelectrospray interface. Xcalibur raw data were transformed to mzXML open source format using the msconvert software in the ProteoWizard 3.0.3706 package (Kessner *et al.*, 2008). A total of 4698 proteins were identified with X!Tandem version 2015.04.01.1 (Craig and Beavis, 2004) by matching peptides against the Pea Genome V1a database (https://urgi.versailles.inra.fr/jbrowse/gmod_jbrowse/, (Kreplak *et al.*, 2019). Enzymatic cleavage parameters were set as trypsin digestion with one possible missed cleavage. Cys carboxyamidomethylation was set as static modification, whereas Met oxidation, N-terminal deamidation, and N-terminal acetylation were set as variable modifications. Precursor mass tolerance was 10 ppm and fragment mass tolerance was 0.02. Identified proteins were filtered and grouped using the X!TandemPipeline software version 3.4.3 (Langella *et al.*, 2017). Peptide and protein e-value cut offs were set to 0.01 and 10^{-5}, respectively, with at least two peptides per protein. The filtered dataset corresponded to 3184 proteins (**Supplementary Table S5**).

The relative quantification of the 3184 proteins was performed using the MassChroQ software version 2.2 (Valot *et al.*, 2011) with quantification of 80% of the theoretical natural isotope profile. The most abundant isotope was selected for peptide quantification. The following criteria were used to analyze the extracted ion chromatograms (XIC): peptides-mz with a

variation of 20s in their retention time were removed, as well as chromatographic peaks wider than 100s, peptide-mz intensities were normalized with the method median.RT, and peptides shared by multiple proteins were removed. All data obtained at each developmental time point were then analyzed separately using the same criteria: peptides-mz present in less than 95% of the samples and which did not show a significant correlation (r>0.5) with the other peptides of the same protein were removed. Proteins with less than two peptides were discarded, then missing peptide-mz intensities and missing protein abundances were imputed.

Differential analysis of proteomics data and gene ontology enrichment analysis

The Limma R package (3.38.3 version) (Ritchie *et al.*, 2015) was used for differential analysis of relative protein abundance between the control and stress conditions at 5, 9 and 12 DAP, and between each time point for the control condition. The lmFit function implemented in limma was used to perform linear fitting, empirical Bayes statistics and Benjamini–Hochberg false discovery rate (FDR) calibration of the P-values. Proteins were considered as differentially accumulated when the adjusted P-value (padj) was <0.05. Gene ontology (GO) enrichment analysis was conducted using the TopGO R package (2.24.0 version) (Alexa and Rahnenfuhrer, 2018). A Fisher's exact test with elim method was used to identify the GO terms significantly enriched in the datasets. Hierarchical clustering of proteins based on their accumulation patterns during seed development was performed using the pheatmap R package (Kolde, 2018). The clustering method was "complete" with "Euclidean" distance measure. Venn diagrams were done using the gplot R package (Warnes *et al.*, 2016).

Inference of a seed protein network

The protein network was constructed from normalized XIC data of the 46 seed samples using the dynamical GEne Network Inference with Ensemble of trees (dynGENIE3) algorithm implemented in R (Huynh-Thu and Geurts, 2018). We have chosen a threshold on the weights > 0.01, which maximized the detection of hub proteins while minimizing the detection of small modules (≤ 3 proteins). The entire network was visualized in Cytoscape (version 3.7.0) (Shannon *et al.*, 2003), then the large module made of 428 proteins was visualized using the "Edge-weighted Spring-embedded" layout of Cytoscape. Subnetworks containing hub

proteins and proteins regulated by stresses were extracted and manually adjusted for ease of visualization of each protein.

RESULTS

Strategy adopted to study the proteome of developing pea seeds and its response to S deficiency combined or not with water stress

In order to provide insights into the proteome of developing pea seeds and its modulation by S deficiency and/or water stress*, Pisum sativum* L. plants (cv. Caméor) were deprived of S from a mid-vegetative stage and subjected to a moderate water stress (WS) for nine days starting at flowering, as described in Henriet *et al.*, (2019). Control plants (well-watered, non-limiting S conditions) and plants subjected to individual stresses (WS or S-) were grown in parallel for comparison. Flowers that opened on the day of water stress imposition were tagged, and developing seeds were harvested from the tagged pods at three time points: during, at the end and 3 days after the drought period (i.e. during re-watering) (**Figure 24A**). These time points corresponded to three key developmental stages: embryogenesis (5 DAP), transition to seed filling (9 DAP) and beginning of seed filling (12 DAP) (Schiltz *et al.*, 2004; Henriet *et al.*, 2019). We observed that the individual seed fresh weight was significantly reduced in the double stress condition as soon as 9 DAP, while the decrease in S- condition was significant at 12 DAP (**Figure 24B**). This was consistent with the data obtained at physiological maturity showing that the combined stress strongly impacted final one-seed weight (Henriet *et al.*, 2019). Seed samples collected at 5, 9 and 12 DAP and in the four conditions (control, S deficiency, drought, and combined stress) were subjected to a quantitative proteome analysis by shotgun. The relative quantification of the proteins was based on peak area integration of Extracted Ion Chromatograms (XIC). In total, 3184 identified proteins were quantified which enabled us to develop the first atlas of the protein complement of developing pea seeds and to analyze: (i) the dynamic changes in the relative abundance of each protein across stages, and (ii) proteome changes occurring at each developmental stage in response to stresses.

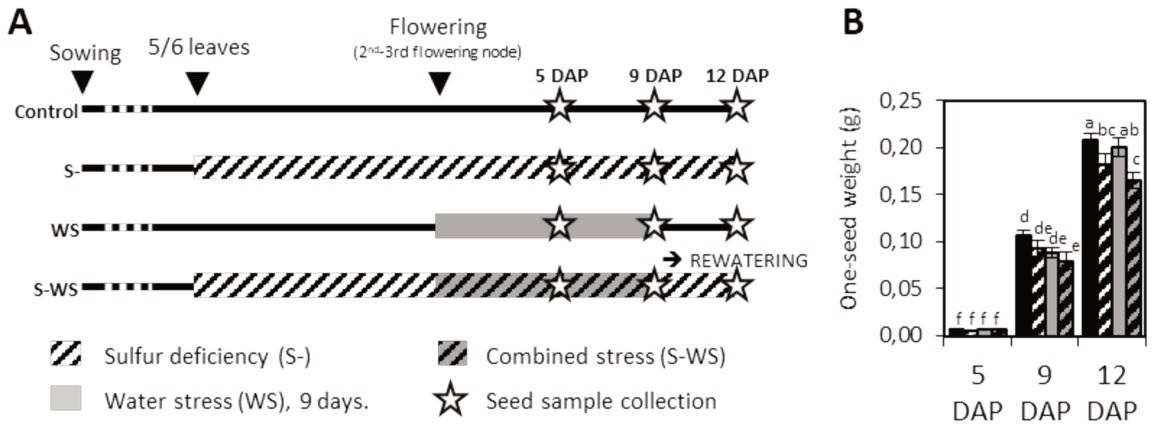


Figure 24. Experimental design for studying the proteome and weight of pea seeds at early stages of seed development under standard, water stress and/or S-deficient conditions. (A) Stress imposition and seed collection. Control, well-watered plants under non-limiting S-condition; S-, plants deprived of S from a mid-vegetative stage; WS, plants subjected to water stress from flowering of the 2nd or 3rd reproductive nodes for 9 days, then re-watered for recovery; S-WS, plants subjected to a combination of the two stresses. Pollinating flower at the beginning of water stress were labelled and developing seeds were collected at three stages: 5, 9 and 12 DAP, corresponding at 5 and 9 days of water stress, and 3 days after re-watering. **(B)** One-seed fresh weight (g) in labelled pod at 5, 9 and 12 DAP. Values are means ± standard errors (n=8 plants). Different letters above each bar represent significant differences (P<0.05, ANOVA followed by a SNK test).

Dynamic of the pea proteome at early stages of seed development

To provide a dynamic view of proteome changes occurring in developing pea seeds during the time course investigated, we first analyzed the seed samples collected in control condition. Of the 3184 proteins quantified, 2210 were detected at the three developmental stages, whereas 214, 101 and 146 proteins were only detected at 5, 9 or 12 DAP, respectively **(Supplementary Table S5 and Figure 25A**). In addition 190, 236 and 87 proteins were specifically detected at 5 and 9 DAP, 9 and 12 DAP, and 5 and 12 DAP, respectively. A GO enrichment analysis from each protein list revealed that different functional classes were over-represented depending on the developmental stage(s) at which the proteins were detected (**Figure 25B, Supplementary Table S6**). For example, the list of proteins specific to 12 DAP (146 proteins) was enriched in GO terms for “ribosome biogenesis” and “lipid storage” (biological process), and for “nutrient reservoir activity” (molecular function). These data and the identification of 16 different

storage proteins (10 vicilins and 6 legumins) in 12 DAP seeds (**Supplementary Table S7**) confirmed that seeds already entered the filling phase 12 DAP.

A

B

GO Term	Description	Annotated	Significant	P-value
Specific to 5 DAP (214 proteins)				
GO:0045039	Protein insertion into mitochondrial inner membrane	3	2	0.00012
GO:0006414	Translational elongation	54	3	0.00484
GO:1901605	Alpha-amino acid metabolic process	279	8	0.00563
GO:0009309	Amine biosynthetic process	183	5	0.00616
GO:0007031	Peroxisome organization	19	2	0.00633
GO:0016120	Carotene biosynthetic process	1	1	0.00633
GO:0006535	Cysteine biosynthetic process from serine	20	2	0.007
GO:0008652	Cellular amino acid biosynthetic process	220	7	0.00828
Specific to 9 DAP (101 proteins)				
GO:0006480	N-terminal protein amino acid methylation	1	1	0.0038
GO:0051016	Barbed-end actin filament capping	2	1	0.0076
Specific to 12 DAP (146 proteins)				
GO:0042254	Ribosome biogenesis	91	6	3.00E-06
GO:0019915	Lipid storage	11	2	0.001
GO:0008283	Cell proliferation	19	2	0.0031
GO:0036265	RNA (guanine-N7)-methylation	1	1	0.0044
GO:0009081	Branched-chain amino acid metabolic process	24	2	0.005
GO:0019725	Cellular homeostasis	178	4	0.0079
GO:0018344	Protein geranylgeranylation	2	1	0.0088
GO:0097576	Vacuole fusion	2	1	0.0088
GO:0009408	Response to heat	34	2	0.0098

Figure 25. Comparison of proteomics data obtained at each stage of seed development. (A) Venn diagram showing the number of proteins identified and quantified at each stage. **(B)** GO term enrichment analysis for proteins specific to one stage of development. Terms in the "Biological Process" category are shown. For each term, the number of genes present in the genome ("annotated") and in the gene lists ("significant") are shown along with the associated *P*-value (Fisher test, threshold of P<0.01).

A statistical analysis of quantitative XIC data obtained for the 2723 proteins detected at two or three developmental stages indicated that the abundance of 2012 proteins varied significantly between at least two stages (Padj < 0.05, **Supplementary Table S5**). A hierarchical clustering grouped these proteins into seven clusters according to their accumulation patterns, and GO enrichment analyses highlighted biological processes and molecular functions enriched in each cluster (**Figure 26, Supplementary Tables S8 and S9**). Cluster 1, which contained 492 proteins preferentially accumulated at 9 and 12 DAP, was enriched in proteins related to translation, intracellular protein transport and cellular carbohydrate metabolic processes. Cluster 4 was the second largest cluster (347 proteins preferentially accumulated 5 DAP) and displayed an over-representation of proteins related to amine and amino acid metabolisms. Cluster 2 contained 314 proteins also preferentially accumulated 5 DAP and enriched in proteins related to DNA replication, protein folding and histidine biosynthesis. Cluster 5 corresponded to 338 proteins preferentially accumulated at 5 and 9 DAP and was enriched in proteins related to carbon and ketone metabolisms, and oxidation-

reduction process. Cluster 3, which contained 193 proteins preferentially accumulated at 5 and 12 DAP, was enriched in proteins related to amine and amino acid metabolisms. Cluster 7 contained 231 proteins preferentially accumulated at 12 DAP and was enriched in proteins related to translation and photosynthesis. Finally, cluster 6 was the smallest cluster with only 97 proteins accumulated at 9 DAP and enriched in proteins with oxidoreductase activity.

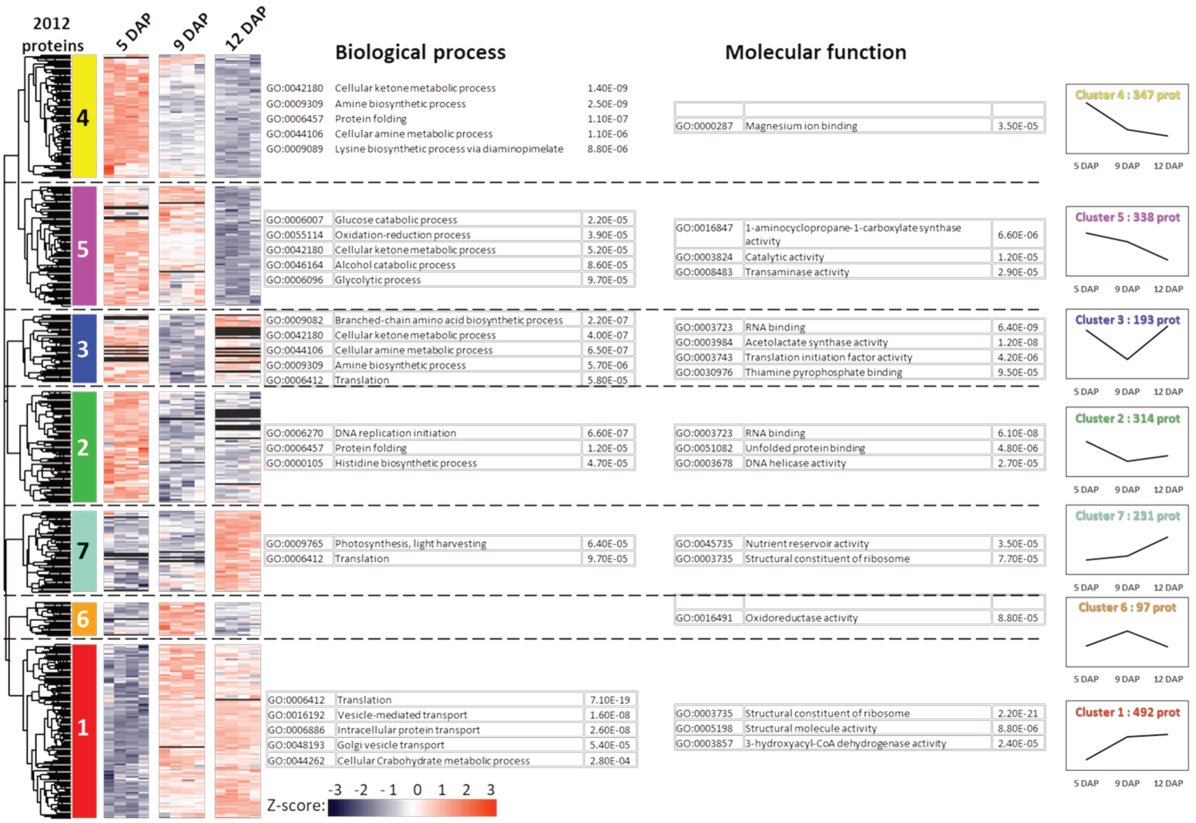


Figure 26. Seed proteome changes during early seed development in pea. The heatmap represents the hierarchical clustering of proteins present at least at two developmental stages and differentially accumulated between stages. Each row represents a gene and each column represents a seed sample. The gradual color change from red to blue represents up- to down-accumulation across stages (Z-score value). Missing data (*i.e.* undetected proteins) are represented by dark grey fields. For each cluster, GO terms enriched are shown for the "Biological Process" and "Molecular Function" categories along with their associated *P*-value (Fisher test, $P<1.00E^{-05}$). Graphs on the right represent the mean variation of protein accumulation across stages for each cluster. The number of proteins in each cluster is indicated.

The proteomic data were then scrutinized for proteins involved in the regulation and modulation of transcription, including transcription factors (TF), chromatin/DNA methylation-related proteins or general transcription regulators (**Table S10**). Out of the 16 TF that were retrieved, four belonged to the Nuclear Factor Y (NF-Y) family and five to the plant-specific B3 superfamily. In particular, LEAFY COTYLEDON1-Like (LEC1-Like, Psat0s2409g0040) was first detected at the switch towards seed filling (9 DAP) and was co-accumulated at 12 DAP with FUSCA3 (FUS3), a master regulator of embryo development and seed maturation in several species (Carbonero *et al.*, 2017).

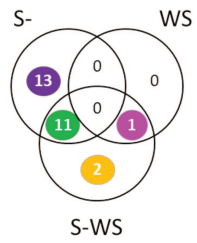
Impact of S deficiency and/or water stress on the pea seed proteome

We next investigated the impact of S deficiency combined or not with water stress on the seed proteome. Differential analyses between control and stress conditions were performed at each developmental stage independently (*cf*. Material and Methods). At 5 DAP, the accumulation of 13 proteins was affected by one or the two stresses, while at 9 and 12 DAP, the abundance of 10 and 17 proteins varied, respectively. Overall, the accumulation of 27 unique proteins varied significantly (padj < 0.05) in response to the single or/and combined stresses compared to the control (**Figure 27**), which represented only 0.85% of the total number of proteins quantified. This indicates that only a subset of proteins are sufficient to adjust seed metabolism in response to S deficiency and/or to a moderate water stress. The abundance of only two proteins varied specifically in response to the combined stress that corresponded to an alcohol dehydrogenase and a histone H2A (down-regulated at 5 and 12 DAP, respectively). Another alcohol dehydrogenase was down-regulated 5 DAP in response to water stress under both S-sufficient and S-deficient conditions. Interestingly, a higher number of proteins varied in response to S deficiency (24 proteins) than in response to the combined stress (14 proteins), reflecting the establishment of specific processes in response to S deficiency alone (**Figure 27**). Most of the proteins that varied specifically in response to S deficiency were regulated at the beginning of the seed filling phase (12 DAP) rather than during embryogenesis (5 DAP). Among the up-regulated proteins were two NADPH-dependent oxidoreductases that are scavengers of reactive carbonyls: alkenal/one oxidoreductase (AOR, Psat3g172160) and 2-alkenal reductase (AER, Psat0s2549g0200) (Mano *et al.*, 2005; Yamauchi *et al.*, 2012).

Of the 11 proteins whose abundance varied in response to S deficiency alone or combined with water stress, only one was down-accumulated (at 5 and 9 DAP). This protein was identified as ATP sulfurylase, the first enzyme of sulfate reduction. Interestingly, among the 10 up-regulated proteins, four play an important function in protecting cells against oxidative damages: a methionine sulfoxide reductase (MSRB, Psat1g185480), which repairs oxidized proteins, two thioredoxins (TRX#1, Psat7g082200; TRX#2, Psat5g207000), and a glutathione S-transferase (GST#1, Psat6g125080) that are part of central antioxidant systems. These results highlight the importance of these processes to help early developing seeds to mitigate the effect of S deficiency combined or not with water stress. By exploiting a previously

published dataset of transcriptomics data obtained from the same seed samples harvested at 9 DAP (Henriet *et al.*, 2019), we could decipher whether changes in the accumulation of these proteins was primarily determined at the transcriptional level.

A



B

Gene ID	Annotation	5 DAP S-	5 DAP WS	5 DAP S-WS	9 DAP S-	9 DAP WS	9 DAP S-WS	12 DAP S-	12 DAP WS/R	12 DAP S-WS/R	Cluster	Stage of regulation
Specific to S- (13 proteins)												
Psat3g172160	Alkenal/one oxidoreductase (AOR)*	**0.60**	0.08	0.16	0.23	0.00	0.35	-	-	-	-	5
Psat6g125840	Legumin	**0.53**	0.02	0.33	-	-	-	-0.33	0.01	-0.48	7	5
Psat3g038120	Poly-adenylate binding protein	**-0.34**	-0.15	-0.26	-0.04	-0.04	0.03	0.04	0.00	-0.17	2	5
Psat7g041960	Cysteine proteinase inhibitor	-	-	-	**-0.32**	-0.02	-0.08	0.03	0.02	0.11	-	9
Psat5g033680	Dirigent-like protein (DIR)*	0.06	-0.02	-0.02	0.27	0.07	0.24	**0.38**	0.02	0.21	4	12
Psat4g158520	Serine acetyltransferase	0.22	0.00	0.12	0.19	0.03	0.19	**0.26**	0.01	0.16	7	12
Psat0s2549g0200	2-alkenal reductase (AER)*	0.02	-0.02	-0.06	0.09	-0.02	0.06	**0.24**	-0.05	0.15	5	12
Psat3g192200	2 +3-bisphosphoglycerate-independent phosphoglycerate mutase	0.00	0.01	-0.02	-0.07	-0.03	-0.03	**-0.17**	-0.07	-0.02	3	12
Psat3g204280	Reversibly glycosylated polypeptide	-0.02	-0.02	0.00	-0.04	0.00	-0.02	**-0.24**	-0.03	0.05	4	12
Psat3g140920	Unknown gene	-0.14	0.06	0.01	-0.17	0.00	-0.13	**-0.35**	-0.02	-0.05	-	12
Psat3g069920	Polygalacturonase inhibiting protein	-0.14	-0.04	-0.15	-0.26	-0.11	-0.25	**-0.36**	0.03	-0.18	-	12
Psat4g112000	Gamma-thionin family (PDF2.2)*	-0.08	-0.04	0.09	-0.30	-0.13	-0.31	**-0.53**	0.14	-0.19	-	12
Psat7g247120	Ferritin	**-0.60**	0.01	-0.03	-0.47	0.26	0.26	**-0.29**	0.12	0.11	3	5, 12
Specific to S- and S-WS (11 proteins)												
Psat1g185480	Methionine sulfoxide reductase (MSRB)*	**0.88**	0.01	0.44	**0.73**	0.25	**0.77**	-	-	-	-	5, 9
Psat5g221480	NADPH:quinone oxidoreductase	**0.53**	0.00	0.32	**0.48**	0.04	**0.44**	0.61	0.09	0.45	4	5, 9
Psat6g202040	ATP-sulfurylase	**-0.38**	-0.01	**-0.63**	**-0.58**	0.04	**-0.64**	0.15	-0.01	0.01	4	5, 9
Psat4g147120	Annexin (ANNAT8)*	0.56	-0.06	0.41	**0.71**	0.19	**0.56**	0.48	0.06	0.29	2	9
Psat3g164320	Zinc-binding dehydrogenase	-0.01	0.00	-0.06	**0.50**	-0.10	0.30	**0.69**	0.00	**0.61**	1	9, 12
Psat7g120800	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase protein	-	-	-	**0.41**	0.01	**0.41**	**0.67**	-0.09	**0.53**	6	9, 12
Psat0s62g0080	DNA-damage-repair/toleration (DRT102)*	0.34	0.04	0.22	**0.30**	-0.04	**0.30**	**0.31**	-0.02	**0.32**	1	9, 12
Psat6g125080	Glutathione S-transferase (GST#1)*	**0.53**	-0.01	0.38	**0.47**	0.04	**0.43**	**0.69**	-0.07	**0.70**	5	5, 9, 12
Psat7g082200	Thioredoxin (TRX#1)*	**0.33**	0.03	0.17	**0.37**	0.04	**0.33**	**0.58**	-0.01	**0.45**	5	5, 9, 12
Psat0s1058g0200	NmrA-like negative transcriptional regulator protein	**0.80**	0.10	**0.47**	0.31	-0.21	0.25	**0.63**	-0.12	0.40	1	5, 12
Psat5g207000	Thioredoxin (TRX#2)*	**0.29**	0.03	0.16	0.25	0.00	0.24	**0.43**	-0.04	**0.40**	4	5, 12
Specific to S-WS (2 proteins)												
Psat5g081400	Alcohol dehydrogenase	-0.16	-0.59	**-0.67**	0.10	-0.23	-0.39	-0.58	-0.12	-0.50	7	5
Psat4g042040	Histone H2A protein	0.23	-0.13	0.26	0.03	0.06	0.02	-0.15	-0.03	**-0.26**	7	12
Specific to WS and S-WS (1 protein)												
Psat5g081320	Alcohol dehydrogenase*	-0.09	**-0.45**	**-0.42**	0.01	-0.25	-0.43	0.02	-0.42	-0.52	5	5

Figure 27. Effect of S deficiency and/or water stress on the proteome of pea seeds at early stages of seed development. A, Venn diagram showing the number of proteins differentially accumulated in response to stresses compared to the control condition. **B,** For each pea protein, the $\log_{10}$ fold change in stress condition compared to the control is given. Expression values in colour indicate significant differences between stress and control conditions, blue indicating a down-regulation and red an up-regulation of protein abundance. For each protein, the cluster (**Figure 26**) to which they belong and the developmental stage at which the abundance varied in response to stresses were indicated. Asterisk indicates the protein was present in the protein network in Figure 5. S-, S-deficiency alone; WS, water stress alone; S-WS, combined stress; WS/R indicates re-watering.

Of the 10 proteins differentially regulated by stresses 9 DAP, nine were encoded by genes similarly regulated in response to the stresses (**Figure 28A**), suggesting that the regulation primarily occurs at the level of transcription. Among these proteins were ATP sulfurylase (down-regulated by stresses), MSRB, GST#1 and TRX#1 (all three being up-regulated). Gene expression and protein abundance data for these regulated proteins were highly correlated (R2 = 0.9) (**Figure 28B**).

A

Gene ID	Annotation	Proteins S-	Proteins WS	Proteins S-WS	ARN S-	ARN WS	ARN S-WS
Psat1g185480	Methionine sulfoxide reductase (MSRB)*	**0.73**	0.25	**0.77**	**2.25**	0.22	**1.82**
Psat6g125080	Glutathione S-transferase (GST#1)	**0.47**	0.04	**0.43**	**2.10**	0.40	**1.74**
Psat7g120800	2-oxoglutarate and Fe(II)-dependent oxygenase*	**0.41**	0.01	**0.41**	**1.64**	0.12	**1.32**
Psat4g147120	Annexin	**0.71**	0.19	**0.56**	**1.75**	0.16	**1.22**
Psat0s62g0080	Ribose/Galactose Isomerase*	**0.30**	-0.04	**0.30**	**1.29**	-0.08	**1.03**
Psat5g221480	NADPH:quinone oxidoreductase	**0.48**	0.04	**0.44**	**1.35**	0.15	**0.97**
Psat7g082200	Thioredoxin (TRX#1)*	**0.37**	0.04	**0.33**	**1.24**	0.07	**0.90**
Psat3g164320	Zinc-binding dehydrogenase*	**0.50**	-0.10	0.30	**1.32**	-0.01	**0.81**
Psat7g041960	Cysteine proteinase inhibitor	**-0.32**	-0.02	-0.08	-0.07	0.16	0.22
Psat6g202040	ATP-sulfurylase*	**-0.58**	0.04	**-0.64**	**-1.19**	0.00	**-1.66**

B

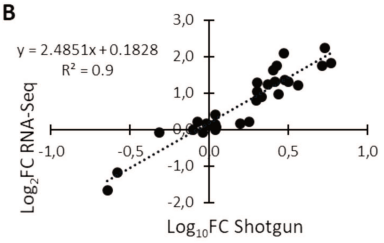
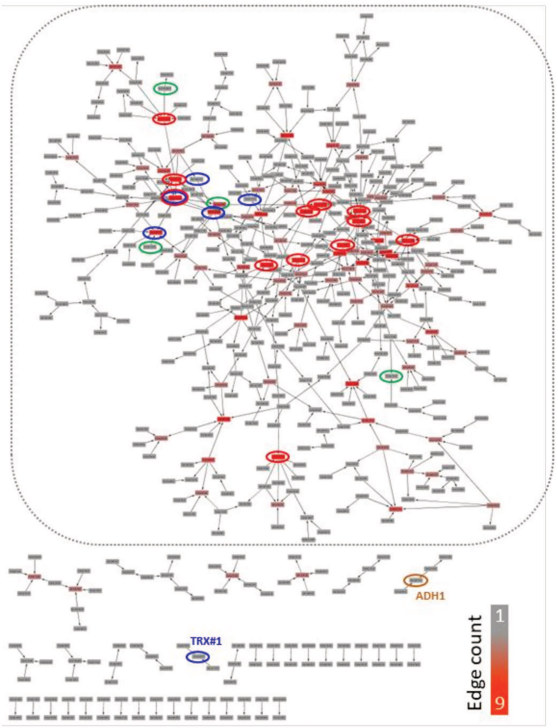


Figure 28. Correlation between protein accumulation and gene expression for proteins regulated by stresses at the 9 DAP stage. (A) List of proteins differentially accumulated in response to stresses at 9 DAP and comparison with RNA-seq data previously obtained at this stage (Henriet et al., 2019). Proteomics data are expressed in $\log_{10}$ fold-change (Log_{10}FC) compared to the control condition. Bold and color values indicated significant differences compared to the control (red and blue indicate respectively an up- and down-regulation). **(B)** Correlations between RNA-Seq data (Log_2FC, mean value) from Henriet *et al.* (2019) and shotgun proteomics data (Log_{10}FC, mean value) obtained for each protein whose accumulation varied in response to stresses 9 DAP.

Inference of a seed protein network - interplay between hub and stress-regulated proteins

To describe protein modules underlying seed metabolism and its response to S deficiency and/or water stress, an inferred protein network was built from XIC data of all individual seed samples (3184 proteins) using dynGENIE3 that enables the exploitation of time series and steady-state data jointly (Huynh-Thu and Geurts, 2018). This led to a seed protein network consisting of 536 nodes. A total of 38 modules with low numbers of connections were identified along with one large module containing 428 proteins (**Figure 29**). The proteins that compose the large module and the number of edge for each protein in the network are indicated in **Supplementary Table 5**. A GO enrichment analysis from these 428 proteins indicated a significant (P<0.001) over-representation of proteins related to the generation of precursor metabolites and energy (GO:0006091), pyruvate metabolic process (GO:0006090), protein neddylation (GO:0045116), cellular ketone metabolic process (GO:0042180), oxidation-reduction process (GO:0055114) and cellular catabolic process (GO:0044248), suggesting an interplay between these biological processes during early seed development.



Seed Protein Network (536 nodes)

- 12 hub proteins (≥ 7 edges)
- 4 proteins regulated by S- (well-watered plants)
- 6 proteins regulated by S- with or without WS
- 1 protein regulated by WS (under S+ and S-)

Large module
428 nodes

GO enrichment analysis:

GO.ID	Term (significant/annotated)	*P* value
GO:0006091	Generation of precursor metabolites and energy (16/349)	0.0002
GO:0006090	Pyruvate metabolic process (9/127)	0.00023
GO:0045116	Protein neddylation (2/2)	0.00026
GO:0042180	Cellular ketone metabolic process (21/562)	0.00037
GO:0055114	Oxidation-reduction process (52/1913)	0.00056
GO:0044248	Cellular catabolic process (26/639)	0.00083

Figure 29. Inference of a protein regulatory network from all proteomics data obtained at early stages of seed development in pea. A, The seed protein network is presented as visualized using Cytoscape. Node colors vary depending on the number of links/edges with other nodes: red indicates highly connected nodes and light grey indicates low number of edges. Hub proteins (<= 7 edges) and proteins regulated by stresses are respectively encircled in red and black. A large module (blue dashed) was identified that contain 428 nodes, of which 9 are hubs and 12 are regulated by stresses. Biological processes significantly enriched (P<0.001, GO enrichment analysis) from these 428 proteins are presented on the right.

The average value for the number of edges per protein (i.e., the connectivity of each protein with other proteins) in the largest module was 2.2. However, we identified proteins with higher connectivity that we refer here as hub proteins (in red in **Figure 29**, also highlighted in **Supplementary Table 5**). To examine whether the stresses could influence the accumulation of hub proteins, subnetworks containing the most connected proteins (≥ 7 edges, 12 hub proteins) and the proteins regulated by stresses (11 proteins in the network) were extracted (**Figure 30**). The 113 proteins that compose these subnetworks are presented in **Supplementary Table 5** along with their relative abundance at each developmental stage under control and stress conditions. The results indicated that four proteins whose abundance varied in response to stresses were hubs or linked to hub proteins. One is a dirigent-like protein (DIR, Psat5g033680) significantly up-regulated in pea seeds in response to S deficiency. DIR was connected to three hub proteins with roles in dephosphorylation (PP2C, protein phosphatase, Psat7g261800), ubiquitination (UPL1, ubiquitin-protein ligase, Psat2g064840), or S-glutathionylation (GST#1). This suggests important post-translational events at early developmental stages and a possible relationship with the DIR protein.

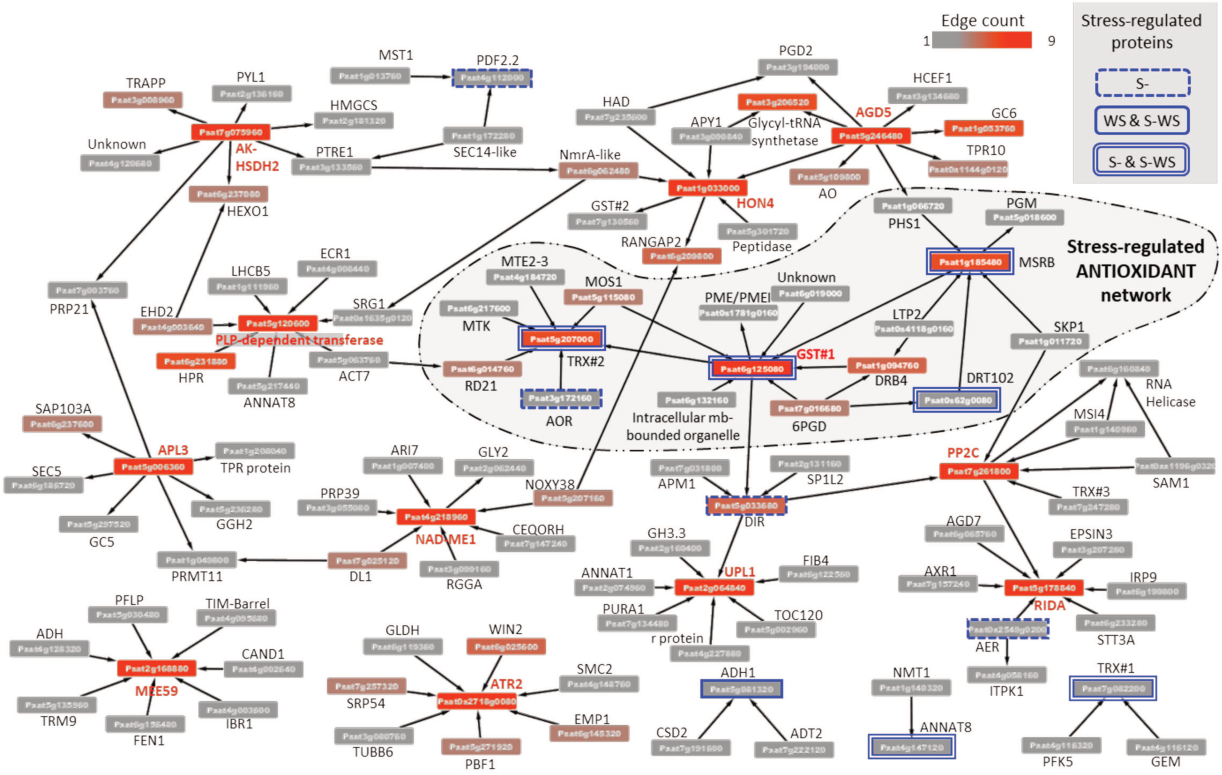


Figure 30. Subnetwork of protein hubs and proteins regulated by stresses in pea seeds. The most highly connected proteins (>=7 edges, red squares) and proteins regulated by stresses (blue) are shown along with their first neighborhoods in the network. Each protein was annotated by homology with genes characterized in other species, mainly Arabidopsis, and the pea accession number (v1a) of the corresponding gene is indicated in the middle of each square. The stress-regulated antioxidant network, containing antioxidant proteins displaying high number of connections and regulated by stresses, is outlined with a dashed black line.

Interestingly, GST#1 was the most connected protein in the network (9 edges) and linked to MSRB (6 edges) and TRX#2 (6 edges), the latter being also connected to AOR (one edge). All these proteins contribute to the anti-oxidative defense of plant cells and were up-regulated in response to S deficiency alone (AOR) or under both S-deficient and combined stress conditions (GST#1, MSRB, TRX#2). These data indicated that proteins with anti-oxidant roles are central actors in the metabolism of developing pea seeds and may help to counteract the effect of S deficiency, combined or not with water stress, upon seed development. This antioxidant-related group of proteins is highlighted in **Figure 30**. The last antioxidant protein regulated by S-deficiency (AER, involved in the detoxification of reactive carbonyls) was not directly connected to this group of proteins, but appeared connected to the hub protein RIDA (Reactive Intermediate Deaminase A, 7 edges). Although no direct relationship between the connected proteins could be established, the knowledge gained using this directed network (i.e. made of outgoing or incoming edges) can be used to propose hypotheses about which protein influences the accumulation of which protein in early developing seeds. These hypotheses are discussed hereafter in light of data available in the literature concerning the role reported for these proteins, mainly in Arabidopsis.

DISCUSSION

In this study quantitative shotgun proteomics was used to unveil the proteome of developing pea seeds at three key stages covering embryogenesis, transition to seed filling, and beginning of storage protein synthesis, and then to investigate how this proteome was influenced by S deficiency and water stress, applied separately or combined. A total of 3184 proteins were identified, the majority of which (78%) varied during the time course investigated (data from **Figures 25 and 26**), while only 27 were affected by the stresses (**Figure 27**). Exploring these proteins contributed to a better understanding of the mechanisms that are important to shift the developmental program of pea seeds to storage protein deposition and of how this program is affected by S deficiency and/or water stress.

Insight into the proteome dynamics of early seed development in pea

At 5 DAP the pea seed is mainly composed of maternally derived tissues, the seed coat and the endosperm, and the embryo is at the globular stage, undergoing high rate of cell division.

Several GO terms with a significantly high occurrence in the list of proteins preferentially accumulated at 5 DAP (Clusters 2 and 4, **Figure 26**) were related to amino acid (AA) metabolism and enzymes involved in the metabolism of lysine, histidine, glutamine, asparagine, methionine or cysteine were preferentially accumulated early in seed development (**Table S11**). In legumes, the seed coat and the endosperm of the young developing seeds play an important role in the transient storage of nutrients coming from the phloem and in their metabolism before their transfer to the embryo (Radchuk and Borisjuk, 2014; Melkus *et al.*, 2009). In pea, mainly glutamine, alanine, and threonine are released from the seed coat (Lanfermeijer *et al.*, 1992). Enzymes involved in the biosynthesis of several AA which are not supplied by the phloem, but indispensable for protein synthesis could be identified and quantified. Cluster 2 was enriched in protein involved in histidine biosynthesis (GO:0000105) and Cluster 4 in enzymes implicated in lysine biosynthesis (GO:0009089). Among these enzymes were an aspartate semialdehyde dehydrogenase (Psat4g004640), which functions at a critical junction in the aspartate-biosynthetic pathway leading to lysine and threonine/S-AA biosynthesis, and a dihydrodipicolinate synthase (DHPDS, Psat3g114440) involved in lysine biosynthesis. In particular, DHPDS showed high homology with the DHDPS-2 protein whose mutation in Arabidopsis decreased lysine level (Craciun *et al.*, 2000). The identification of such proteins in pea seeds open prospects for manipulating lysine metabolism in view of improving the AA balance of pea seeds, which are rich in lysine but poor in sulfur AAs (Burstin *et al.*, 2011).

As mentioned above, embryogenesis is associated with high rate of cell divisions and in legumes final seed size depends on the number of cells produced during this phase of development. This dependency implies a tight control of cell division and cell cycle. Cluster 2 (**Figure 26**), which contained 314 proteins preferentially accumulated at 5 DAP was enriched in proteins related to DNA replication initiation (Biological process, GO:0006270) and DNA helicase activity (Molecular function, GO:0003678). In particular, members of the MINICHROMOSOME MAINTENANCE 2–7 (MCM2–7) complex implicated in the S-phase and cell cycle progression could be detected and quantified (**Table S9**). This complex acts as a DNA helicase, unwinding double-stranded DNA ahead of the rest of the replication machinery. In Arabidopsis, the MCM2 gene was reported to be essential for embryo development (Ni *et al.*, 2009). In pea, six *MCM* genes have been identified (Tuteja *et al.*, 2011), and five protein members were detected in the present study in early developing seeds (**Table S9**). In addition,

a pea protein (Psat1g164200) showing high homology with the A-type cyclin-dependent kinase CELL DIVISION CONTROL2 (AT3G48750, CDKA;1) from Arabidopsis was detected at 5 DAP specifically. Cyclin-dependent kinases (CDKs) are at the heart of eukaryotic cell-cycle control and the A-type CDKs function during S-phase and at the G1/S and G2/M transition. In Arabidopsis null *cdka;1* embryos show delayed development and drastically altered cell numbers and sizes (Nowack *et al.*, 2012). Whether in pea PsCDKA;1 could control embryogenic cell proliferation as in Arabidopsis remains to be investigated.

Controlling the switch from cell division to maturation is of particular interest for crop's seeds because it is a key step in the initiation of storage metabolism. Clustering of our proteomic data (**Figure 26**) identified a small cluster with proteins detected at the transition toward filling 9 DAP (Cluster 6) and a larger one composed of proteins showing higher abundance at 9 and 12 DAP (Cluster 1, **Figure 26**). The GO terms with the highest occurrence in Cluster 1 were related to translation, intracellular protein transport and vesicle-mediated transport (**Figure 26 and Table S9**), which is consistent with the appearance of globulins between 9 and 12 DAP (**Table S7**). Although the importance of intracellular protein trafficking in seed storage protein accumulation is well known (Herman and Larkins, 1999; Gallardo-Guerrero *et al.*, 2016), the underlying molecular mechanisms are not well described. Our quantitative proteomic analysis allow us to identify a number of proteins potentially implicated in the transport of globulins from the endoplasmic reticulum (ER), where they are newly synthesized, to the Golgi. In particular, two of the three components of the COPII vesicles which are implicated in ER to Golgi transport (Xiang *et al.*, 2013), SEC23/SEC24 (Psat0S3087g0080) and a small SAR1-like GTPase (Psat3g012960) were identified and quantified in the pea seed proteome (**Table S9**). Interestingly, a recent study aiming at discovering new molecular determinants underlying seed protein composition in *Medicago truncatula* by combining proteomics and genome wide association studies, identified polymorphisms in genes encoding the Medicago orthologs of these pea genes, as being associated with major forms of 7S and 11S globulins (Le Signor *et al.*, 2017). These genes were proposed as good candidates for storage protein trafficking and deposition. The identification of the pea orthologs in our seed proteomic data set and their co-accumulation with the newly made globulins reinforces this hypothesis, and suggests that pea allelic variants for this genes could represent an attractive material for seed quality improvement.

Cluster 1 was also enriched in GO terms related to cellular carbohydrate process (GO: 0044262) including two sucrose synthases, Psat4g019440 and Psat1g39760, and a sucrose-phosphate phosphatase, Psat6g108920. It is well established that in legume seeds, the transition towards reserve accumulation is characterized by a metabolic switch from a high hexose status, which promotes embryo growth by cell division, to a high sucrose environment. This is associated with changes from an invertase to a sucrose synthase pathway (Weber *et al.*, 1995; Weber *et al.*, 1996; Weber *et al.*, 2005). Increased sucrose uptake by embryo, concomitantly with the differentiation of transfer cells, is indeed a key step for storage initiation, with sucrose being channelled to starch biosynthesis (pea seeds accumulate 40% of starch). Intriguingly, our proteomic data did not reveal cell wall invertases (CwINV) preferentially accumulated during early seed development. The only CwINV identified in our proteome dataset accumulated throughout the studied period and was not restricted to early seed development (CwINV2, Psat6g084360, **Table S5**). However, it was interesting to observe that several invertase inhibitors were in Cluster1, in particular Psat0S2823g0080, the pea homolog of the soybean invertase inhibitor GmCIF1 (Tang *et al.*, 2017), which strongly accumulated at 9 and 12 DAP (**Table S5**). Invertase inhibitors are small inhibitory proteins involved in the post-translational control of acidic invertases. In soybean, silencing of GmCIF1 resulted in marked increase of CwINV activity and in bigger seeds with higher accumulation of proteins and starch (Tang *et al.*, 2017). Similarly in tomato, silencing of CIF led to a delay in leaf senescence and improvement of seed filling and fruit size (Jin *et al.*, 2009). We hypothesized that the seed-specific invertase inhibitors identified in the present study could regulate cell wall invertase activity in pea seeds. They are thus interesting candidates for improving seed size and quality in this species.

The appearance of 7S and 11S globulins between 9 and 12 DAP (**Table S7**) prompted us to search for TF that could have a role in the initiation of seed storage protein synthesis. Shotgun proteomics enabled the quantification of 16 TF, indicating that these TFs accumulate in non-negligible amounts during early seed development (**Table S10**). Among these, L1L and FUS3 are well known for their role in embryo development and maturation (Parcy *et al.*, 1997; Kwong *et al.*, 2003; Yamamoto *et al.*, 2009; Lepiniec *et al.*, 2018; Jo *et al.*, 2019). The accumulation of PsL1L in pea seeds preceded that of PsFUS3 and first accumulated at the transition to seed filling (9 DAP). In Arabidopsis, L1L is the most closely related NF-YB member to the master transcriptional regulator LEC1, and is expressed preferentially during seed

development (Kwong *et al.*, 2003). L1L can complement the *lec1* mutation when expressed under the control of the *LEC1* promoter suggesting redundancy between the NF-YB regulators, although their expression during seed development is slightly shifted (Jo *et al.*, 2019). L1L is a regulator of embryo development (Kwong *et al.*, 2003) and has been shown, like LEC1, to activate the promoters of the 12S globulins and of SUCROSE SYNTHASE 2 (SUS2) in combination with NF-YC subunit (NF-YC2, Yamamoto *et al.*, 2009). This transcriptional regulation of protein storage by LEC1/L1L has been proposed to act through the regulation of FUS3 (Kagaya *et al.*, 2005) or by direct binding (Jo *et al.*, 2019; Yamamoto *et al.*, 2009). Whether L1L could have similar function in pea remains to be determined.

Specific antioxidant systems are activated in developing pea seeds under S deficiency

In this study, we also investigated the effect of S deficiency and water stress, applied individually or combined, on the proteome of developing pea seeds. The results showed that the seed response to these stresses relies on a small set of proteins and that the most substantial proteome changes occurred when S deficiency was applied alone (**Figure 27**). This is consistent with data obtained at the transcriptome level of 9 DAP seeds, showing that genes related to similar biological processes were regulated in response to S deficiency and to the combined stress, but that the number of related genes was greater under S deficiency applied individually (Henriet *et al.*, 2019). In the present study, 13 proteins specifically up- or down-accumulated in response to S deficiency alone were identified. Because S deficiency impacted less individual seed weight than the combined stress (**Figure 24** and Henriet *et al.*, 2019), we hypothesized that some of these proteins might help to mitigate negative impacts on seed growth. This mitigation is likely to involve two oxidoreductases, which were specifically up-regulated in response to S deficiency, either at 5 DAP (AOR) or 12 DAP (AER) (**Figure 27**). Their Arabidopsis orthologs (AT1G23740 for AtAOR, and AT5G16990 for AtAER) play key roles in the antioxidant response by scavenging lipid-derived reactive carbonyl species that lead to the carbonylation of molecules, notably proteins (Yamauchi *et al.*, 2011; Yamauchi *et al.*, 2012; Mano *et al.*, 2005). In Arabidopsis, suppression of AtAOR causes oxidative stress, which affects both the expression and activity of enzymes related to carbon metabolism in rosette leaves, leading to growth retardation (Takagi *et al.*, 2016). Although a role of AOR and AER during seed development has not been reported yet, our data suggest the establishment of specific

anti-oxidant systems in developing seeds enabling the scavenging of lipid-derived carbonyls under S deficiency. This would avoid these carbonyl species to react with proteins and alter their activity and expression, and may thus contribute to maintain early growth of the embryo.

Protein carbonylation occurs during seed germination, ageing, dormancy alleviation, and late seed maturation in several species, including *Arabidopsis thaliana* and *Medicago truncatula* (Satour *et al.*, 2018; Rajjou *et al.*, 2008). During late seed maturation, protein carbonylation might contribute to decrease metabolic activities by modifying or denaturing enzymes such as those involved in glycolysis (Job *et al.*, 2005). However, there are presently no report on the impact of protein carbonylation at early stages of seed development associated with intense metabolic activities. Depending on their abundance, reactive carbonyls can either act as signal mediators, notably in response to environmental changes (Islam *et al.*, 2016), or induce cellular damages associated to oxidative stress (Bailly *et al.*, 2008). Hence, studying the mechanisms controlling the abundance of these reactive carbonyls in seeds, and how these mechanisms are influenced by environmental changes, is of key importance. Our results provide a basis for further investigations in this area, with AOR and AER as key candidates for scavenging lipid-derived reactive carbonyls in early developing seeds.

The protein network highlighted a group of antioxidant proteins that are likely to interplay during seed development and in response to stresses

A notable result arising from the seed protein network is the identification of three connected proteins in the largest module that play detoxification roles by repairing oxidized methionine residues in proteins (MSRB, **Figure 30**) or by controlling the redox balance (GST#1, TRX#2) (Vieira Dos Santos and Rey, 2006; Dixon *et al.*, 2005). The three proteins have been shown in several species to be induced by environmental constraints that generate oxidative stress (Rouhier *et al.*, 2006; Hasanuzzaman *et al.*, 2017; Vieira Dos Santos and Rey, 2006). Their up-accumulation in developing pea seeds in response to S deficiency alone or combined with water stress is therefore likely indicative of an oxidative stress response. During the time course investigated, MSRB was only detected at 5 and 9 DAP, and TRX#2 and GST#1 were in clusters 4 and 5, respectively, indicating that these proteins act during seed embryogenesis associated with intense cell divisions. These proteins shared connections between them and with five to seven other proteins not necessarily regulated by stresses, indicating a possible

role during embryogenesis. By examining the links between the different proteins in the antioxidant network, we could propose hypotheses as how the proteins could interplay to control the redox state of early developing seeds, notably under stressful conditions.

In the seed protein network, GST#1 was found to share connections with nine proteins, including MSRB, whose homologs in plants have been shown to play an important role in oxidative stress tolerance by regenerating methionine from oxidized methionine (C.,-W., Li *et al.*, 2012; Rouhier *et al.*, 2006). In Arabidopsis, GSTs were among the proteins of which methionine get oxidized upon oxidative stress (Jacques *et al.*, 2015), and specific GSTs were identified as substrates of MSRB, which reversed the oxidation of GSTs to confer tolerance to oxidative stress (Lee *et al.*, 2014). These data and the observation that MSRB is likely to influence the accumulation of GST#1 (i.e., incoming edge towards GST#1) suggest that MSRB could repair GST#1 within the developing seed in order to maintain active the glutathione-dependent antioxidant system. Of the other proteins predicted to influence the accumulation of GST#1 was 6-Phosphogluconate dehydrogenase (6PGD, Psat7g016680), which produces NADPH with role as a cofactor in the glutathione reduction system. In fact, NADPH enables the reduction of glutathione disulfide (GSSG) to glutathione in a reaction catalyzed by glutathione reductase. Glutathione can then be conjugated with other molecules via GST, as part of the antioxidant system. Hence, the accumulation of 6PGD might provide NADPH for activating the glutathione-dependent antioxidant system in developing pea seeds.

GST#1 was predicted by the network to influence the accumulation of two pea proteins, one of which, TRX#2, may also play a fundamental role in oxidative damage avoidance. TRX#2 has no clear homologs in Arabidopsis but was homologous to Clot thioredoxins from several species, including *Medicago truncatula* and *Phaseolus vulgaris*. Clot thioredoxins are present in only one copy in plants (Chibani *et al.*, 2009), suggesting a non-redundant function that remain to be elucidated. The cross talk between GST#1 and TRX#2 can possible occur through glutathionylation, as previously proposed in Arabidopsis and poplar, where glutathionylation has been shown to modulate the activity of peculiar TRXs (Michelet *et al.*, 2005; Gelhaye *et al.*, 2004). Among the five additional proteins that may influence the accumulation of TRX#2 were a subunit of mitochondrial pyruvate dehydrogenase (MTE2-3, Psat4g184720), which is a potential TRX target (Balmer *et al.*, 2004), and the AOR oxidoreductase proposed above as a candidate for scavenging reactive carbonyls in early developing seeds.

Proteins with phosphorylation/dephosphorylation roles and acting in ABA signaling are likely to interplay in connection with the antioxidant network during seed development

Several hub proteins appeared connected to the antioxidant network (**Figure 30**), one of which was homologous to PP2C phosphatases. One PP2C has been shown in soybean to control seed weight by increasing cell size in the integuments through the activation of seed trait-related genes (Lu *et al.*, 2017). Hence, understanding how PP2C accumulates in seeds might help to improve and stabilize seed size. The abundance of PP2C is supposed to be influenced by several proteins, including SKP1 (S-phase kinase-associated protein 1, Psat1g011720), a component of the Skp1-Cullin1-F-box (SCF) complex that ubiquitinates target proteins for degradation (Zhao *et al.*, 1999; Pauwels *et al.*, 2015). SKP1 has been shown to act as a positive regulator of abscisic acid (ABA) signaling, presumably through SCF-mediated protein degradation (C.,-W., Li *et al.*, 2012). It is noteworthy that PP2C phosphatases, which are negative regulators of ABA signaling (Merlot *et al.*, 2001; Ni *et al.*, 2019), have been proposed to be the target substrates of the SCF-mediated protein degradation (C., Li *et al.*, 2012). This reinforces the present hypothesis, based on the seed protein network, that PP2C accumulation in developing pea seeds could be influenced by SKP1. Functional analysis of SKP1 proteins indicated that they positively influenced seed germination and seedling growth under abiotic stress conditions, possibly by modulating the accumulation of reactive oxygen species (Rao *et al.*, 2018). The precise role of this protein at early stages of seed development remains to be determined. Owing to its link with the antioxidant network (SKP1 is linked to MSRB), it would be of particular interest to investigate its role in relation to the redox state of developing seeds. In this connection, it has been demonstrated that the activity of PP2C phosphatases decreased in response to oxidative stress induced by ABA due to oxidation of its cysteine residues leading to intramolecular dimers (Ni *et al.*, 2019; Meinhard and Grill, 2001). It was therefore interesting to observe that PP2C had an incoming edge from a thioredoxin (TRX#3, Psat7g247280), which could control the reduction of intermolecular disulfide bonds during seed development, thus avoiding the inhibition of PP2C activity.

Putative regulators of the redox state in early developing seeds

Two putative regulators were identified in the antioxidant network that are candidates to coordinate the redox state of developing seeds (**Figure 30**). One, Psat1g094760, was

homologous to DRB4, a double-stranded RNA-binding protein involved in the biogenesis of different classes of small RNA (sRNA) whose targets remain to be identified (Marrocco *et al.*, 2012). DRB4 was not regulated by stresses but connected to GST#1 and 6PGD, suggesting a relationship with the glutathione system that remains to be investigated. The second putative regulator, Psat5g115080, was homologous to MOS1 (MODIFIER OF snc1-1, AT4G24680), which regulates plant immunity and cell-cycle progression (Zhang *et al.*, 2018; Li *et al.*, 2010). The direct targets of MOS1 controlling the cell cycle have not been identified yet. In pea seeds, MOS1 was not regulated by stresses but, in the protein network, it carried out an outgoing edge directed towards GST#1 and TRX#2, suggesting that MOS1 could influence their accumulation during seed development. Studying the relationship between MOS1 and these redox-sensitive enzymes merits further investigations, notably in the context of cell cycle progression. Indeed, reactive oxygen species and low molecular weight antioxidants, such as nuclear glutathione, modulate progression through the mitotic cell cycle, probably via post-translational modifications of cysteine residues in the nucleus (Foyer *et al.*, 2018; Diaz Vivancos *et al.*, 2010). Interestingly, the unique Clot thioredoxin of Arabidopsis fused to the N terminus of a reporter protein was detected in the cytosol, but also in the nucleus (Chibani *et al.*, 2012). Hence, post-translational modifications of cysteine residues in nuclear proteins by Clot thioredoxins, like TRX#2, could possibly occur. Our data open up new perspectives to investigate the relationship between TRX#2, GST#1, MOS1 and cell cycle progression in pea seeds, especially in response to the combined stress which dramatically affected the individual seed weight as early as 9 DAP.

CONCLUSION

We have shown here that the response of developing seeds to S-deficiency combined or not with drought relies on a subset of detoxification proteins that may repair or modify protein residues (e.g. MSRB, GST, TRXs). In particular, of the proteins specifically induced in response to S deficiency were scavengers of reactive carbonyl species (AOR, AER) that might contribute to maintain early growth of the embryo compared to the combined stress condition. By building a seed protein network, we highlighted antioxidant proteins that are linked together and are likely to play a central role during seed development since they shared many connections with proteins not necessarily regulated by stresses. Other proteins connected to

this antioxidant network were highlighted that might play key roles in modulating the redox-state of early developing seeds (SKP1, PP2C), notably in relation to cell cycle progression (DRB4, MOS1). Although the direct relationship between the pea seed proteins identified for the first time in this study has not been investigated yet, these data advanced our understanding of the redox network in early developing seeds, and open numerous perspectives for investigating the molecular basis of the putative interactions. Moreover, it suggested that extensive post-translational modifications of seed proteins (e.g. disulfide bridge formation, carbonylation, methionine oxidation) occur during early seed development and in response to stresses that could be the subject of future work.

SUPPLEMENTARY INFORMATION

Supplementary data with big data set will be available online once published.

<u>Chapitre IV :</u> Etude multi-omique des feuilles de pois au cours des stades précoces de la phase reproductive : impact d'une carence en soufre combinée à un stress hydrique

<u>Chapitre IV :</u> Etude multi-omique des feuilles de pois au cours des stades précoces de la phase reproductive : impact d'une carence en soufre combinée à un stress hydrique

Approches

Mesures phénotypiques à 5 stades précoces de la phase reproductive chez le pois cultivé dans 4 conditions : non limitante en soufre et en eau, carencée en soufre, stress hydrique modéré (ou ré-arrosage au 5ème stade), combinaison des stress.

Mesures physiologiques à ces 5 stades sur les dernières feuilles formées (contenu relatif en eau et potentiel osmotique) et sur les feuilles des deux premiers nœuds reproducteurs (teneur en chlorophylle et conductance stomatique)

Analyse multi-omique (transcriptome, protéome, métabolome, et ionome) des feuilles des deux premiers nœuds reproducteurs aux 5 stades précoces de la phase reproductive, et étude de l'effet des stress simples et combinés.

Principaux résultats

- Les stress simples et combinés affectent le développement des feuilles de la partie reproductive, et les caractéristiques physiologiques des feuilles (potentiel osmotique, teneur en chlorophylle et conductance stomatique) varient en réponse à une carence en soufre et au double stress.

- Le double stress induit une accumulation de métaux dans les feuilles aux derniers stades étudiés. Ces métaux sont susceptibles d'augmenter la sensibilité du pois au double stress, et d'expliquer pourquoi un double stress prolongé conduit à la nécrose des feuilles.

- Une reprogrammation transcriptionnelle en réponse au double stress a lieu dans les feuilles aux premiers et derniers stades étudiés. Cette reprogrammation fait intervenir des gènes impliqués dans les processus de modifications post-traductionnelles et de transport des protéines.

- Des changements dans le protéome des feuilles ont lieu à la fin du double stress et lors du ré-arrosage, mettant en lumière des protéines qui jouent un rôle clé dans les processus de détoxification, notamment liés à l'accumulation des métaux.

Multi-omic study of pea leaves during the early stages of the reproductive phase: impact of sulfur deficiency combined with water stress

Henriet *et al*., in preparation

INTRODUCTION

In legumes, seed quality relies on protein content which, in pea, is about 23% (Burstin *et al.*, 2011). Nitrogen (N) accumulation by seeds during the filling phase depends on nitrate uptake by roots and/or symbiotic fixation of atmospheric N_2 within nodules, which represent the two complementary modes of N nutrition in legumes. However, this exogenous N is usually insufficient to fulfil the seed high-N demand, and endogenous N previously accumulated in the plant vegetative part needs to be remobilized to sustain seed protein accumulation (Salon *et al.*, 2001). In pea (var. *Caméor*), N remobilization from vegetative parts contributes to 71% of the total N in mature seeds borne on the first two nodes (Schiltz *et al.*, 2005). Leaf proteins constitute the principal source of remobilized N. In particular the highly abundant proteins from the photosynthetic machinery are degraded, ultimately leading to leaf senescence including chlorophyll breakdown, leaf yellowing and finally death. In pea, the transcriptional reprogramming of source leaves at stages preceding the sharp decrease in chlorophyll breakdown was recently investigated (Gallardo *et al.*, 2019). Transcriptomic changes occurring in leaves during early seed development included an up-regulation of genes encoding transporters, in particular sulfate transporters that might sustain sulfur metabolism in leaves of the reproductive part (Gallardo *et al.*, 2019).

Drought and sulfur deficiency are two abiotic stresses that may occur simultaneously in fields due to climate change, sulfur emission regulations and low-input agricultural practices. In pea, drought stress is particularly acute during the reproductive phase, although its overall effect depends on intensity and duration (Ney *et al.*, 1994; Guilioni *et al.*, 2003; Prudent *et al.*, 2015). Early water stress response is characterized by ABA-mediated stomatal closure and by a decrease in cellular expansion (Chaves *et al.*, 2003; Anjum *et al.*, 2011). While stomatal closure reduces water loss, it can also decrease plant photosynthesis and biomass accumulation

(Chaves *et al.*, 2003). Many studies have investigated the effect of drought at the transcriptome and metabolome levels and a common trend in the plant response's includes the regulation of hormones, sugars and amino-acid metabolism, the synthesis of osmoprotectants and detoxifying enzymes, and the establishment of anti-oxidant systems to remove excess levels of ROS and re-establish the redox balance of the cell (Chaves *et al.*, 2009). Previous studies on water stress have suggested that S nutrition could play a key role in the plant response, in particular *via* the synthesis of S-containing metabolites implicated in redox (glutathione), osmoprotectant (choline-O-sulfate) or repair (S-adenosylmethionine) processes (Chan *et al.*, 2013). Sulfate is also involved in cysteine synthesis which is required for the production of ABA (Cao *et al.*, 2014; Malcheska *et al.*, 2017).

We previously showed that drought combined with sulfur deficiency greatly affected seed yield and quality in pea (Henriet *et al.*, 2019). This could be associated with the accumulation in the young developing seeds of specific proteins implicated in the maintenance of the redox-homeostasis to avoid cellular damages in response to stresses (**Article 2, Chapitre III**). In this study, we aimed at analysing the physiological changes occurring in the leaves, source of nutrients for the developing seeds, and to identify the molecular mechanisms established in these source organs in response to the single or combined stresses. Because plant stress responses are dynamic and involve complex cross-talk between regulatory processes such as adjustment of metabolism and gene expression, we developed a multi-omics analysis which included metabolomics, proteomics, transcriptomics and ionomics using a sampling time-series spanning the combined stress period and a post-stress sampling point. This comprehensive description of the pea leaf response to sulfur deficiency combined or not with water stress highlighted the establishment of a rapid transcriptional reprogramming in leaves in response to the combined stress and molecular processes that are likely to protect cells against ROS damages, notably due to the accumulation of metal ions at later stages of the combined stress.

MATERIALS AND METHODS

Stress imposition, leaf sampling and measurements

Pea plants (*Pisum sativum* L., 'Caméor' genotype) originate from the same experiment as described in Henriet *et al.* (2019). Plants were subjected to S deficiency (S-) after three weeks of growth and water stress (WS) was imposed in the 4PMI platform at flowering of the 2nd or 3rd flowering node until soil water content reached 50% of the maximum water-holding capacity (100%) of the substrate. After 9 days (d), plants were re-watered normally. Control plants, grown in parallel, were well supplied with sulfur (S) and water throughout development. Leaves of the first two reproductive nodes were collected just before water stress imposition (T0), 2, 5, and 9 days after water stress imposition (T2, T5, T9), and during the re-watering period of the WS and double-stressed plants (i.e 12 days after the beginning of water stress imposition, T12) (**Figure 31**). For each condition and time point, leaves from the first and second flowering node were collected. Four biological replicates were sampled, each replicate consisting of a pool of two leaves from two plants. The day before leaf collection, we measured (in the 4PMI platform) the relative chlorophyll content of these leaves using a SPAD–502 chlorophyll meter (Minolta Camera Co. Ltd, http://www.konicaminolta.eu/) and stomatal conductance using a portable photosynthesis measurement system (LI-6400. LI-COR, Inc. Lincoln, NE, USA). After collection, leaves were immediately frozen in liquid nitrogen and stored at -80°C until RNA and protein extraction, elemental analysis and sulfate measurement (see below). The number of vegetative and reproductive nodes was recorded on each individual plant and plants were scanned for image analysis to calculate leaf area of the vegetative and reproductive nodes. Leaflets from the last fully-expanded leaves were collected for estimating relative water content (RWC, three leaflets used) as described in Smart (1974) and osmotic potential (three leaflets) as described in (Sorin *et al.*, 2015). The remaining leaves and stems from the vegetative and reproductive parts were then dried at 80°C for dry weight measurements.

Sulfate measurement

Anions were extracted from 30 mg of freeze-dried plant material and were initially mixed with 1.5 ml of a 50% ethanol solution. After incubation at 40°C for 1h, the extract was centrifuged at 12 000g for 20 min and the supernatant was collected. This step was repeated on the pellet

and the resulting supernatant obtained was pooled with the previous one. All these operations (*i.e.* incubations and centrifugations) were repeated twice, but with 1.5ml of ultra-pure water and incubation at 95°C. All the supernatants were pooled and evaporated (Univapo100H, UniEquip, Fraunhoferstr, Martinsned) to remove ethanol and then the supernatants were lyophilized (Beta1-8 LD Christ Bioblock Scientific, Illkirch, France). The dry residue was suspended in 1.5 ml of ultra-pure water. Then, sulfate content was determined by high performance liquid chromatography with a conductivity detector (Integrion HPIC, Thermo Scientific-Dionex, Villebon-sur-Yvette, France). The eluent solution for anion analysis consisted of 9 mM Na_2CO_3 and was pumped isocratically over an analytical column (AS9-HC 4*250mm). Anion measurements were done using the Chromatography Data System software Chromelon 7 (software version 7.2, Thermo Scientific-Dionx, Villebon-sur-Yvette, France).

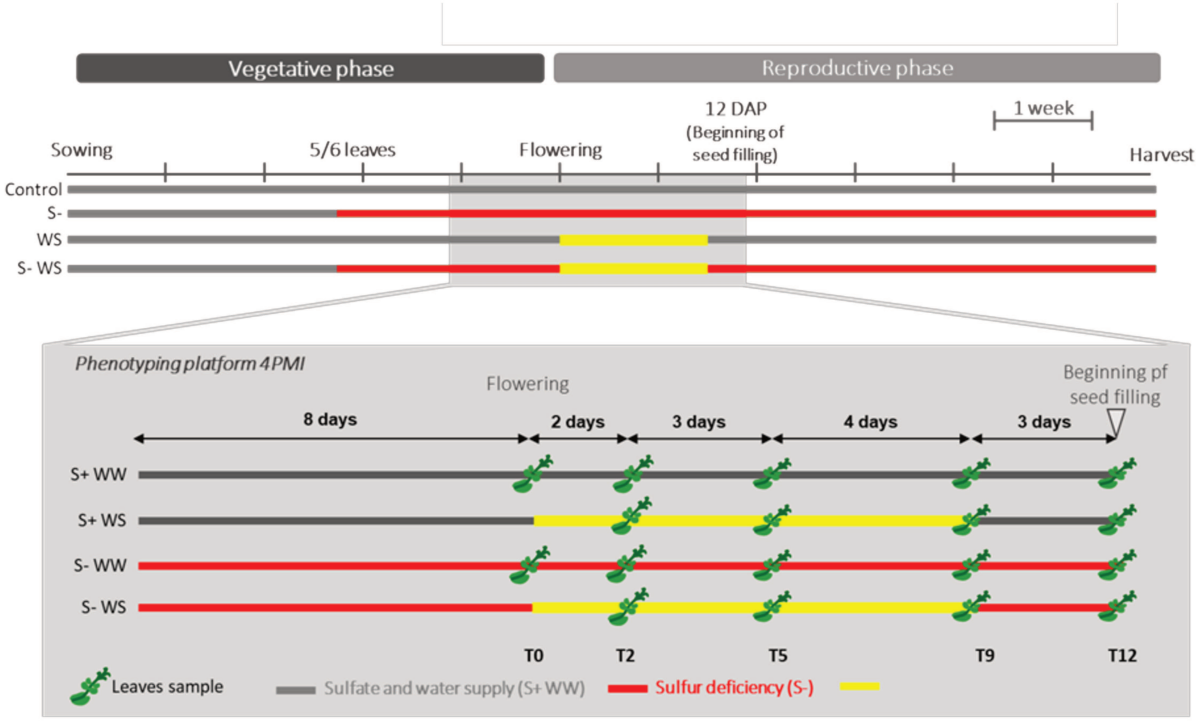


Figure 31. Experimental design for studying the leaves responses under standard, water stress and/or S-deficient conditions. Stress imposition and leaf collection. Control, well-watered plants under non-limiting S-condition; S-, plants deprived of S from a mid-vegetative stage; WS, plants subjected to water stress from flowering of the 2nd or 3rd reproductive nodes for 9 days, then re-watered for recovery; S-WS, plants subjected to a combination of the two stresses. The leaves from the first two reproductive nodes were collected at five stages: 0 days (before the application of the water stress); 2, 5 and 9 days (after the beginning of the water stress); and 12 days (corresponding at 3 days after re-watering).

Elemental analyses

S, C and N content was first determined on dried, ground leaf samples (four biological replicates) using the Dumas method (Allen *et al.*, 1974) on a Carlo Erba elemental analyzer NC2500 (Thermo Fisher Scientific, http://www.thermofisher.com/) adapted with a multi-separation column (polytétrafluoroéthylène, 2 mm length, internal and external diameters of 5 and 6 mm, respectively; CE Elantech, http://www.ceelantech.com/). Prior to analysis, 2 mg vanadium pentoxide was added to 5 mg tissues. Second, elemental analyses in the pea leaf samples were performed in collaboration with the PLATIN platform (Plateau d'Isotopie de Normandie, Caen Normandy University). Macronutrients (P, K, S, Ca, Mg), micronutrients (Zn, Mn, B, Cu, Mo, Fe, Ni), beneficial elements (Se, Co, V) and exposure markers or contaminants (Cd, Pb, As) were quantified by high-resolution inductively coupled plasma mass spectrometry (HR ICP-MS, Thermo Scientific, Element 2^{TM}). Dry matter of each sample (40 mg) was re-suspended in 800 µl of concentrated HNO_3, 200 µl of H_2O_2 and 1 ml of Milli-Q water. All samples were then spiked with three internal standard solutions containing gallium, rhodium and iridium with final concentrations of 5, 1 and 1 µg l^{-1}, respectively. After microwave acidic digestion (Multiwave ECO, Anton Paar, les Ulis, France), all samples were diluted with 50 ml of Milli-Q water to obtain solutions containing 2.0% (v/v) nitric acid. Before HR ICP-MS analysis, samples were filtered at 0.45 µm using a teflon filtration system (Digifilter, SCP Science, Courtaboeuf, France). Quantification of each element was performed using external standard calibration curves and concentrations were expressed in µg g^{-1} of leaves. We also determined carbon, nitrogen and S contents of leaves following the procedure described in **Chapter II**.

Phytohormone and Metabolite quantification

Phytohormones and metabolites were extracted from leaf tissue which corresponded to the same samples as those used for RNA, protein and elemental analyses (4 replicates per time and treatment). Salicylic acid and jasmonic acid were extracted and quantified by reverse-phase ultra-performance liquid chromatography mass spectrometry as described in (Lemarié *et al.*, 2015). Analysis of amino acids (AA), organic acids (OA) and sugars were performed by liquid chromatography (AA) or gas chromatography coupled to a flame ionization detector (GC-FID, OA and sugars) as described in (Ourry *et al.*, 2018).

Statistical analyses of phenotypic, physiologic, ionomic and metabolomic data

Statistical analyses of data were conducted using the R software (version 3.5.1) (R Core Team, 2018). Significant differences (P<0.05) were identified by one-way ANOVA followed by a Student-Newman-Kheul (SNK) post-hoc test.

Proteomics and transcriptomics of leaves

The four replicates of leaf samples in **Figure 31** were subjected to transcriptomics and proteomics. Transcriptomics was carried out in collaboration with the GeT core facility (http://get.genotoul.fr) and proteomics was carried out in collaboration with the PAPPSO platform (http://pappso.inra.fr/). RNA extraction, RNA-seq and statistical analysis of transcriptomics data were performed as described in the Materials and Methods of **Chapter II**. Protein extraction, shotgun proteomics and statistical analysis of the data were carried out as described in Materials and Methods of **Chapter III,** except that the proteomics data were not separated according to the developmental stages.

Hierarchical clustering based on their expression/accumulation patterns was performed using the pheatmap R package (Kolde, 2018). The clustering method was “complete” with “Euclidean” distance measure. Venn diagrams were done using the gplot R package (Warnes *et al.*, 2016).

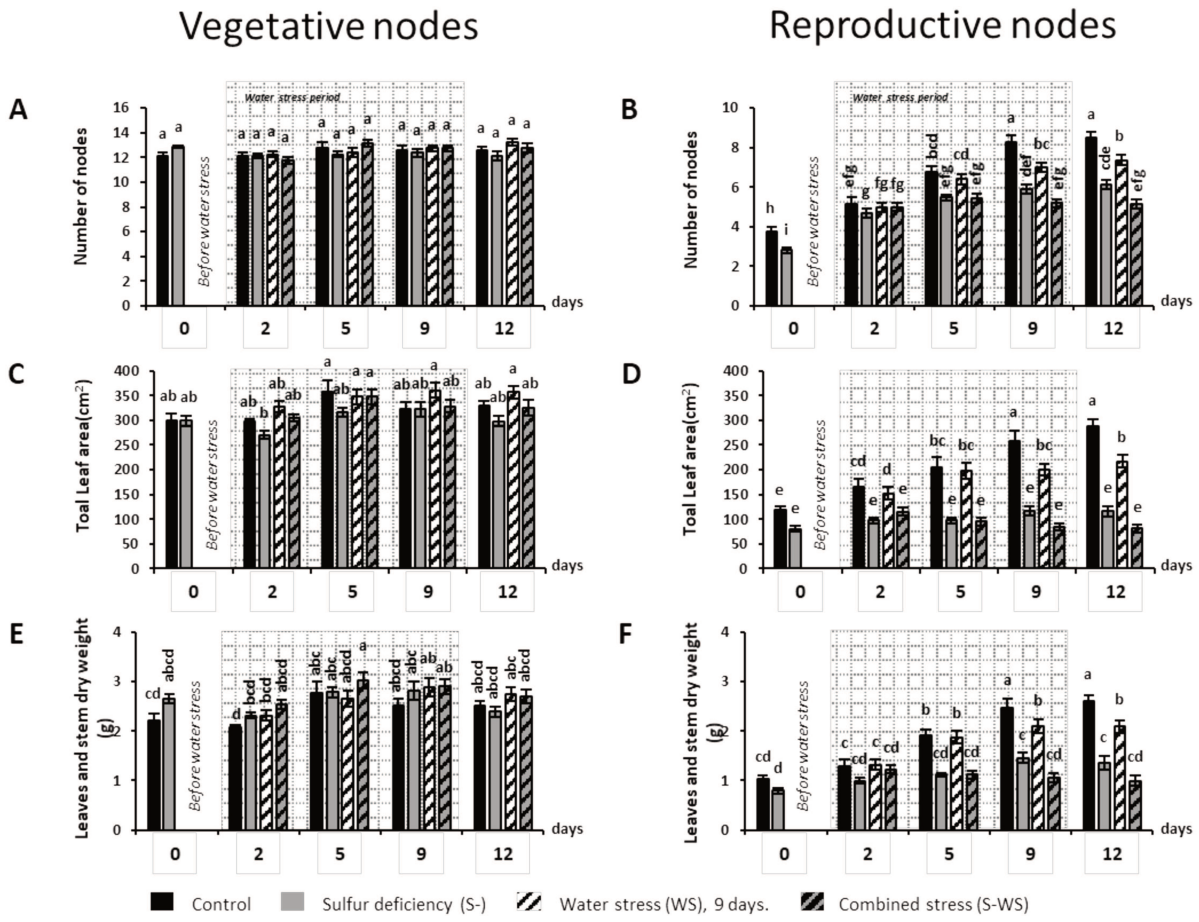


Figure 32. Effects of water stress combined with S deficiency applied during the reproductive phase on phenotypic characteristics in pea. Vegetative nodes (below the first flowering node) and reproductive nodes (nodes with flowers/pods) were treated separately. **(A-B)** Number of nodes. **(C-D)** Total leaf area (cm^2). **(E-F)** Dry weight (g) of the leaves and stem. Grid indicates the water stress period. Values are means ± standard errors (n=8 plants). Different letters above each bar represent significant differences ($P<0.05$, ANOVA followed by a SNK test).

RESULTS AND DISCUSSION

Leaf development was affected by the single and combined stresses

To study the impact of water stress and/or S deficiency on pea leaf development, three stress conditions were used (**Figure 31**): S-deficiency (S-) applied from the mid-vegetative stage, a moderate water stress (WS) for nine days during the early reproductive phase, and the two stresses combined (S-WS). For comparison, control plants were grown in parallel under sufficient water and S supply. Phenotypic measurements were recorded at five time points: before the imposition of water stress (T0); two (T2), five (T5) and nine (T9) days after the beginning of water stress; and 3 days (T12) after the end of the water stress period (*i.e.* during re-watering) (**Figure 31**). Phenotypic measurements carried out on eight plants per treatment and time point revealed few or no changes in the number of nodes (**Figure 32A**), leaf area (**Figure 32C**) and leaf dry weight (**Figure 32E**) from the vegetative part in response to the single or combined stresses. This indicated that S deficiency applied at a mid-vegetative stage did not affect the development of the vegetative part, presumably due to the presence of sulfate in the vacuole that can fulfil S requirements in case of reduced S uptake (Kataoka *et al.*, 2004). In contrast, water stress and S deficiency, alone or combined, induced significant changes in the development of leaves on the reproductive part. There were less reproductive nodes in response to all stress conditions compared to control plants at T5, T9 or T12, depending on the conditions (**Figure 32B**). The double-stressed plants were the most impacted with a decrease of -40% in the number of reproductive nodes at T9 and T12. Re-watering until maturity did not overcome this strong effect of the combined stress since the number of nodes remained strongly affected at maturity (Henriet *et al.*, 2019). This decrease in the number of flowering nodes is explained by an early arrest of flowering of the stressed plants. This was accompanied by an important decrease in total leaf area (-25% WS, -60% S-, -72% S-WS, **Figure 32D**) and dry weight (-20% WS, -48% S-, -62% S-WS, **Figure 32F**) of the reproductive nodes, thus demonstrating the negative effect of the single stresses on leaf development and growth on the reproductive part and additive effects in response to the combined stress.

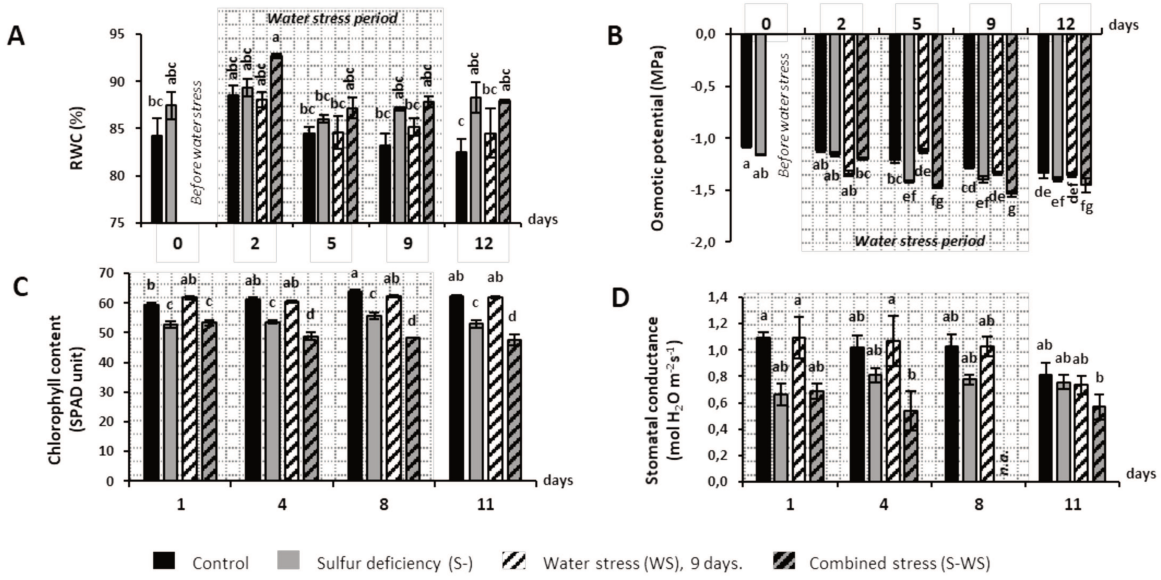


Figure 33. Effects of water stress combined with S deficiency applied during the reproductive phase on physiological characteristics of pea leaves. (A) Relative Water Content (RWC, %) and **(B)** osmotic potential (Mpa) were measured on the last fully expanded leaf before water stress imposition (0), during the water stress (2, 5 and 9 days) and after three days of re-watering (12 days). (n=4 samples). **(C)** Chlorophyll content (SPAD unit) (n=8 plants) and **(D)** stomatal conductance ($MolH_20.m^{-2}.s^{-1}$) (n=4 samples) were measured on the leaf of the first reproductive node during the water stress (1, 4 and 8 days) and after 2 days of re-watering (11 days). Grid indicates the water stress period. Values are means ± standard errors. Different letters above each bar represent significant differences (P<0.05, ANOVA followed by a SNK test); n.a., non available.

Sulfur deficiency combined with water stress improved leaf water potential but affected leaf photosynthesis.

Physiological measurements were performed on the last fully expanded leaves collected at the five different time points from T0 to T12. No significant changes were observed for the relative water content (RWC), neither for the WS plants, which is in accordance with the moderate effect of the water stress applied (Henriet *et al.*, 2019), nor for the S- and S-WS plants (**Figure 33A**). The osmotic potential presented no significant change in response to water stress but significantly decreased at T5 and T9 in S-deficient and combined stress conditions (**Figure 33B**). Decrease in osmotic potential in response to S-deficiency has been reported in *Brassica* (Sorin *et al.*, 2015). The authors suggested that this could be due to metabolic disorders, such as a release of free AA from protein degradation.

The relative chlorophyll content and stomatal conductance of the leaf samples, measured the day before sample collection, from T1 to T11 (**Figure 33C**), showed no impact of the moderate water stress alone. In contrast, S deficiency affected leaf chlorophyll content, as previously shown in *Medicago truncatula* (Zuber *et al.*, 2013), where a negative effect of S deficiency on the photosynthetic activity of leaves was proposed. This impact was exacerbated when S deficiency was combined with water stress (from T4 to T11, **Figure 33C**), suggesting low photosynthetic rates in leaves of the double-stressed plants. The leaf stomatal conductance of S-deficient plants subjected or not to water stress was reduced compared to control plants (**Figure 33D**). A similar effect of S deficiency on stomatal conductance was observed in rapeseed (Lencioni *et al.*, 1997) or sugar beet plants (Kastori *et al.*, 2000), and in the latter case this could be related to a decrease in leaf stomatal density (Kastori *et al.*, 2000). The decrease in stomatal conductance in pea leaves could also be explained by stomatal closure in S- or S-WS conditions. S nutrition has been proposed to be one of the factors regulating plant photosynthesis. It is therefore possible that the reduction of leaf chlorophyll content in response to S deficiency resulted from leaf stomatal closure, which could decrease endogenous CO_2 and affect photosynthesis at early reproductive stages (Chaves, 1991).

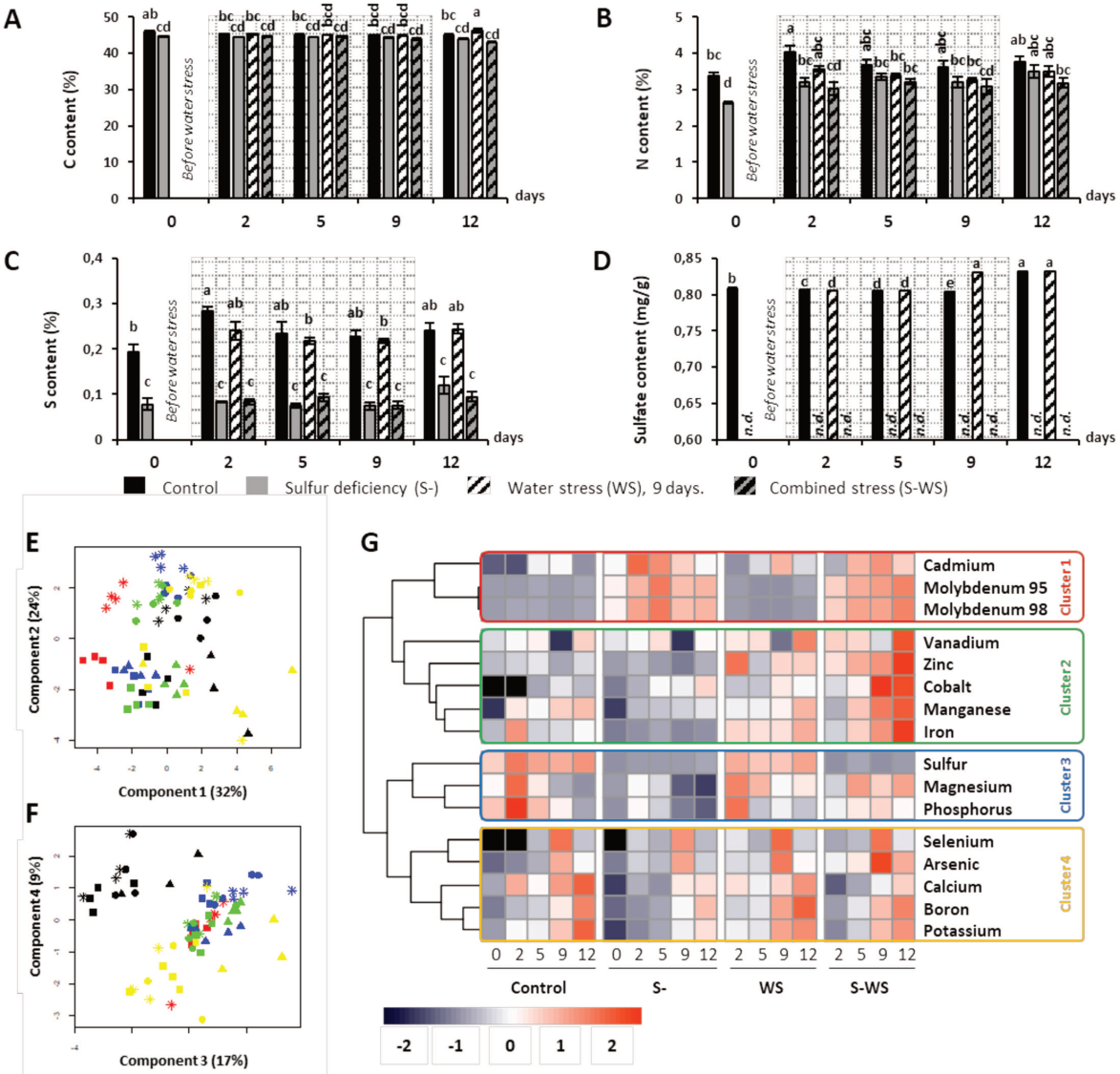


Figure 34. Elemental analysis in response to water stress and/or sulfur deficiency in the leaves before (0) or during, (2, 5 and 9) the water stress, and 3 days after the re-watering (12) in pea.

(A-C) Nitrogen (N), Carbone (C) and Sulfur (S) content (%) of first two reproductive nodes. **(D)** Sulfate content in the dry biomass (mg per g of dry biomass) of the first two reproductive nodes (n.d., not detected). Grid indicates the water stress period. Values are means ± standard errors (n=4 samples). Different letters above each bar represent significant differences (P<0.05, ANOVA followed by a SNK test). **(E-F)** Principal component analysis of differentially accumulated macro and micro-elements at each stage (0 day [red], 2 days [blue], 5 days [green], 9 days [black], 12 days [yellow]) in different environmental conditions (control [star], sulfur deficiency [square], water stress [circle], and combined stress [triangle]). **(G)** Heatmap representing the hierarchical clustering of macro and micro-elements differentially accumulated in response to stresses (n=4 samples). The gradual color change from red to blue represents up- to down-accumulation across stages and conditions (Z-score value).

S-, S-deficiency alone; WS, water stress alone at 2,5 and 9 days, re-watering at 12 days; S-WS, combined stress.

Analysis of the pea leaf ionome identified elements with possible toxic roles that accumulate specifically in response to the combined stress

We first measured the level of S, N, and C in leaves of the first two reproductive nodes collected at the different time points from T0 to T12 using the Dumas method (Allen *et al.*, 1974) (**Figure 34**). C content did not vary significantly, except at T12 for the WS condition (the re-watering period), where a slight but significant increase in C content was observed (**Figure 34A**). This possibly reflects an increased concentration of sugars that could help plants to recover from water stress. Indeed, sugars, such as sucrose, have been shown to accumulate in plants for osmotic adjustment in response to abiotic stresses (Slama *et al.*, 2015), and specific sucrose synthase genes were found to be up-regulated in Arabidopsis leaves in response to drought that might help to meet the increased glycolytic demand (Déjardin *et al.*, 1999). Concerning leaf N content, it decreased significantly in response to S deficiency at T0 and T2 (**Figure 34B**), suggesting a reduced or delayed loading of N in leaves of the reproductive part during early flowering or a higher remobilization of N to sink tossues. As expected, the most marked changes were in leaf S content, which was significantly reduced in response to S deficiency alone and to the combined stress (**Figure 34C**). It is noteworthy that under control condition, leaf S content increased significantly between T0 and T2, which was not the case in response to S deficiency combined or not with water stress: under these conditions, S content remained low and stable during the studied period. This suggests that at T0, the sulfate store has been already used to sustain growth and development. Accordingly, the concentration of sulfate in leaves of S-deprived plants was too low to be detected, whereas non-negligible amounts of sulfate (~0.8 mg/g on a dry weight basis) were identified in leaves of plants well-supplied with S (**Figure 34D**). The data suggest that S remaining in leaves of S-deprived plants might be bound to molecules, such as proteins.

We next enlarged the elemental analysis to other macro- and micro-elements by subjecting pea leaf samples to a HR ICP-MS analysis. A total of 19 elements were quantified and 16 presented significant variations in their accumulation in responses to stresses. A principal component analysis was performed from the quantitative data of these 16 metabolites (**Figure 34E-F**). The first principal component explained 32% of the data variance compared to 24% for the second component. The pea leaf samples were mainly separated on the first component with regards to the developmental stage (T0, red; T2, blue; T5, green; T9, black;

T12, yellow) and on the second component with regard to S nutrition (Control, star; WS, circle; S-, square; S-WS, triangle). The third and fourth axes explained 17% and 9% of total variation, respectively, separating the pea leaf samples according to the developmental stage (T9, T12, and the others). A hierarchical clustering grouped the elements into four clusters according to their accumulation patterns during the reproductive phase (**Figure 34G and Figure 35**). Interestingly, the ionome signature in cluster 1 was characteristic of S deficiency combined or not with water stress, while that in cluster 2 was characteristic of the combined stress condition. Cluster 1 was composed of cadmium and molybdenum (Mo), whose accumulation significantly increased from the T2 stage under S- and S-WS conditions. S assimilation has been shown to be crucial for cadmium stress tolerance in crop plants (Gill and Tuteja, 2011), enabling the synthesis of glutathione (GSH), which protects the plant from various abiotic stresses, including heavy metals. Hence, the increased amount of cadmium in S-deficient leaves of pea might be a consequence of reduced S assimilation and GSH synthesis. High levels of Mo accumulation in tissues of *Brassica* plants under S- conditions has been reported (Schiavon *et al.*, 2012; Maillard, Etienne, *et al.*, 2016). Mo and Sulfate are chemical analogs and Mo can be transported by sulfate transporters (Fitzpatrick *et al.*, 2008). The increase of sulfate transporter activity/expression under S-deficiency (in order to improve sulfate uptake, Maillard *et al.,* 2016), may indirectly increase Mo concentration in pea leaves, as suggested for *Brassica Napus* (Maillard *et al.*, 2016). Interestingly, cluster 2 contained five elements (vanadium, zinc, cobalt, manganese, and iron) that accumulated in pea leaves specifically in response to the combined stress (from T9 and/or during re-watering at T12, **Figure 35**). In particular, cobalt amount was increased by about four times in double-stressed leaves, while it remained similar to the control in S- plants (**Figure 35**). The accumulation of this metal ion can induce toxic effects, as shown in tomato plants where high concentrations of cobalt (or manganese) induced the yellowing of leaves, which later developed brown necrosis (Bakr Ahmed and Twyman, 1953). It is noteworthy that leaves of S-deprived plants experiencing a 3-d longer period of water stress (12 days) turned brown and dried out (**Figure 17A**). It is therefore possible that the accumulation of metal ions specifically in response to the combined stress increased the sensibility of S-deprived plants to water stress, leading to leaf necrosis.

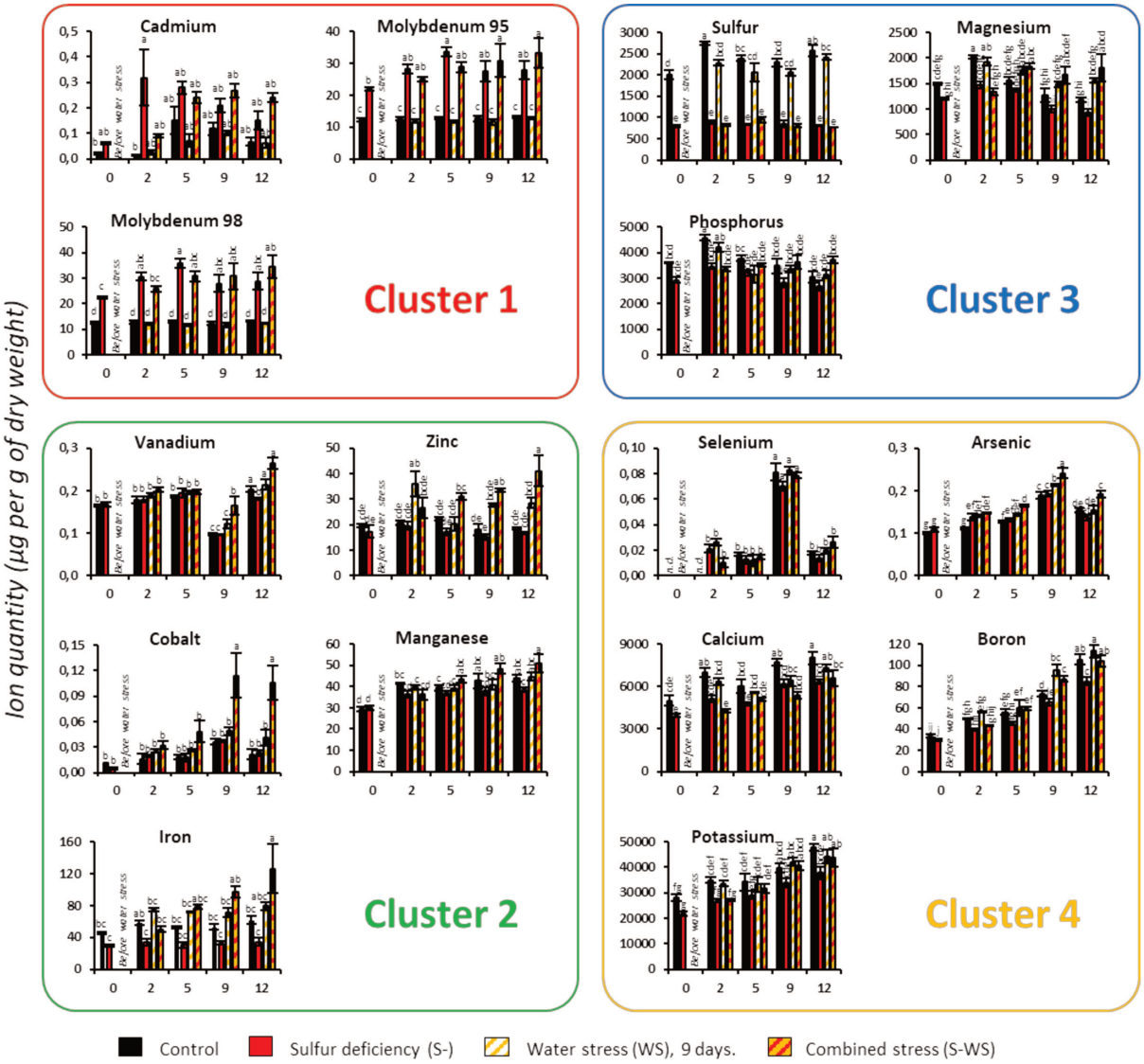


Figure 35. Elements quantity in µg per g of leaf dry weight (n=4 samples).
Values are means ± standard errors. Different letters above each bar represent significant differences (P<0.05, ANOVA followed by a SNK test). S-, S-deficiency alone; WS, water stress alone at 2,5 and 9 days, re-watering at 12 days; S-WS, combined stress.

Metabolome changes in response to sulfur deficiency alone or combined with water stress highlighted metabolites differentially accumulated in response to stresses

A targeted metabolome analysis of the leaf samples enabled to quantify 38 metabolites of which 19 presented significant variations in response to stresses. Data for these 19 metabolites are available in **Figures 36 and Annex 8**. A principal component analysis was performed from these data (**Figure 36A-B**). The first axis explained 37% of the variation and separated the leaf samples with regards to S nutrition (S- and S-WS, squares and triangles on the right; Control and WS Control, stars and circles on the left). The second and third axes explained 21% and 15% of the variation, respectively, and separated the leaf samples with regards to the developmental stages (T0, red; T2, blue; T5, green; T9, black; T12, yellow). A hierarchical clustering grouped these metabolites into five clusters according to their accumulation patterns (**Figure 36C**). Cluster 1 contained metabolites whose accumulation in leaves increased in response to S-deficiency combined or not with water stress. Of these, asparagine, aspartate, and serine, are among the amino acids predominantly found in the phloem (Tegeder, 2014). Interestingly, aspartate and asparagine were much more induced in response to S deficiency than in response to the combined stress, especially at later stages (T9, T12), reflecting a mitigating effect on their accumulation of the combined stress compared to S deficiency alone (**Annex 8**). These data suggest a higher availability of these amino acids for phloem loading and source to sink translocation under S deficiency alone, which might help to provide seeds with N. Accordingly, seed yield along with the quantity of N in the mature seed compartment was significantly higher in S-deprived plants compared to double-stressed plants (Henriet *et al.*, 2019). Cluster 2 was composed of glutamine, one amino acid transported by the phloem to seeds (Tegeder and Masclaux-Daubresse, 2018), and phenylalanine. The accumulation of these two amino acids also increased in response to S deficiency, particularly at early stages (T0 for glutamine, and T5 for phenylalanine). Cluster 3 contained metabolites whose accumulation increased at early reproductive stages (T2 and/or T5), either specifically under S deficiency combined or not with water stress (lysine and valine) or in response to water stress combined or not with S deficiency (tryptophan and isoleucine). Finally, metabolites in clusters 4 and 5 decreased in response to S-deficient conditions. For instance, the quantity of spermidine was significantly reduced in S-deprived leaves at T0,

which could be explained by the central role of the S metabolism in polyamine biosynthesis (*S*-adenosylmethionine is the precursor of polyamine synthesis) (Sauter *et al.*, 2013). This study was complemented by the measurement of the level of two phytohormones (jasmonic acid and salicylic acid) in the pea leaf samples (**Annex 9**). In control condition, both hormones were differentially accumulated during the time course: jasmonic acid accumulated at early stages (T0 to T5), whereas salicylic acid accumulated later on (From T5 to T12). Interestingly, the level of salicylic acid was much higher in S-deprived leaves compared to water-stressed and double-stressed leaves, suggesting a need to maintain the level of this hormone in leaves specifically in response to S deficiency. The underlying mechanisms remain to be investigated.

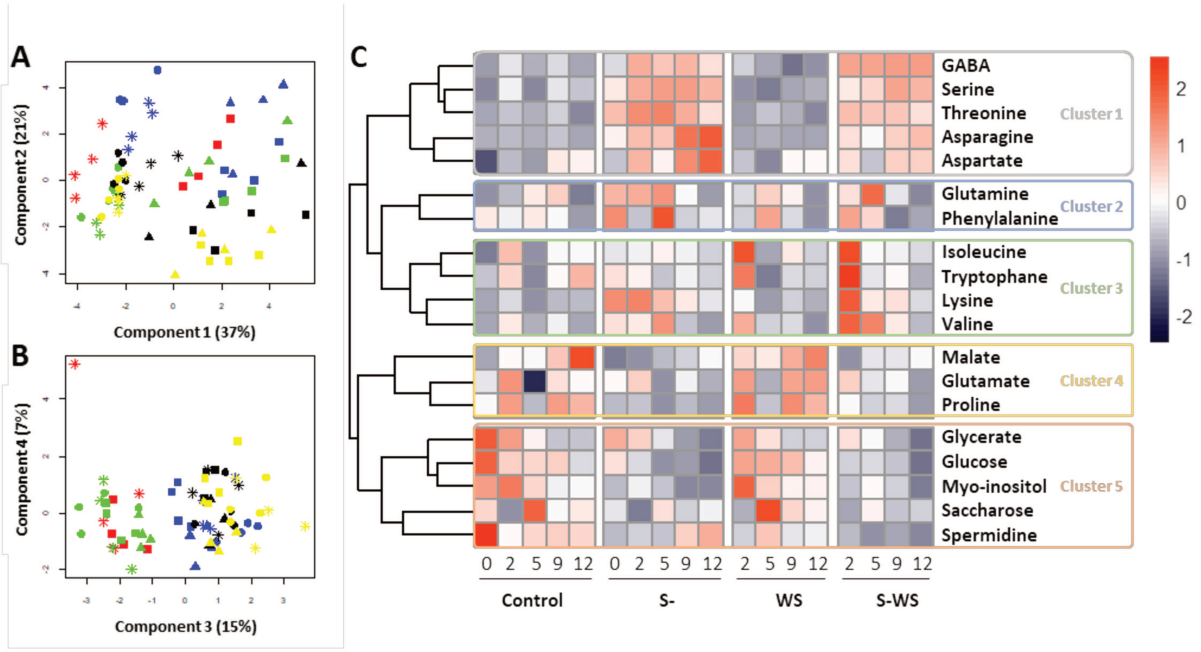
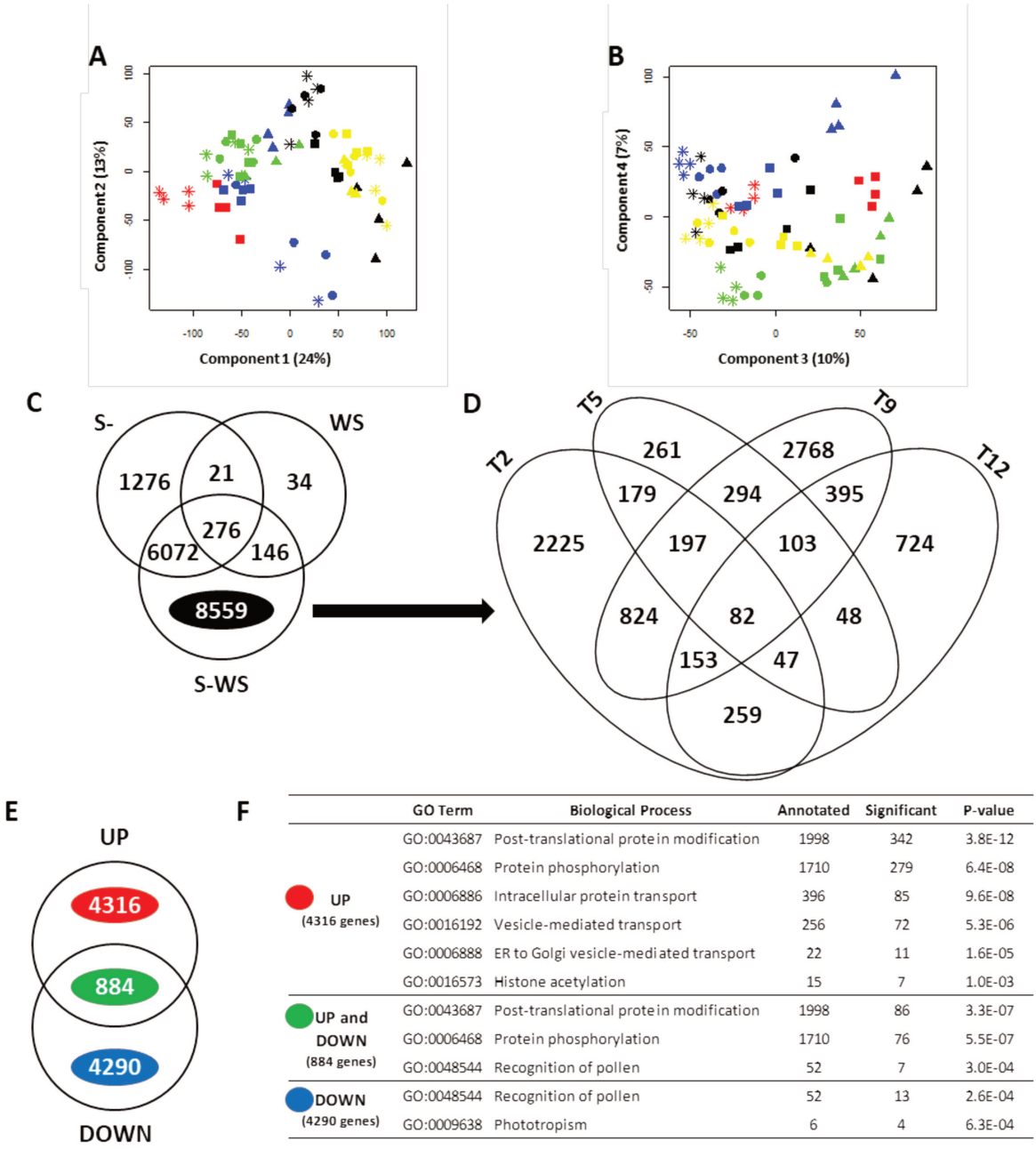


Figure 36. Clustering analysis of differentially accumulated metabolites in response to water stress and/or sulfur deficiency in the leaves before (0) or during, (2, 5 and 9) the water stress, and 3 days after the re-watering (12) in pea. (A-B) Principal component analysis of differentially accumulated metabolites at each stage (0 day [red], 2 days [blue], 5 days [green], 9 days [black], 12 days [yellow]) in different environmental conditions (control [star], sulfur deficiency [square], water stress [circle], and combined stress [triangle]). **(C)** Heatmap representing the hierarchical clustering of metabolites differentially accumulated in response to stresses (n=4 samples) (P<0.05, ANOVA followed by a SNK test). The gradual color change from red to blue represents up- to down-accumulation across stages (Z-score value). S-, S-deficiency alone; WS, water stress alone at 2,5 and 9 days, re-watering at 12 days; S-WS, combined stress.



F

	GO Term	Biological Process	Annotated	Significant	P-value
UP (4316 genes)	GO:0043687	Post-translational protein modification	1998	342	3.8E-12
	GO:0006468	Protein phosphorylation	1710	279	6.4E-08
	GO:0006886	Intracellular protein transport	396	85	9.6E-08
	GO:0016192	Vesicle-mediated transport	256	72	5.3E-06
	GO:0006888	ER to Golgi vesicle-mediated transport	22	11	1.6E-05
	GO:0016573	Histone acetylation	15	7	1.0E-03
UP and DOWN (884 genes)	GO:0043687	Post-translational protein modification	1998	86	3.3E-07
	GO:0006468	Protein phosphorylation	1710	76	5.5E-07
	GO:0048544	Recognition of pollen	52	7	3.0E-04
DOWN (4290 genes)	GO:0048544	Recognition of pollen	52	13	2.6E-04
	GO:0009638	Phototropism	6	4	6.3E-04

Figure 37. Analysis of differentially expressed genes in response to water stress and/or sulfur deficiency in pea. (A-B) Principal component analysis from expressed data of differentially expressed genes at each stage (0 day [red], 2 days [blue], 5 days [green], 9 days [black], 12 days [yellow]) in different environmental conditions (control [star], sulfur deficiency [square], water stress [circle], and combined stress [triangle]). **(C)** Venn diagram showing the numbers of genes differentially expressed in response to stresses compared to the control. **(D)** Venn diagram showing the time repartition of the 8559 genes specifically regulated in combined stress condition (P-adjust<0.05). **(E)** Venn diagram showing the regulation (up- or down-) of the 8559 genes specifically regulated in combined stress condition. **(F)** GO term enrichment analysis for the genes specifically regulated in response to the combined stress. Terms in the 'Biological Process' category are shown for genes up-, up- and down-, and down-regulated in response to the combined stress. For each term, the number of genes present in the genome ('annotated'), the number of genes present in the gene lists ('significant'), and the associated P-value (Fisher's test, threshold of P<0.001) are given. S-, S-deficiency alone; WS, water stress alone at 2,5 and 9 days, re-watering at 12 days; S-WS, combined stress.

Transcriptomics highlighted the role of post-translational protein modifications and intracellular protein transport in helping leaves to cope with the combined stress

Leaf samples collected in the different conditions at T0, T2, T5, T9 and T12 were subjected to paired-end RNA-seq. In between 18–38 million paired-end reads were generated per sample after adapter trimming and filtering of low-quality reads, and 88% were uniquely mapped to the pea reference genome (**Supplementary Table S12**). Of the 29681 genes expressed in the leaf samples, 16384 were differentially expressed (DEG) in response to the stresses (*P*-adjust <0.05), representing 55% of the total number of genes quantified. This result contrasted with previous data obtained on seeds of the same plants where only 11% of the genes were differentially regulated by stresses (Henriet *et al.*, 2019). This indicated that the stresses trigger important molecular regulations in pea leaves, which might help to maintain them in a healthy state to ensure seed production and seed quality for future germination. Annotation and expression values of these differentially expressed genes (DEGs) are given in **Supplementary Table S13.** A principal component analysis was performed from the list of DEG genes (**Figure 37A-B**). The first two components explained 37% of the variations and separated the leaf samples with regards to the developmental stages. In particular, the first axis separated the leaf samples at early stages (T0, red; T2, blue; T5, green) to those at late stages (T9, black; T12, yellow). The third axis explained only 10% of the variation and separated the leaf samples according to S nutrition (S-, square; S-WS, triangle; on the right - Control, star; WS, circle; on the left).

The transcriptomics data enabled the identification of 8559 genes whose expression varied in leaves specifically in response to the combined stress (**Figure 37C**), representing 52% of the total number of genes regulated by the single or combined stresses. Most of the regulations specific to the combined stress mainly occurred at the T2 and T9 stages, with 2225 genes specifically regulated at T2 and 2768 genes specifically regulated at T9 (**Figure 37D**). These data are indicative of a rapid establishment of adaptive processes at the beginning of the water stress period under S deficiency and of profound transcriptional changes by the end of the water stress period that might help to maintain the metabolism of S-deficient leaves. To explore the molecular mechanisms specifically regulated by the combined stress, the expression pattern of the 8559 genes specifically regulated in response to the combined stress

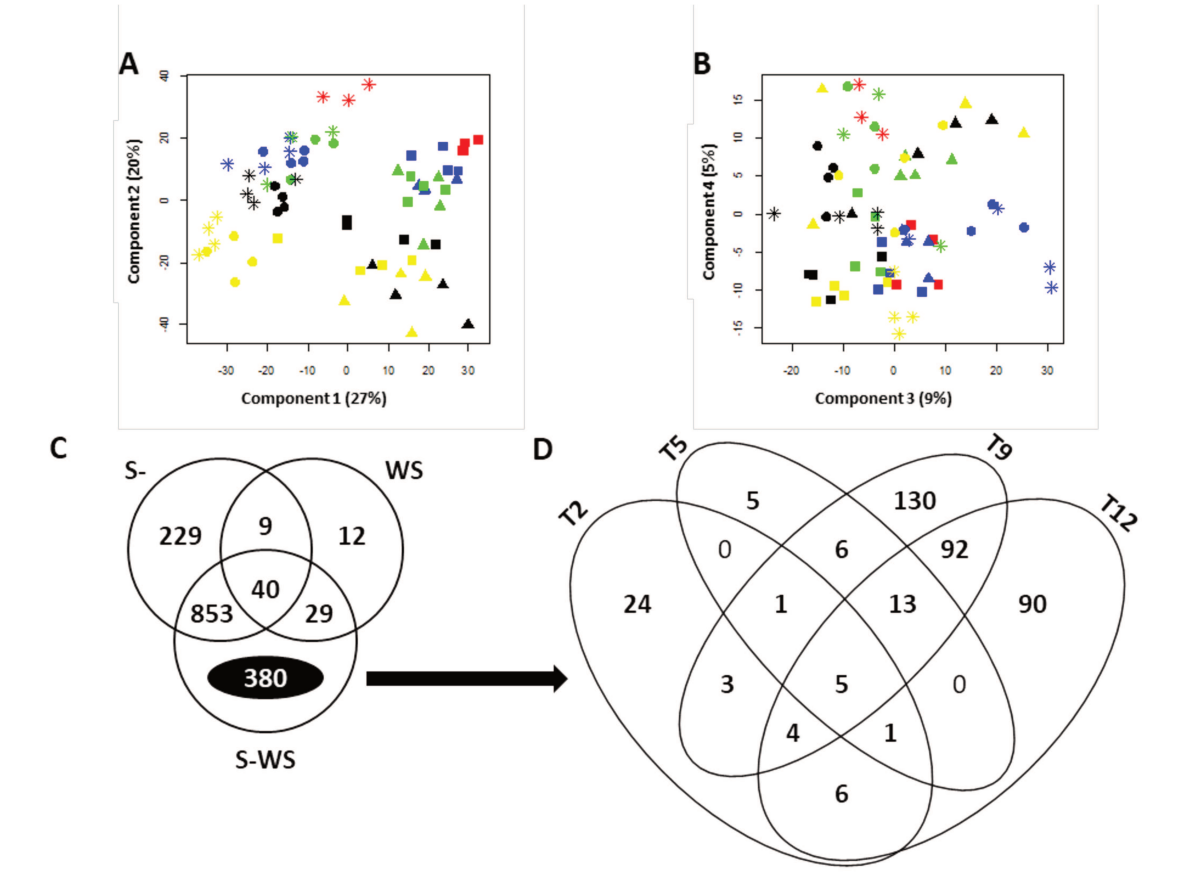
was examined. This revealed 4316 and 4290 genes respectively up- and down-regulated under this condition (**Figure 37E**). Moreover, 884 genes were up- or down-regulated, depending on the developmental stages. GO enrichment analyses revealed a significant over-representation (Elim method/Fisher's test, P<0.001) of genes related to post-translation protein modification and protein transport in the list of genes up-regulated in response to the combined stress (**Figure 37F** and **Table S14**). This highlights a potential role of post-translational processes for the regulation of protein quantity and activity in pea leaves subjected to the combined stress. In particular, genes related to protein phosphorylation (GO:0006468, encoding protein kinases) were enriched in the list of genes up-regulated by the combined stress (**Figure 37F**). Phosphorylation events contribute to rebalancing cellular ion homeostasis in response to stresses in plants, and proteins involved in the translocation of ions have been identified as kinase targets (Haak *et al.*, 2017). Hence, our data furnishes a list of genes that will serve to better understand the signal transduction pathways involving protein kinases governing the leaf response to S deficiency combined with water stress (**Table S15**). The list of genes down-regulated in response to the combined stress was enriched in genes related to recognition of pollen (GO:0048544) and phototropism (GO:0009638) (**Figure 37F**). The 13 genes related to recognition of pollen encode lectin protein/receptor kinases. These proteins are located at the plant cell surface and known to play roles during plant development and in the perception of biotic and abiotic stresses (Bellande *et al.*, 2017). Hence, their down-regulation in pea leaves might contribute to regulate the molecular response to the combined stress. Of the four genes related to phototropism, three were homologous to phytochrome kinase substrate 1 in Arabidopsis, which is a phototropin 1 binding protein required for phototropism (Lariguet *et al.*, 2006). Their down-regulation in response to the combined stress suggests a reduction of light perception in leaves of the reproductive part.

Leaf proteomics highlighted the role of regulators of the redox state in response to combined stress

To go further in dissecting the molecular response of pea leaves to the combined stress, a quantitative proteome analysis (shotgun) of the same leaf samples as used for transcriptomics was performed. Of the 2268 quantified proteins, 1552 were differentially accumulated in response to the stresses (P-adjust <0.05), representing 68% of the total number of quantified proteins. Annotation and accumulation data of these differentially accumulated proteins are

given in **Supplementary Table S13.** Like observed with the transcriptomics, this result differed from the data obtained for early developing seeds (Henriet *et al.,* 2019; **Chapter III**) where only 0.85% of the total number of proteins quantified were differentially accumulated in response to the single or combined stresses. These results indicated that a greater stress is perceived by leaves leading to profound changes in their proteome. A principal component analysis was performed from the quantitative data of the 1552 proteins regulated by the stresses (**Figure 38A-B**). The first two components explained most of the variations (27% and 20%, respectively), separating the leaf samples according to S nutrition (first axis; Control, star; WS, circle; S-, square; S-WS, triangle) and the developmental stages (second axis: T0, red; T2, blue; T5, green; T9, black; T12, yellow). Of the 1552 proteins regulated by stresses, the abundance of 380 proteins varied specifically in response to the combined stress (24% of the regulated proteins) (**Figure 38C**). Most of them were differentially accumulated at the end of the water stress period (T9) or three days after re-watering (T12) (**Figure 38D**). This contrasted with transcriptomics data showing profound changes at the beginning of the water stress period, suggesting that early transcriptome changes contributed to mitigate the effect of the combined stress on the leaf proteome.

Most of the proteins differentially accumulated specifically in the combined stress were either up- (207 genes) or down-regulated (244 genes) (**Figure 38E**). Only two proteins corresponding to a zinc-binding dehydrogenase (Psat6g091760) and a methionine aminopeptidase (Psat2g085800) were up- or down-regulated according to the developmental stages (**Figure 38E**). A GO enrichment analysis from the list of proteins up- and down-regulated was performed that revealed an enrichments of proteins related to specific biological processes (Elim method/Fisher's test, $P<0.001$) (**Figure 38F** and **Table S16**). The list of down-regulated proteins was enriched in proteins related to photosynthesis and metabolism (**Figure 38F**). These data are consistent with the decrease in chlorophyll content and stomatal conductance, suggesting reduced photosynthesis (**Figure 33C-D**). Interestingly, the up-regulated proteins were enriched in proteins related to oxidation-reduction processes, proteolysis, and superoxide metabolic processes (**Figure 38F**). This indicated that the leaf response to the combined stress relied on proteins involved in the regulation of the redox balance and detoxification processes, which is reminiscent to the data obtained for early developing seeds that accumulated similar proteins in response to the combined stress (**Chapter III**).



F

	GO Term	Biological Process	Annotated	Significant	P-value
UP (207 proteins)	GO:0055114	Oxidation-reduction process	1913	34	3.6E-06
	GO:0006508	Proteolysis	877	17	5.2E-04
	GO:0006801	Superoxide metabolic process	23	3	7.5E-04
DOWN (244 proteins)	GO:0009765	Photosynthesis, light harvesting	24	6	2.0E-08
	GO:0042180	Cellular ketone metabolic process	562	19	4.8E-08
	GO:0044106	Cellular amine metabolic process	392	15	4.9E-06
	GO:0006542	Glutamine biosynthetic process	22	4	2.1E-05
	GO:0016114	Terpenoid biosynthetic process	30	4	7.4E-05
	GO:0055114	Oxidation-reduction process	1913	35	2.0E-04
	GO:0006457	Protein folding	285	9	3.2E-04
	GO:0006429	Leucyl-tRNA aminoacylation	4	2	3.4E-04
	GO:0006979	Response to oxidative stress	178	7	4.2E-04
	GO:0006419	Alanyl-tRNA aminoacylation	5	2	5.6E-04
	GO:0009773	Photosynthetic electron transport in photosystem I	6	2	8.4E-04

Figure 38. Analysis of differentially accumulated proteins in response to water stress and/or sulfur deficiency in pea. (A-B) Principal component analysis of accumulation data of the differentially accumulated proteins at each stage (0 day [red], 2 days [blue], 5 days [green], 9 days [black], 12 days [yellow]) in different environmental conditions (control [star], sulfur deficiency [square], water stress [circle], and combined stress [triangle]). **(C)** Venn diagram showing the numbers of proteins differentially accumulated in response to stresses compared to the control. **(D)** Venn diagram showing the time repartition of the 380 proteins differentially accumulated in combined stress only (P-adjust<0.05). **(E)** Venn diagram showing the regulation (up- or down-) of the 380 proteins differentially accumulated in combined stress only. **(F)** GO term enrichment analysis for the differentially accumulated proteins in response to combined stress only. Terms in the 'Biological Process' category are shown for proteins up- and down-regulated in response to the combined stress. For each term, the number of genes present in the genome ('annotated'), the number of genes present in the gene lists ('significant'), and the associated P-value (Fisher's test, threshold of P<0.001) are given. S-, S-deficiency alone; WS, water stress alone at 2,5 and 9 days, re-watering at 12 days; S-WS, combined stress.

Among the 34 up-regulated proteins related to oxidation-reduction processes were two ascorbate peroxidases (Psat2g069720, Psat7g223000), glutathione peroxidase (Psat7g032560), two copper/zinc superoxide dismutases (Psat3g008560, Psat7g191600), and a methionine sulfoxide reductase (MSR, Psat4g135160) (**Table S17**). All these proteins play a key role in protecting plant tissues by scavenging ROS (peroxidases, superoxide dismutase) or by repairing oxidized proteins (MSR). Another isoform of MSR (Psat1g185480) was up-accumulated in early developing seeds in response to the combined stress, indicating a tissue-specific regulation of different MSR isoforms whose understanding merits further investigations. Interestingly, targeted overexpression of a copper/zinc superoxide dismutase and ascorbate peroxidase in chloroplasts of transgenic tall fescue plants conferred protection against heavy metal-induced oxidative damage (Lee *et al.*, 2007). Hence, the up-regulation of these proteins in pea leaves might help to combat the negative effects of the combined stress, which includes the accumulation of metals (e.g. zinc) in response to the combined stress (**Figure 34G and Figure 35**).

CONCLUSION

This integrative study provides a first global view of the physiological and molecular processes underlying the response of pea leaves to S deficiency and water stress. Important changes in the leaf transcriptome were observed when the two stresses were combined, especially at the beginning (T2) and at the end (T9) of the double stress period. This is indicative of rapid transcriptional reprogramming to water stress in pea leaves deprived of S. This reprogramming at T2 is likely to maintain leaf metabolism under the combined stress condition since few proteins varied at this stage. The transcriptomics data pointed out post-translational processes, especially protein phosphorylation, and processes regulating protein transport as central actors in this adaptive response. Moreover, the data revealed an over-accumulation of metal ions, such as zinc and cobalt, in pea leaves at later stages of the combined stress period (T9) and during re-watering (T12). This might generate ROS and consequently have detrimental effects on leaf metabolism. Most proteome changes in response to the combined stress occurred at T9 and T12 and the list of proteins up-regulated was enriched in proteins with roles in ROS scavenging. This might contribute to limit the

oxidative damages and maintain leaf metabolism for seed production on the reproductive nodes. Future work will be directed towards the integration of the omics data obtained to better understand the regulatory mechanisms governing the pea response to S deficiency and water stress.

SUPPLEMENTARY INFORMATION

Supplementary data with big data set will be available online once published.

Chapitre V : Conclusions générales et perspectives de la thèse

L'objectif principal de ces travaux de thèse était de développer des connaissances et une meilleure compréhension : (i) de l'interaction entre une carence en soufre et un stress hydrique modéré lors des stades précoces de la phase reproductive sur la productivité et la qualité des graines, et (ii) des réponses moléculaires associées à ces stress au sein des feuilles et des graines en développement, dans le but d'identifier des gènes cibles pour l'amélioration de la tolérance du pois à ces stress.

Le premier objectif visait à étudier l'impact du stress hydrique et d'une carence en soufre appliqués séparément ou combinés sur le rendement et la qualité des graines matures (**Chapitre II**). Cette étude a permis de mettre en évidence l'impact synergique du stress combiné sur le développement du pois et les composantes du rendement. En effet, le double stress a impacté de manière plus importante ces caractéristiques comparé aux stress simples. Au contraire, le stress combiné n'a pas induit de changement plus important dans la composition protéique de la graine mature de pois comparé à la carence en soufre (Chandler et al., 1984). L'effet de la carence en soufre sur les quantités relatives des globulines, à savoir l'augmentation des globulines 7S (pauvres en soufre) et la diminution des globulines 11S (riches en soufres), est atténué en condition de double stress. L'effet du stress combiné est tel que le ratio 7S/11S n'est pas significativement différent de la condition contrôle. Nous avons mis en évidence que le ratio 7S/11S était corrélé au ratio N/S dans la graine, et que celui-ci variait en fonction du nombre et de la taille des graines produites par rapport à la quantité de soufre et d'azote disponibles. L'accumulation des globulines plus ou moins riches en acides aminés soufrés est régulée au niveau transcriptionnel (Evans et al., 1984; Evans et al., 1985). Il serait donc intéressant d'identifier les mécanismes impliqués dans la perception d'un manque de soufre au niveau des graines, conduisant à l'accumulation de protéines pauvres en acides aminés soufrés pour le maintien de la teneur en protéines. Nos résultats suggèrent que ces mécanismes font intervenir des modifications post-traductionnelles au niveau des facteurs de transcription régulant l'expression des gènes de globulines. En effet, l'analyse transcriptomique des graines au stade de transition entre l'embryogenèse et le remplissage a mis en évidence des SUMO-ligases susceptibles de réguler l'activité d'ABI5, un régulateur de la synthèse de vicilines chez le pois (**Chapitre II**). Ce travaille ouvre des perspectives d'étude des modifications post-traductionnelles de type sumoylation dans la régulation de ABI5 au sein de la graine en développement.

Le second objectif visait à identifier les mécanismes moléculaires mis en jeu dans les graines (organes puits de nutriments) au cours de leur embryogenèse et de leur passage en mode remplissage, et leur modulation en réponse à une carence en soufre et/ou un stress hydrique (**Chapitre III**). La production d'un atlas des protéines accumulées au cours de l'embryogénèse et au début du remplissage des graines de pois a permis de décrypter les évènements métaboliques associés à ces stades précoces du développement de la graine. Par ailleurs, ce travail a mis en évidence peu de changements dans le protéome de ces graines en réponse aux stress. De manières intéressantes, les quelques protéines régulées sont bien connues pour leur rôle antioxydant. En effet, l'analyse différentielle des données protéomiques a mis en évidence le rôle central d'enzymes impliquées dans les processus de détoxification dans la réponse aux stress. La construction d'un réseau protéique à partir de l'ensemble des données protéomiques a permis de découvrir que ces protéines se trouvent au centre du réseau. Elles sont connectées entre elles et avec d'autres protéines non régulées pas les stress, suggérant qu'elles jouent un rôle central dans le métabolisme de la graine en développement, notamment en réponse aux stress. Une perspective est l'étude plus approfondie des protéines impliquées dans les processus de détoxification au sein des graines de pois. Il a précédemment été suggéré que le monoxyde d'azote (NO) produit pendant un stress régule par S-nitrosylation la détoxification cellulaire en modulant l'activité des enzymes du cycle de l'ascorbate-glutathion, de la superoxyde dismutase, de la glutathion S-transférase et de la glyoxalase I (Sehrawat and Deswal, 2014). Il serait donc intéressant d'étudier les modifications post-traductionnelles des protéines identifiées dans notre étude en réponses aux stress au cours du développement de la graine.

Le troisième objectif consistait à identifier les mécanismes moléculaires mis en jeu dans les feuilles issues des premiers nœuds reproducteur (organes sources de nutriments) au cours des stades précoces de la phase reproductive, et leur modulation en réponse à une carence en soufre combinée à un stress hydrique (**Chapitre IV**). L'analyse ionomique a révélé une forte accumulation d'ions métalliques, tels que le zinc et le cobalt, dans les feuilles de pois à la fin de la période de stress combiné (T9) et lors du ré-arrosage (T12). Cette accumulation induirait une toxicité dans les feuilles qui pourrait être la cause de la mortalité des plantes doublement stressées plus de 9 jours (Henriet et al., 2019, **Chapitre II**). Cela est cohérent avec les résultats protéomiques obtenus, mettant en évidence que la plupart des modifications du protéome

en réponse au stress combiné se sont produites à T9 et T12, et montrant que la liste des protéines régulées positivement et spécifiquement en réponse au double stress était enrichie en protéines jouant un rôle dans les mécanismes de détoxification. Cette réponse « tardive » est susceptible de limiter les dommages oxydatifs et de maintenir le métabolisme de la feuille pour la production de graines sur les nœuds reproducteurs. Il serait intéressant de mesurer les ROS et le niveau de carbonylation des protéines pour estimer l'état oxydatif des feuilles au cours du stress combiné. De même, des modifications importantes du transcriptome foliaire ont été observées en réponse au stress combiné, en particulier au début (T2) et à la fin (T9) de la période de double stress. Cela indique une reprogrammation transcriptionnelle rapide en réponse au stress hydrique dans les feuilles de pois dépourvues de soufre. Cette reprogrammation est susceptible de mettre en place des mécanismes de tolérance au stress. Cependant, peu de variations d'accumulation des protéines n'est observée à T2 et les données transcriptomiques ont mis en évidence un enrichissement des processus post-traductionnels, en particulier la phosphorylation des protéines, et les processus régulant le transport des protéines. Il est donc probable que les mécanismes de détoxification soient régulés par des modifications post-traductionnelles au niveau des protéines/enzymes impliquées, favorisant ou inhibant leur activité, et/ou permettant leur translocation dans leurs compartiments d'action. Ces résultats posent de nouvelles questions et ouvrent de nombreuses perspectives de recherche. Il sera intéressant d'explorer les données omiques obtenues pour les feuilles seulement en condition contrôle afin de décrire et caractériser les processus moléculaires qui la composent, puis regarder finement leur modulation en condition de stress. Ces données pourront aussi faire l'objet d'une étude intégrative *via* l'utilisation d'outils d'intégration de données omiques tel que l'approche DIABLO du package R mixOmics (Singh et al., 2019; Rohart et al., 2017). De même, une recherche de régulateurs des processus métaboliques clés pourra être réalisée *via* la construction de réseaux (Huynh-Thu et al., 2010; Huynh-Thu and Geurts, 2018).

Les perspectives de ce travail de thèse seront de rechercher des mutants TILLING pour les gènes et protéines susceptibles de jouer un rôle dans la tolérance du pois aux stress et de les tester en expérimentation. Comme l'objectif est d'améliorer et de stabiliser le rendement et la qualité des graines de pois, des allèles favorables des gènes candidats pourront être recherchés et introgressés dans des fonds agronomiques performants.

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Annexe 1

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RESEARCH PAPER

Water stress combined with sulfur deficiency in pea affects yield components but mitigates the effect of deficiency on seed globulin composition

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Abstract

Water stress and sulfur (S) deficiency are two constraints increasingly faced by crops due to climate change and low-input agricultural practices. To investigate their interaction in the grain legume pea (*Pisum sativum*), sulfate was depleted at the mid-vegetative stage and a moderate 9-d water stress period was imposed during the early reproductive phase. The combination of the stresses impeded reproductive processes in a synergistic manner, reducing seed weight and seed number, and inducing seed abortion, which highlighted the paramount importance of sulfur for maintaining seed yield components under water stress. On the other hand, the moderate water stress mitigated the negative effect of sulfur deficiency on the accumulation of S-rich globulins (11S) in seeds, probably due to a lower seed sink strength for nitrogen, enabling a readjustment of the ratio of S-poor (7S) to 11S globulins. Transcriptome analysis of developing seeds at the end of the combined stress period indicated that similar biological processes were regulated in response to sulfur deficiency and to the combined stress, but that the extent of the transcriptional regulation was greater under sulfur deficiency. Seeds from plants subjected to the combined stresses showed a specific up-regulation of a set of transcription factor and SUMO ligase genes, indicating the establishment of unique regulatory processes when sulfur deficiency is combined with water stress.

Keywords: Abiotic stresses, drought, nutrient partitioning, *Pisum sativum*, sulfur, seed quality, seed transcriptomics, storage proteins.

Introduction

Legumes are able to accumulate large amounts of proteins in their seeds even in the absence of nitrogen (N) fertilizer thanks to their ability to interact symbiotically with soil-borne Rhizobiaceae that fix atmospheric dinitrogen. In pea (*Pisum sativum*), one of the most cultivated pulse crops, seeds contain between 18–34% protein on a dry-weight basis

(Burstin *et al.*, 2011). As in other legumes, pea seed proteins are poor in the sulfur (S) amino acids methionine and cysteine (S-AA). However, being rich in lysine, they complement the protein intake from cereals, which are in contrast poor in lysine (Burstin *et al.*, 2011). About 70% of seed proteins in legumes are seed-storage proteins consisting of 7S (vicilins, convicilins) and 11S (legumins) globulins (Boulter and Croy, 1997). These abundant proteins differ in their nutritional properties. Notably, 11S globulins contain higher levels of S-AA than 7S globulins (1.5% and 0.6%, respectively, in *Medicago truncatula*) (Zuber *et al.*, 2013). Hence, variations in the 7S/11S ratio (which has a mean value of 1 in the pea cultivar 'Caméor'; Bourgeois *et al.*, 2009) could influence the nutritional quality of legume seeds by affecting the dietary intake of methionine and cysteine. Variations in this ratio could also influence the functionality of protein isolates such as their solubility, foaming, and emulsifying capacities (Dagorn-Scaviner *et al.*, 1986; Rangel *et al.*, 2003), or their textural properties (Mujoo *et al.*, 2003).

Variations in environmental conditions influence the final seed yield and the content and composition of proteins in pea (Karjalainen and Kortet, 1987; Bourgeois *et al.*, 2009). One of the environmental constraints affecting crop productivity is water stress (WS), which is predicted to occur more frequently and severely with climate change. In pea, water stress is a major yield-limiting factor (Fougereux *et al.*, 1997), and its impact depends on the intensity of the stress and its duration, and on the phenological stage at which it occurs (Ney *et al.*, 1994; Guilioni *et al.*, 2003; Prudent *et al.*, 2015). Previous studies on water stress have suggested that S nutrition could play a key role in the plant response (Chan *et al.*, 2013, and references therein). Under water stress, sulfate concentration increases in the xylem sap (Ernst *et al.*, 2010; Malcheska *et al.*, 2017) and enhances stomatal closure in the shoot by promoting the expression of genes involved in abscisic acid (ABA) synthesis (Malcheska *et al.*, 2017). Moreover, a rapeseed cultivar with improved efficiency of S uptake and utilization is more resistant to water stress (Lee *et al.*, 2016), supporting the importance of S in tolerance to water stress.

Due to controls on emissions, areas with S-deficient soils are increasing (Mcgrath *et al.*, 2003) and molecular indicators are being developed to diagnose S deficiency (Etienne *et al.*, 2018). In legumes, the S-starvation response can be reduced through mycorrhizal colonization under high phosphate levels (Sieh *et al.*, 2013). S deficiency reduces photosynthesis through a negative effect on both chlorophyll content and the rate of photosynthesis per unit chlorophyll (Terry, 1976), and affects nitrogen fixation and assimilation (Scherer and Lange, 1996; Zhao *et al.*, 1999; Varin *et al.*, 2010). S deficiency also affects the quality of legume seeds, such as germination capacity and the accumulation of 11S globulins, which is compensated by an increased accumulation of 7S globulins (Blagrove *et al.*, 1976; Chandler *et al.*, 1983, 1984; Spencer *et al.*, 1990; Zuber *et al.*, 2013). Variations in legume seed globulins have been shown to be primarily controlled at the transcriptional level, with the changes in transcript abundance closely reflecting the pattern of synthesis of the corresponding globulins (Chandler *et al.*, 1984; Gallardo *et al.*, 2007).

Since water stress may occur simultaneously with S deficiency in cropping systems with low fertilizer input, the impact of deficiency combined with a water-stress period is clearly very important but has not previously been studied. Recent studies have highlighted the fact that the plant response to a combination of two abiotic stresses is unique and that the outcome of the interaction cannot be extrapolated from the effects of the individual stresses (Pandey *et al.*, 2015; Zhang and Sonnewald, 2017). Plants adapt their responses to combined stress factors, displaying unique and/or common responses compared to single stresses. The interaction between two stresses can be either additive (adding the impacts of two hypothetical stressors) or synergistic (a response that is greater than the additive response) and, in some cases, mitigation strategies have been revealed (Pandey *et al.*, 2015). In this study, we provide a first overview of the combined effects of water stress and S deficiency on pea productivity, nutrient partitioning between plant parts, and seed quality traits. Moderate water stress was applied at the beginning of the reproductive phase, a period during which this stress frequently occurs in the field. Clear differences in the plant and seed characteristics in response to the combined stress compared to the individual stresses were observed, highlighting synergistic effects on reproductive processes and mitigating effects on seed globulin composition, which could be explained by changes in seed sink strength for N. By studying the transcriptome of developing seeds, specific molecular signatures for S deficiency and for the combined stress were identified, some of which could play key roles in the transcriptional regulation of globulin accumulation.

Materials and methods

Plant growth conditions

Seeds of *Pisum sativum* L. ('Caméor' genotype) were pre-germinated for 5 d in a Fitoclima S600 germinator (Aralab, Rio de Mouro, Portugal) at 20 °C in the dark. The germinated seeds were sown individually in 2-l pots containing a mixture of perlite/sand (3/1, v/v) at a day/night temperature of 19/15 °C, with a 16-h photoperiod with artificial lighting (250 µmol m^{-2} s^{-1}). The seedlings were irrigated with a nitrate- and S-rich nutrient solution (S+) as previously described [Zuber *et al.*, 2013; 2 mM $Ca(NO_3)_2$ as the only modification] for 3 weeks (to the 5-/6-node stage). Half of the plants were then subjected to S deficiency (S–) by using the same solution lacking $MgSO_4.7H_2O$ but containing 1.16 mM $MgCl_2$, after rinsing the substrate twice with deionized water and then twice with the S– solution. After 8 d of S deficiency, plants at the 8-node stage (on the primary branch: emerging secondary branches were removed throughout development) were transferred to an automated Plant Phenotyping Platform for Plant and Micro-organism Interactions (4PMI, Dijon, France). Plants were automatically weighed and watered four times a day in order to maintain a soil relative water content corresponding to the maximum (100%) water-holding capacity of the substrate. At flowering of the second/third flowering node, irrigation was stopped for half of the plants until the soil water content reached 50% of the maximum water-holding capacity of the substrate. Once this target value was reached it was maintained for 9 d. Plants were then re-watered normally with their appropriate solution (S+ or S–) until maturity (Fig. 1A).

Physiological measurements and collection of seed samples

The midday leaf water potential was measured at the end of the water-stress period on the last fully expanded leaf of four plants per treatment in a C52 sample chamber coupled to a HR-33T Dew Point Microvoltmeter

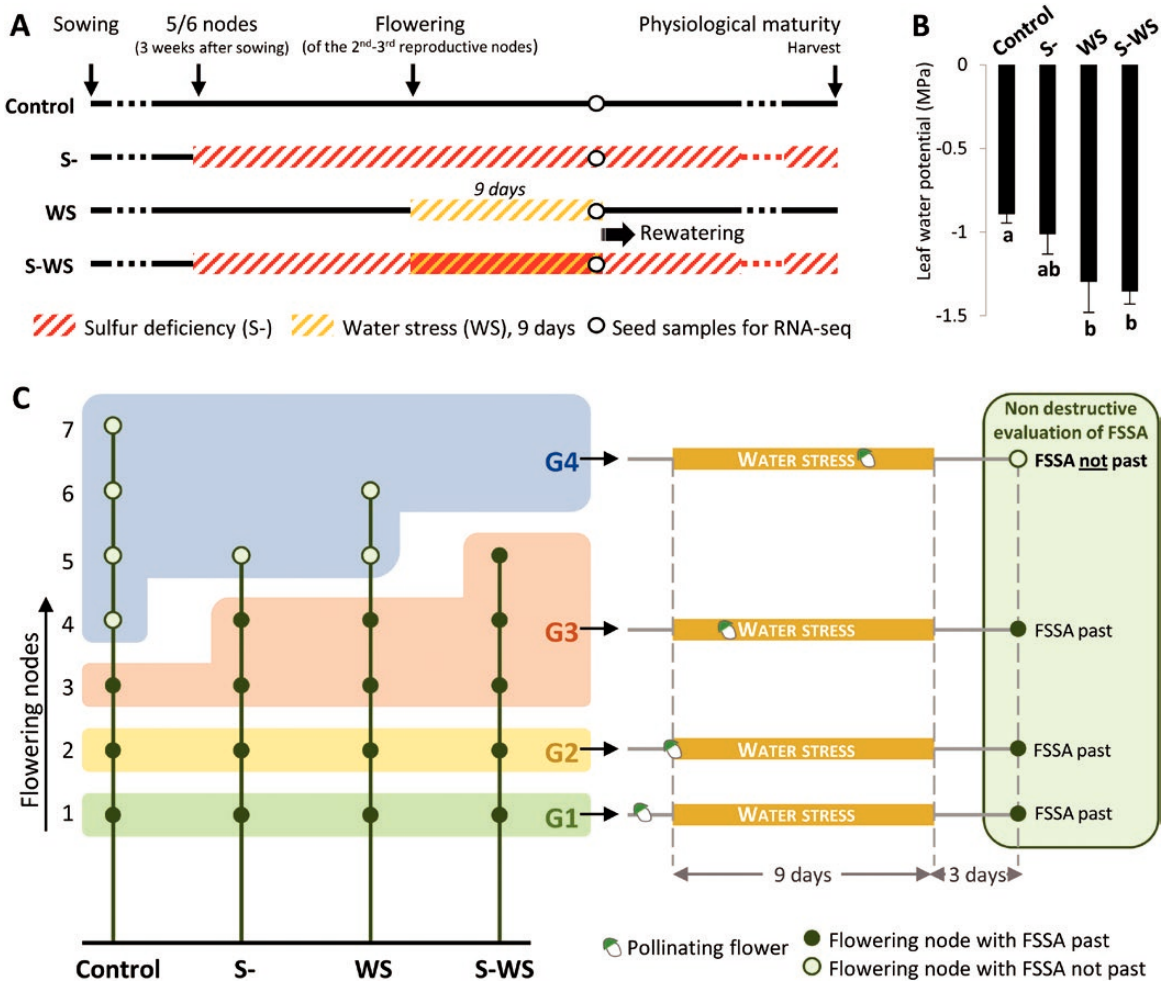


Fig. 1. Experimental design for studying the interaction between water stress and S deficiency in pea (cv. 'Caméor'). (A) Developmental stages at which stresses were imposed. Control, plants were well watered under non-limiting S conditions; S–, plants were deprived of S from the mid-vegetative stage until harvest; WS, plants were subjected to water stress from flowering of the 2nd or 3rd reproductive node for 9 d, then re-watered for recovery; S–WS, plants were subjected to a combination of the two stresses. Developing seeds were collected at the end of the water-stress period for transcriptome analyses, and other plants were harvested at maturity for phenotyping and analyses of seed composition. (B) Leaf water potential measured at the end of the water-stress period. Data are means (±SE), n=4 plants. Different letters indicate significant differences as determined by ANOVA followed by a SNK test (P<0.1). (C) Schematic representation of how mature seeds were collected. For each plant, seeds from each individual flowering node (dots on the left diagram) were collected at maturity and pooled into four groups (G1–G4). G1 seeds corresponded to flowering nodes that flowered before the beginning of the water stress period; G2 seeds corresponded to the flower that opened on the day that water stress was imposed; G3 and G4 seeds corresponded to nodes that flowered during the water-stress period. G1–G3 seeds had passed the final stage of seed abortion (FSSA) 3 d after the end of the water-stress period, while G4 seeds had not. Note that no G4 seeds developed on the double-stressed plants.

(Wescor Inc.). The chlorophyll content of the leaf from the first reproductive node of 7–10 plants per treatment was estimated throughout the reproductive period using a SPAD-502 chlorophyll meter (Minolta).

For each plant, the flower that opened on the day that water stress was applied was tagged, thus allowing us to separate seeds at harvest that corresponded to flowers that opened before water stress, on the day it was applied, and during the stress period. A pea pod sizer developed by Arvalis-Institut du Végétal was used to non-destructively estimate whether or not the seeds of the plants had reached the final stage of seed abortion (FSSA, i.e. the stage at which seeds no longer abort: pod thickness >0.7 cm) (Munier-Jolain *et al.*, 2010). FSSA measurements were carried out 3 d after the end of the water-stress period, which corresponded to 12 d after pollination (DAP) for the tagged pod. Mature seeds were harvested in four groups (G1–G4) according to the date of anthesis of the corresponding flower, with G3 and G4 being further defined according to the FSSA measurements (Fig. 1C). For germination tests, 20 mature seeds (G1–G4 pooled) from each of three replicate plants per treatment were germinated in the dark at 20 °C as described by Benamar *et al.* (2003). A seed was considered as germinated when the radicle protruded through the seed coat.

For transcriptomics, developing seeds were harvested from tagged pods at 9 DAP, corresponding to 9 d after the beginning of the WS period (Fig. 1A). The experiment was carried out in a randomized complete block design with either three (S– and WS treatments) or four (control and S–WS treatments) biological replicates per treatment, each replicate consisting of two plants pooled together.

Measurements of S, N, and C contents in mature seeds and plant parts

S, N, and carbon (C) contents were determined on dried (80 °C for 48 h), ground tissues from four plants per treatment. Samples included seeds (separated into four groups as described before), pod walls, roots, vegetative parts (leaves and stems developed before flowering, i.e. below the first reproductive node), and reproductive parts (leaves and stems of the reproductive nodes). N and C contents were determined from 5 mg of ground tissue using the Dumas procedure on a Flash 2000 Elemental Analyzer (ThermoFisher Scientific) with two technical replicates per sample. S content was determined from 20 mg of ground tissue mixed

with 5 mg of tungsten trioxide on an elemental PYRO cube analyser (Elementar), with two technical replicates per sample. Using these data, the quantity of elements in each plant part was calculated by multiplying the element concentrations by the total biomass of each part. Nutrient partitioning between plant parts was calculated as a percentage relative to the total quantity of each element in the entire plant.

Extraction, separation, and relative quantification of seed proteins using 1-D electrophoresis gels

Total soluble proteins were extracted as previously described (Gallardo *et al*., 2007) from four biological replicates of mature seeds using 500 µl of urea/thiourea buffer for 10 mg of seed powder. Protein concentration was determined according to Bradford (1976) using a Bio-Rad Protein Assay. For each seed sample, 10 µg proteins were separated by one-dimensional electrophoresis (1-DE) in a SDS polyacrylamide gel using the XCell4 *Surelock*™ Midi-Cell system (Life Technology) [Resolution gel: 4.1 mM Tris-HCl pH 8.8, 40% (v/v) acrylamide/bisacrylamide (30%/0.8%), 1% (g/v) SDS, 0.05% (g/v) ammonium persulfate, 0.05% (v/v) TEMED; Concentration gel: 0.6 mM Tris-HCl pH 6.8, 13% (v/v) acrylamide/bisacrylamide (30%/0.8%), 1% SDS, 0.05% ammonium persulfate, 0.05% TEMED; Electrophoresis buffer: 50 mM Trizma base, 380 mM glycine, 0.1% (g/v) SDS]. After staining with Coomassie Blue R250 (Bio-Rad), gels were scanned using an Odyssey Infrared Imaging System (LI-COR) with an intensity of 7.5 and a resolution of 84 µm. Protein band detection and quantification were performed using Phoretix 1D (v11.2, TotalLab Limited). In each well, the quantitative data were normalized by dividing the volume of each protein band by the total band volume, and the molecular weight (kDa) of each band was calculated using a low-range protein ladder (Bio-Rad).

Identification and annotation of mature seed proteins

Protein bands were excised from the gel and protein digestion was carried out according to Labas *et al.* (2015). The resultant peptides were extracted in 5% formic acid (10 min sonication, supernatant removed and saved), followed by incubation in 100% acetonitrile/1% formic acid (1:1, 10 min), and a final incubation with 100% acetonitrile (5 min, supernatant was again removed and saved). These two peptide extractions were pooled and dried using a SPD1010 speedvac system (Thermosavant, Thermofisher Scientific). The resultant peptide mixtures were analysed by nanoflow liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) for protein identification. Peptide separation and data acquisition were performed according to Labas *et al.* (2015) on a LTQ Orbitrap Velos mass spectrometer (ThermoFisher Scientific) coupled to an Ultimate® 3000 RSLC Ultra High Pressure Liquid Chromatographer (Dionex) controlled by Chromeleon Software (v6.8 SR11; Dionex). MS/MS ion searches were performed using Mascot (v2.3.2, Matrix Science) via Proteome Discoverer 2.1 software (ThermoFisher Scientific) against the pea gene atlas (Alves-Carvalho *et al*., 2015). The search parameters included trypsin as a protease with two allowed missed cleavages and carbamidomethylcysteine, methionine oxidation, and acetylation of N-term protein as variable modifications. The tolerance of the ions was set to 5 ppm for parent and 0.8 Da for daughter-fragment ion matches. Peptides and proteins identified by Mascot were validated using Scaffold (v4.8.3, Proteome Software): protein identifications were accepted when they contained at least two identified peptides and when the probability was at least 95% and 99% as specified by the PeptideProphet and ProteinProphet algorithms, respectively (Keller *et al*., 2002). To annotate each protein band, we used the quantitative values obtained by mass spectrometry. These values corresponded to the Normalized Weighted Spectra counts calculated by Scaffold 4.8.3 (Supplementary Table S1 at *JXB* online) that reflected the abundance of each protein identified within a band. We assigned a protein name to a band only when the Normalized Weighted Spectra count indicated a high abundance for this protein (i.e. a major protein).

RNA sequencing, read mapping, and differential analyses

Total RNAs were extracted from 9-DAP seeds using an RNeasy Plant Mini Kit (QIAGEN), treated with an RNase-Free DNase Set (QIAGEN), and then purified by lithium chloride precipitation. Quantification of RNA was performed using a spectrophotometer NanoDropTM 2000 (ThermoFisher Scientific) and RNA quality was assessed on a 2100 Bioanalyzer (Agilent Technologies). The same RNA pools were used for RNA sequencing (RNA-seq) and reverse-transcription quantitative PCR (RT-qPCR). RNA-seq was performed on an Illumina HiSeq3000 to generate 150-nucleotide-long paired-end reads. RNA-seq libraries were prepared using an Illumina TruSeq Stranded mRNA sample prep kit according to the manufacturer's instructions. Briefly, mRNAs were selected using poly-T beads, fragmented to generate double-stranded cDNA, and ligated to adapters. Eleven cycles of PCR were applied to amplify the libraries. Library quality was assessed using a Fragment Analyzer (Agilent Technologies) and libraries were quantified by qPCR using a Kapa Library Quantification Kit (Roche). RNA-seq experiments were performed on an Illumina HiSeq3000 using a paired-end read length of 2×150 pb with Illumina HiSeq3000 sequencing kits. The sequence quality of the raw data was assessed using the FastQC v0.11.2 software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and raw data were corrected using the k-mer-based error correction software Rcorrector (*-k 31*) (MacManes, 2015, Preprint; Song and Florea, 2015). The corrected data were trimmed for low-quality and adapter sequences using Trimmomatic v0.32 (Bolger *et al*., 2014) with the following parameters: ILLUMINACLIP:TruSeq3PE2:2:40:15, LEADING/TRAILING:2, SLIDINGWINDOW:4:15, and MINELN:25. Trimmed reads with less than 25 bp and unpaired reads were discarded. The corrected and trimmed reads were aligned against the Pea Reference Genome sequence v1 (https://urgi.versailles.inra.fr/Species/Pisum/Pea-Genome-project) using the HISAT2 v2.0.5 alignment program (Kim *et al*., 2015) with the specific parameter *--dta* for output compatibility with StringTie 1.2.2 (Pertea *et al*., 2015). For each library, more than 90% of the reads were uniquely mapped to the genome (Supplementary Table S2). A protocol was developed based on Pertea *et al.* (2016) to annotate new transcripts. New transcript assembly was performed using StringTie 1.2.2 with default parameters and with a file containing reference gene models as a guide. For each library, a StringTie prediction was made based on HISAT2 mapping files. Assemblies from the different samples were then merged using the StringTie *merge* function. The Gffcompare v0.9.9e software (https://github.com/gpertea/gffcompare) was used to classify the predicted transcripts compared to the reference, and only new transcripts were kept (class code U, i.e. the predicted transcript is intergenic in comparison to known reference transcripts). To remove potential transposable elements (TEs) from our set of new assembled transcripts, transcript and TE coordinates were compared using the Bedtools intersect v2.26.0 software (Quinlan and Hall, 2010) with the option *--wao*. Transcripts with more than 50% coverage with a TE were removed from our data set. Long non-coding transcripts were identified using the FEElnc v0.1.0 software (Wucher *et al*., 2017) with default parameters. When a coding protein gene had at least one of its alternative transcripts identified as a long non-coding RNA (lncRNA), all alternative sequences were mapped against the protein databases of *Medicago truncatula* v4 (http://www.medicagogenome.org/) and *Arabidopsis thaliana* TAIR10 (https://www.arabidopsis.org/). When a match (e-value<0.001) was obtained for at least one of the transcripts, the gene and its alternative transcripts were considered as coding RNAs. Coding transcripts were translated into proteins using the *load*, *longestorf*, and *translate* commands from the JCVI toolbox (Tang *et al*., 2015). The entire dataset was mapped against the *Medicago truncatula* proteome (v4) and the *Arabidopsis thaliana* proteome (TAIR10) using Blastp with an e-value of 0.001. For each species, the best hit for both coverage and identity was selected. Protein domain characterization and assignment of gene ontology (GO) terms were performed using InterProScan5 (Jones *et al*., 2014). Mapped reads were assigned to the pea reference genome v1 enriched with the new transcripts using FeatureCounts v1.5.0-p3 (Liao *et al*., 2014). Only paired reads that mapped once and that were correctly mapped on the same chromosome were counted (*-p -B -C*). Reads were assigned to their overlapping meta-features. Counts of genes were pre-filtered to keep only rows that had at least 10 reads total. Pairwise differential gene expression between control and stress treatments was obtained using the DESeq2 R package (1.12.3 version) (Love *et al*., 2014). Genes were considered

as differentially expressed when the false discovery rate (FDR)-adjusted *P*-value was ≤0.05. GO term enrichment analysis was conducted using the TopGO R package (2.34.0 version; Alexa and Rahnenführer, 2018), with the Elim method and Fisher's exact test ($P<0.001$).

RT-qPCR

Samples of 5 µg of DNAse-treated RNA were reverse-transcribed with an iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's protocol in a final volume of 20 µl. Quantitative real-time PCR was carried out on a LightCycler 480 apparatus (Roche) as described by Noguero *et al.* (2015) with GoTaq qPCR Master Mix (Promega), using 3 µl of 50× diluted cDNA and 0.2 µM of each primer in a final volume of 10 µl. Reactions were performed in duplicates from each biological replicate. Expression levels relative to the housekeeping reference genes *Actin* (Psat5g063760) and *Histone* (Psat6g056720) were calculated using the $\Delta\Delta C_T$ method (Schmittgen and Livak, 2008). Primers used for the reference genes and deregulated genes are given in Supplementary Table S3.

Statistical analyses

Statistical analyses of phenotypic and physiological data, 1-DE gel-derived protein data, and CNS content, quantities, and partitioning were conducted using Statistica Version 7.0 (StatSoft). ANOVAs followed by Student–Newman–Kheul (SNK) *post hoc* tests were used to determine significant differences ($P<0.05$, except for leaf water potential where $P<0.1$).

Results

S deficiency combined with water stress dramatically affects seed yield components

To investigate the interplay between S nutrition and the response to water stress, *Pisum sativum* plants (cv. 'Caméor') deprived of S from the mid-vegetative stage (to minimize vacuolar storage of sulfate) were subjected to a moderate water stress (WS) for 9 d during the early reproductive phase, i.e. at flowering of the second or third reproductive nodes (S–WS treatment, Fig. 1A). Control plants (well-watered, in non-limiting S conditions) and plants subjected to individual stresses (WS or S–) were examined for comparison. Leaf water potential measurements confirmed that the water stress was moderate (–1.3 MPa, Fig. 1B). The effect of individual and combined stresses was evaluated at maturity by measuring plant, pod, and seed characteristics (Table 1). Water stress did not significantly affect seed yield, although there was a slight decrease in individual seed weight and in the number of reproductive nodes. In contrast, S deficiency alone or combined with water stress significantly decreased yield by 38% and 65%, respectively. The stronger impact of the combined stress could be explained by a marked decrease of both individual seed weight (–34%) and seed number per plant (–48%). In addition, the combined stress increased seed abortion (+58% compared to the control), leading to fewer seeds per pod. These effects were not observed in response to the individual stresses, thus demonstrating the dramatic effect of S starvation combined with moderate water stress on seed production. Furthermore, pea plants subjected to the same S deficiency but experiencing a 3-d longer period of water stress (12 d) did not survive: the leaves dried out even though the water stress was moderate (Supplementary Fig. S1A).

To determine whether the stresses affected early- and late-produced seeds differently, mature seeds were collected in four different groups (G1–G4) depending on (i) the time of pollination of the corresponding flower (before, at the time water stress was imposed, or during the water-stress period) and (ii) whether or not seeds had reached the FSSA 3 d after the end of the water-stress period (see Methods; Fig. 1C). G1 seeds corresponded to flowering nodes that flowered before the beginning of the water stress period. G2 seeds corresponded to the flower that opened on the day that water stress was imposed. Both G3 and G4 seeds corresponded to flowering nodes that flowered during the water stress period but they could be distinguished according to the FSSA measurements: G3 seeds had reached FSSA 3 d after the end of the water-stress period, while G4 seeds were still in embryogenesis at this time. Plants subjected to the individual or combined stresses produced more nodes carrying G3 seeds and fewer nodes carrying G4 seeds than control plants (Table 1), indicating accelerated seed production. Interestingly, in the combined stress treatment, seeds from all reproductive nodes had reached FSSA (i.e. there were no G4 seeds), while seeds from the last reproductive nodes of plants grown in control or single-stress conditions were still in embryogenesis. In addition, all groups of seeds grown under the combination of water stress and S deficiency displayed a significant decrease in individual seed weight compared to the control treatment. Hence, the combined stress negatively affected individual seed weight whatever the developmental stage of the seeds exposed to water stress.

Seed protein composition is less affected by combined stress than by S deficiency alone

To examine the influence of the individual and combined stresses on seed protein composition, total proteins of mature seeds of each group (G1–G4) in the different treatments were separated by 1-DE. A total of 27 individual bands were detected and quantified (Fig. 2A), and 19 could be annotated following nanoLC-MS/MS analyses (Supplementary Table S1). The relative abundance of each protein band for each group of seeds is given in Supplementary Table S4 and expressed as a ratio to the control in Fig. 2B. These data showed that the short and moderate water stress alone did not significantly modify seed protein composition. In contrast, S deficiency drastically affected the accumulation of major proteins (i.e. storage proteins), as seen in Fig. 2A. As expected, the major proteins whose relative abundance increased in response to S deficiency corresponded to 7S globulins (vicilins and convicilins, 0.47–0.86% S-AA in their sequence), and the major proteins whose relative abundance decreased corresponded to 11S globulins (legumins, 2.31% S-AA) and 2S albumins (3.03% S-AA). This resulted in a significantly higher 7S/11S ratio in G1–G4 seeds from S– plants compared to the control (by 6.25- to 7.81-fold). Interestingly, fewer differences in the accumulation of 7S and 11S globulins were observed when S deficiency was combined with water stress, especially for G1 seeds (Fig. 2), and the 7S/11S ratio was not significantly affected by the combined stress (Fig. 2B, Supplementary Table S4). These data clearly demonstrated that the storage protein composition of

Table 1. *Effect of water stress combined or not with S-deficiency on phenotypic characteristics at harvest in pea.*

Phenotypic characteristics at maturity	Well-watered							Water-stressed						
	Sulfur supply			Sulfur deficiency				Sulfur supply				Sulfur deficiency			
	Control			S-				WS				S- WS			
	Mean	SE	SNK test	Mean	SE	SNK test	Ratio to Control	Mean	SE	SNK test	Ratio to Control	Mean	SE	SNK test	Ratio to Control
Plant characteristics at harvest															
Seed yield per plant (g)	12.83	0.650	a	7.96	0.471	b	**0.62 ↘ -38%**	11.63	0.463	a	0.91	4.45	0.547	c	**0.35 ↘ -65%**
Total plant biomass (g)	23.03	0.798	a	14.67	0.831	b	**0.64 ↘ -36%**	21.32	0.967	a	0.93	10.46	0.617	c	**0.45 ↘ -55%**
Harvest index	0.56	0.018	a	0.54	0.011	a	0.97	0.55	0.007	a	0.98	0.42	0.026	b	**0.74 ↘ -26%**
Number of vegetative nodes	12.86	0.404	a	12.57	0.481	a	0.98	12.60	0.163	a	0.98	12.80	0.200	a	1.00
Number of reproductive nodes	8.86	0.340	a	6.43	0.202	c	**0.73 ↘ -27%**	7.30	0.213	b	**0.82 ↘ -18%**	5.70	0.153	d	**0.64 ↘ -36%**
Number of nodes with pods	6.71	0.244	a	4.86	0.189	c	**0.72 ↘ -28%**	5.70	0.474	b	**0.85 ↘ -15%**	4.80	0.211	c	**0.71 ↘ -29%**
Pod and seed characteristics at harvest (G1 to G4 seeds pooled)															
Seed number per plant	48.57	2.349	a	33.71	1.742	b	**0.69 ↘ -31%**	48.20	1.718	a	0.99	25.50	2.535	c	**0.53 ↘ -48%**
Pod number per plant	9.86	0.404	a	7.29	0.421	b	**0.74 ↘ -26%**	9.40	0.452	a	0.95	7.00	0.298	b	**0.71 ↘ -29%**
One-seed weight (g)	0.26	0.003	a	0.24	0.005	b	**0.89 ↘ -11%**	0.24	0.005	b	**0.91 ↘ -9%**	0.17	0.008	c	**0.66 ↘ -34%**
Seed number per pod	5.66	0.494	a	4.85	0.216	a	0.86	5.56	0.242	a	0.98	3.24	0.354	b	**0.57 ↘ -43%**
Number of aborted seeds per pod	2.62	0.521	a	2.24	0.276	a	0.85	2.08	0.245	a	0.79	4.15	0.426	b	**1.58 ↗ +58%**
Gmax (%)	100	0	a	73.33	26.67	a	0.73	100	0	a	1.00	71.67	14.81	a	0.72
Number of reproductive nodes															
G1	0.71	0.244	a	1.43	0.488	a	2.00	1.10	0.158	a	1.54	1.30	0.242	a	1.82
G2	1.00	0.000	a	1.00	0.000	a	1.00	1.00	0.000	a	1.00	1.00	0.000	a	1.00
G3	1.00	0.408	b	1.43	0.636	ab	1.43	1.60	0.422	ab	1.60	2.30	0.412	a	**2.30 ↗ +130%**
G4	4.00	0.289	a	0.86	0.535	c	**0.21 ↘ -79%**	2.00	0.577	b	**0.50 ↘ -50%**	n/a	n/a	n/a	n/a
Seed number per group															
G1	10.7	1.667	ab	13.5	2.255	a	1.27	10.5	1.443	a	0.98	4.75	0.250	b	0.45
G2	6.75	0.854	a	5.50	0.500	a	0.81	7.00	0.408	a	1.04	2.00	0.408	b	**0.30 ↘ -70%**
G3	16.5	2.327	ab	11.8	3.924	b	0.71	24.3	2.016	a	1.47	20.3	2.839	ab	1.23
G4	19.8	3.425	a	7.00	n/a	a	0.35	8.67	1.856	a	0.44	n/a	n/a	n/a	n/a
One-seed weight (g)															
G1	0.25	0.009	a	0.24	0.006	a	0.96	0.21	0.012	a	0.85	0.13	0.017	b	**0.54 ↘ -46%**
G2	0.27	0.009	a	0.24	0.002	a	0.90	0.23	0.013	a	0.85	0.11	0.018	b	**0.42 ↘ -578%**
G3	0.27	0.002	a	0.24	0.008	a	0.89	0.24	0.012	a	0.90	0.19	0.011	b	**0.69 ↘ -31%**
G4	0.28	0.005	a	0.23	n/a	b	**0.84 ↘ -16%**	0.25	0.010	ab	0.90	n/a	n/a	n/a	n/a

The harvest index corresponds to the ratio of seed yield to total dry biomass, and Gmax to the maximum percentage of germinated seeds after 96 hours. The ratios of values for stressed plants versus control plants are indicated. For each trait, different letters indicate significant differences ($P<0.05$, ANOVA followed by a SNK test, $7<n<10$ for all traits except for seed number and weight per group from G1 to G4 where $n=4$). For Gmax determination, G1 to G4 seeds were pooled and 20 seeds per plant (3 plants per condition) were used. The colored bold values indicate whether the ratio decreased (dark blue scale) or increased (red) significantly. When the differences were statistically significant, the percentage of increase or decrease relative to the control is indicated. SE, standard error. Note that no G4 seeds developed on the double-stressed plants. n/a, not applicable.

all groups of seeds was much less affected by the combined stress than by S deficiency alone. The possible impact of these changes on seed germination capacity was investigated using three biological replicates of seeds pooled from all the groups G1–G4. Although the results were not statistically significant, the maximum percentage of germinated seeds after 96 h of imbibition (G_{max}, Table 1) was lower for S– and S–WS plants compared to control seeds, which achieved 100% germination after 68 h.

N/S balance in seeds subjected to S deficiency and/or water stress

The mitigating effect on seed globulins of the combined stress compared to S deficiency alone prompted us to measure the S, N, and C concentrations in the same seed samples. The absolute quantity of each element per seed was calculated as well as the N/S and C/N ratios (Supplementary Tables S5, S6, summarized in Table 2). In mature pea seeds, N is predominantly stored in the form of proteins (N content multiplied by 5.4 represents the protein content according to Mariotti *et al.*, 2008) while C is mainly stored as starch (Bastianelli *et al.*, 1998). Hence, the variations observed in this study for seed N and C contents reflected modifications in protein and starch contents in the seeds. The data indicated that the moderate water stress did not result in major changes in the SNC contents (%) and quantities (mg seed^{-1}). In contrast, S deficiency alone or combined with water stress significantly decreased the S content and quantity per seed for all groups, and increased the N content in seeds in groups G1–G3. Interestingly, the N and C quantities per seed did not vary in response to S deficiency alone, whereas they decreased significantly in response to the combined stress (Table 2). Therefore, it is likely that the seeds of plants subjected to the combined stress had a reduced N and C demand compared to S– plants, which was consistent with their lower weight (–11% in S– versus –34% in S–WS, Table 1). Consequently, the seed N/S ratio only increased by 2.5-fold in response to the combined stress, whereas it increased by 4-fold in response to S deficiency (Table 2). Significantly, the seed N/S ratio was strongly correlated with the 7S/11S ratio (R=0.9, P<0.001, Supplementary Fig. S2), suggesting that the N/S ratio can be used as an indicator of globulin composition in pea seeds.

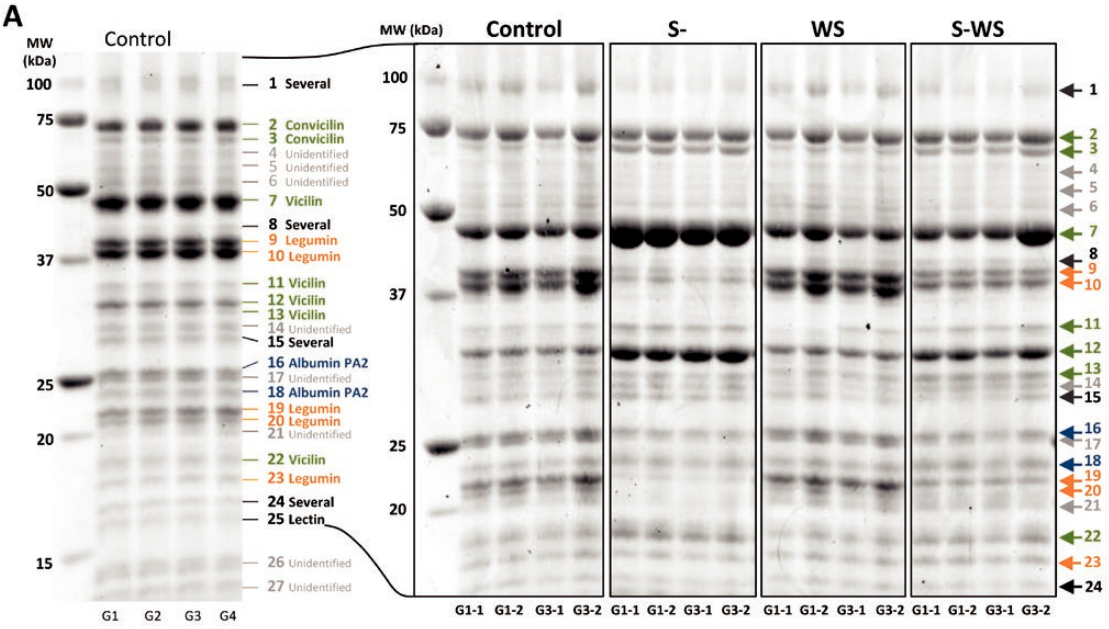
Differences in nutrient allocation between tissues of stressed plants

To link the seed characteristics with nutrient content and partitioning in different plant parts, SNC contents were measured in pod walls, roots, and reproductive and vegetative plant parts (Fig. 3A), and the absolute quantity (Fig. 3B–D) and partitioning (Fig. 3E–G) of each element was calculated. There was a sharp decrease in S content and total S quantity in all compartments of the plants deprived of S (Fig. 3A, B) and the partitioning data indicated a reduced proportion of S in roots of these plants (Fig. 3E), suggesting a major utilization of the root S reserves. In contrast, the proportion of S in pod walls increased significantly, suggesting a higher transfer of S into the pod walls and/or defects in S remobilization from them to the seeds (Fig. 3E). Specifically, when S deficiency was combined with water stress, the proportion of N and C accumulated in the seed compartment decreased while it increased in pod walls, roots, and vegetative plant parts (Fig. 3F, G). These results reflected a decreased allocation of N and C to seeds in response to the combined stress. In support of this, higher chlorophyll contents were observed in leaves of the combined-stressed plants by the end of the reproductive phase (Supplementary Fig. S1), suggesting later senescence.

Transcriptome changes in developing seeds subjected to drought and/or S deficiency

To gain insights into the molecular processes occurring in developing seeds at the end of the combined stress period, a transcriptome analysis was carried out for G2 seeds that experienced the combined stress throughout their early development (0–9 DAP, Fig. 1). Samples collected from the different treatments at 9 DAP were subjected to paired-end RNA-seq. Between 15–33 million paired-end reads were generated per sample after adapter trimming and filtering of low-quality reads, and 91% were uniquely mapped to the pea reference genome (Supplementary Table S2). Of the 28 100 genes expressed in the seed samples, 2976 were differentially expressed in response to the stresses (P-adjust<0.05). Annotation, sequences, and expression values of these differentially expressed genes (DEGs) are given in Supplementary Table S3. The reliability of the Illumina RNA-seq data was validated for 11 genes using quantitative RT-PCR. A strong correlation between the RNA-seq and RT-qPCR data was obtained (R^2=0.97), thus validating our differential analysis (Supplementary Fig. S3). In total, 1394 and 1584 genes exhibited significantly higher and lower expression, respectively, in stress conditions compared to the control (Fig. 4A), and about 40% of the transcriptomic changes (1199 out of 2976 DEGs) were shared between the S– and S–WS treatments. Only two genes (out of 2976) had opposite regulation in response to the different stresses: a ferritin gene (Psat7g247120) was down-regulated in response to S deficiency but up-regulated in response to the combined stress, while a gene encoding an unknown protein (Psat5g068840) was up- and down-regulated in S– and S–WS conditions, respectively (Supplementary Table S3). Interestingly, the number of DEGs was significantly higher in S– (2523 genes) compared to S–WS (1652 genes), indicating important transcriptional modifications in response to S deficiency alone (Fig. 4A). In contrast, moderate water stress did not induce a strong reprogramming of the transcriptome in developing seeds since only 11 genes were differentially expressed in the WS treatment (four genes induced and seven repressed; Fig. 4A, Supplementary Table S3). Among the genes down-regulated by water stress were two legumins (Psat0s1923g0200 and Psat6tg055080), which were also down-regulated in the S– and S–WS treatments. In total, eight globulins were among the DEGs: their expression decreased in response to S deficiency with or without water stress (Supplementary Table S7). Further examination of the RNA-seq data revealed 21 globulin transcripts expressed at

A



B

Type of regulation	Band number	MW (kDa)	Annotation	Ratio to Control (Relative protein abundance) S- G1	S- G2	S- G3	S- G4	WS G1	WS G2	WS G3	WS G4	S- WS G1	S- WS G2	S- WS G3	S-AA %	Lys %	Trp %
↗ S-WS & S-	3	64	Convicilin 7S	**1.89**	**2.13**	**2.67**	**2.54**	0.83	0.89	1.06	1.06	**1.89**	**1.82**	**2.40**	0.70	7.71	0.53
	7	48	Vicilin 7S	**1.50**	**1.73**	**1.63**	**1.77**	0.83	0.85	0.88	0.82	0.95	0.98	**1.39**	0.86	7.56	0
	11	34	Vicilin 7S	**1.71**	1.58	**2.21**	1.63	0.76	0.84	0.82	0.59	1.37	1.29	**1.98**	0.86	7.56	0
	12	32	Vicilin 7S	**2.49**	**2.27**	**2.43**	**2.50**	1.13	1.10	1.03	0.96	1.76	**1.54**	**1.78**	0.47	6.99	0
	22	19	Several proteins	**2.57**	**2.39**	**2.44**	**2.86**	1.14	1.09	1.21	0.89	1.38	1.29	**2.07**			
	24	17	Several proteins	1.16	**1.36**	1.26	**1.80**	1.09	1.03	0.95	1.01	**1.76**	**1.44**	1.31			
	25	16	Lectin	**4.02**	**3.31**	**3.27**	**3.20**	1.45	1.01	1.06	1.23	**1.88**	**2.24**	**2.22**			
	26	14	unidentified	**2.19**	**2.06**	**2.09**	**2.10**	1.10	0.92	1.12	0.90	**1.39**	**1.57**	**1.60**			
↗ S-WS	14	30	unidentified	1.38	0.79	0.72	0.56	1.50	0.99	0.70	0.89	**2.16**	1.31	1.00			
	18	25	Albumin PA2 2S	1.40	1.00	0.89	0.75	1.27	1.06	0.86	1.07	**1.84**	1.33	1.18	3.03	6.93	0.43
	23	18	Legumin 11S	1.20	1.08	1.19	1.03	1.17	1.09	1.14	1.16	**1.45**	**1.50**	**1.47**	1.40	4.56	0.70
↘ S-WS & S-	6	51	unidentified	**0.33**	**0.39**	**0.37**	**0.22**	0.98	1.09	1.44	1.03	**0.34**	**0.45**	0.73			
	8	43	Several proteins	**0.14**	**0.13**	**0.18**	0.20	1.17	1.21	1.11	1.42	1.28	1.22	**0.47**			
	9	40	Legumin 11S	**0.14**	**0.15**	**0.18**	**0.19**	0.98	0.98	1.02	1.15	**0.67**	**0.68**	**0.43**	2.31	4.24	0.58
	10	38	Legumin 11S	**0.22**	**0.27**	**0.31**	**0.27**	0.96	0.98	0.89	0.99	**0.60**	**0.69**	**0.47**	2.31	4.24	0.58
	16	26	Albumin PA2 2S	**0.56**	**0.52**	**0.52**	0.66	1.07	0.81	0.99	1.04	**0.45**	**0.37**	**0.52**	3.03	6.93	0.43
	19	23	Legumin 11S	**0.23**	**0.17**	**0.20**	**0.11**	1.23	1.31	1.04	1.14	0.81	**0.78**	**0.41**	2.31	4.24	0.58
	20	22	Legumin 11S	**0.16**	**0.05**	**0.09**	**0.08**	1.03	0.75	0.80	1.19	0.60	**0.56**	**0.26**	2.31	4.24	0.58
	27	13	unidentified	**0.32**	**0.44**	**0.51**	**0.25**	1.21	1.29	1.30	1.16	0.45	0.87	0.46			
↘ S-	17	26	unidentified	0.50	0.70	**0.70**	0.61	0.90	1.37	1.18	0.73	0.81	0.85	0.91			
↗ S-WS & ↘ S-	21	21	unidentified	**0.34**	**0.27**	**0.41**	0.23	1.18	1.18	1.29	1.16	1.41	**1.77**	1.06			
Non-regulated by stresses	1	97	Several proteins	1.10	0.56	0.54	0.51	0.99	1.13	1.09	0.89	0.61	0.38	0.48			
	2	70	Convicilin 7S	0.97	0.96	1.01	0.97	0.92	0.95	1.00	0.97	0.98	1.01	0.99	0.48	7.92	0.65
	4	59	unidentified	0.51	0.62	1.37	0.79	0.80	1.03	2.84	1.26	1.10	0.90	2.23			
	5	55	unidentified	0.32	0.42	0.51	0.36	0.63	0.93	2.25	0.73	0.47	0.51	1.54			
	13	31	Vicilin 7S	2.22	1.43	1.47	1.44	1.48	1.18	1.21	1.51	1.80	1.66	1.34	0.47	6.99	0
	15	29	Several proteins	1.24	1.23	1.25	1.27	1.02	1.00	0.93	0.85	1.28	1.19	1.23			
			2S	0.89	**0.72**	**0.69**	0.71	1.15	0.91	0.93	1.05	0.99	0.78	0.83			
			7S	**1.57**	**1.63**	**1.66**	**1.69**	0.91	0.93	0.96	0.91	**1.17**	**1.16**	**1.42**			
			11S	**0.23**	**0.24**	**0.28**	**0.24**	1.03	1.01	0.96	1.09	**0.70**	**0.73**	**0.48**			
			All globulins: 11S + 7S	0.94	0.97	0.99	0.99	0.97	0.97	0.96	1.00	0.95	0.96	0.96			
			7S/11S	**7.81**	**7.57**	**6.25**	**7.06**	0.89	0.92	1.01	0.84	1.69	1.66	3.01			

Fig. 2. Effects of water stress combined with S deficiency on protein composition of mature pea seeds from seed groups G1–G4. (A) Protein profiles of mature seeds from plants with or without stresses. Representative one-dimensional electrophoresis (1-DE) protein patterns for mature control seeds from G1–G4 are shown on the left. The 27 individual bands that were detected and quantified in all the seed groups are shown. Protein annotation refers to major proteins in each band, according to the Normalized Weighted Spectra counts (Supplementary Table S1). Six protein bands corresponded to 7S globulins (vicilins and convicilin, in green) and five to 11S globulins (legumins, in orange). One 1-DE gel illustrating qualitative changes in the accumulation of the 7S and 11S globulins between the different treatments is shown on the right for two biological replicates of G1 (G1-1, G1-2) and G3 (G3-1, G3-2). (B) Quantitative variations in response to stresses. Significant changes in the relative abundance of the protein bands are shown for each group of seeds compared to the control. The ratio of values for stressed plants versus control plants is indicated for each group. Note that no G4 seeds could be harvested for S–WS plants. The 7S/11S ratio corresponds to the relative quantity of 7S divided by that of 11S. The colors indicate whether the ratio decreased (grey scale) or increased (red scale) when values were significantly different to that of control seeds, as determined by ANOVA followed by a SNK test ($P<0.05$, $n=4$ plants). The percentage of amino acids in the sequence of storage-proteins is indicated (S-AA, sulfur amino acids, methionine and cysteine). S–, S deficiency alone; WS, water stress alone; S–WS, combined stresses.

Table 2. *S, N, and C contents and quantities, and N/S and C/N ratios in mature seeds from seed groups G1–G4*

SNC measurements in seeds	Ratio to Control										
	S–				WS				S–WS		
	G1	G2	G3	G4	G1	G2	G3	G4	G1	G2	G3
Content (%)											
Sulfur	**0.31**	**0.30**	**0.31**	**0.30**	1.05	**0.92**	0.99	1.08	**0.58**	**0.50**	**0.40**
Nitrogen	**1.21**	**1.16**	**1.12**	1.07	1.04	0.96	1.00	1.03	**1.32**	**1.23**	**1.14**
Carbon	1.00	0.99	**0.99**	**0.99**	1.01	1.00	1.00	1.00	0.99	1.00	**0.98**
Absolute quantity in one seed (mg)											
Sulfur	**0.29**	**0.27**	**0.28**	**0.25**	0.89	**0.78**	0.89	0.97	**0.31**	**0.22**	**0.28**
Nitrogen	1.17	1.05	0.99	0.89	0.89	0.82	0.90	0.92	**0.70**	**0.51**	**0.79**
Carbon	0.96	0.90	0.88	0.82	0.86	0.85	0.90	0.90	**0.53**	**0.42**	**0.68**
N/S	**4.04**	**3.91**	**3.61**	**3.65**	0.99	1.05	1.00	0.96	**2.30**	**2.49**	**2.86**
C/N	**0.83**	**0.85**	**0.88**	0.92	0.97	1.04	1.00	0.98	**0.75**	**0.81**	**0.86**

The ratios of values obtained for stressed plants versus control plants are indicated for each group of seeds from G1–G4. Bold indicates that values were significantly different compared to control plants as determined by ANOVA followed by a SNK test ($P<0.05$, $n=4$ plants). The colors indicate whether the ratio decreased (grey scale) or increased (red). The N/S and C/N ratios correspond to the absolute quantity of nitrogen (N) or carbon (C) divided by that of sulfur (S) or N. S–, Sulfur deficiency alone; WS, water stress alone; S–WS, combined stresses.

low levels (Supplementary Table S7), indicating that, under our conditions, the G2 seeds at 9 DAP were at a transition stage towards storage protein accumulation.

Biological processes regulated in developing seeds in response to stresses

A GO enrichment analysis (Elim method/Fisher's test, $P<0.001$) of the DEGs indicated that several biological processes were similarly enriched in the list of genes responding specifically to S– and in the list of genes responding to both S– and S–WS (Fig. 4B, Supplementary Table S8). These were related to 'Translation' and 'Photosynthesis' (down-regulated genes), and to 'Oxidation reduction processes' and 'Negative regulation of catalytic processes' (up-regulated genes). This indicated that similar biological processes were regulated in response to S deficiency alone and to the combined stress, but that these processes were regulated to a greater extent under S deficiency alone.

GO terms specifically enriched in the set of genes up-regulated in response to S– and S–WS only (525 genes) were related to proteolysis, and S metabolism and transport (Fig. 4B). These data and our observations that mature seeds produced under S– or S–WS conditions had different N/S ratios prompted us to examine the transcriptional regulation of genes for S and N transport and metabolism in response to the stresses. The expression of genes for 20 of the 26 enzymes in Fig. 5 varied in response to S deficiency and/or to the combined stress, and the fold-change in expression was in most cases higher in response to S deficiency. Most of the genes involved in nitrate reduction and assimilation, such as nitrate reductase and glutamine synthetase, and in S metabolism, such as adenosine 5′-phosphosulfate (APS) reductase and methionine synthase, were up-regulated in response to S deficiency with or without water stress. One exception was the first enzyme of sulfate reduction (ATP sulfurylase 1, ATPS1), the expression of which was down-regulated under both conditions, probably because of the low amounts of sulfate in S–WS and S– seeds. In connection with this, the expression of a sulfite oxidase, which synthesizes sulfate from sulfite, increased in these seeds, reflecting a need to provide sulfate from other S sources. Genes encoding sulfate, nitrate, and amino acid transporters were differentially expressed in response to the stresses (Fig. 5). The most up-regulated was the homolog of *AAP8* (Psat1g164680), which in Arabidopsis plays a role in supplying the developing embryo with amino acids (Schmidt *et al.*, 2007).

Interestingly, the GO terms 'Protein Sumoylation' and 'Regulation of Transcription' were over-represented in the list of genes up-regulated in response to the combined stress (260 genes, Fig. 4B). This suggested the establishment of translational and transcriptional regulation processes in response to the combined stress. A list of the genes encoding transcription factors (TFs) and SUMO-ligases specifically induced in the S–WS treatment is given in Supplementary Table S9. The closest Arabidopsis homologs of the pea TFs are mainly involved in embryogenesis, seed development/filling, and stress responses, and four of them have been described as being involved in abscisic acid (ABA)-related processes, including *ABA Insensitive 5* (*ABI5*).

Discussion

This study investigated the effects of two major constraints for crop growth and yield that are expected to occur increasingly in the near future in the context of climate change and low fertilizer input, namely water stress and S deficiency. The combined stress was applied during the flowering period, a key phase during which seed yield and quality are both established (Ney *et al.*, 1994). By comparing nutrient contents and partitioning with changes in seed yield components and

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Nutrient content and ratio in the different compartment	Well-watered							Water-stressed							
	Sulfur supply			Sulfur deficiency				Sulfur supply				Sulfur deficiency			
	Control			S-				WS				S-WS			
	Mean	SE	SNK test	Mean	SE	SNK test	Ratio to Control	Mean	SE	SNK test	Ratio to Control	Mean	SE	SNK test	Ratio to Control
Sulfur Content (%)															
Seeds	0.19	0.001	a	0.05	0.003	c	0.29 ↘	0.19	0.003	a	0.99	0.08	0.002	b	0.42 ↘
Pod walls	0.07	0.002	a	0.04	0.003	c	0.54 ↘	0.06	0.008	ab	0.88	0.05	0.003	bc	0.66 ↘
Reproductive plant parts	0.23	0.023	a	0.05	0.006	c	0.23 ↘	0.18	0.035	a	0.80	0.07	0.001	c	0.30 ↘
Vegetative plant parts	0.29	0.030	a	0.06	0.002	b	0.20 ↘	0.26	0.026	a	0.91	0.05	0.004	b	0.18 ↘
Roots	0.57	0.066	a	0.07	0.003	b	0.13 ↘	0.64	0.034	a	1.13	0.07	0.002	b	0.12 ↘
Nitrogen Content (%)															
Seeds	4.54	0.036	b	4.96	0.081	a	1.09 ↗	4.48	0.073	b	0.99	5.20	0.128	a	1.15 ↗
Pod walls	0.38	0.012	a	0.38	0.049	a	1.02	0.35	0.008	a	0.92	0.88	0.158	b	2.33 ↗
Reproductive plant parts	1.33	0.052	a	1.02	0.070	a	0.77	1.19	0.076	a	0.90	1.21	0.088	a	0.91
Vegetative plant parts	1.60	0.030	a	1.34	0.122	a	0.84	1.48	0.054	a	0.93	1.75	0.190	a	1.10
Roots	3.28	0.035	a	3.08	0.133	a	0.94	2.91	0.173	a	0.89	3.20	0.187	a	0.98
Carbon Content (%)															
Seeds	45.56	0.059	a	45.09	0.094	b	0.99 ↘	45.60	0.040	a	1.00	44.92	0.115	b	0.99 ↘
Pod walls	43.83	0.066	a	44.26	0.203	a	1.01	42.89	0.632	a	0.98	44.16	0.081	a	1.01
Reproductive plant parts	40.27	0.098	a	41.08	0.133	a	1.02	40.66	0.504	a	1.01	41.22	0.446	a	1.02
Vegetative plant parts	42.43	0.063	a	42.50	0.051	a	1.00	42.71	0.106	a	1.01	42.64	0.203	a	1.00
Roots	41.08	0.502	a	37.66	0.211	b	0.92 ↘	38.49	0.426	b	0.94 ↘	38.69	0.844	b	0.94 ↘
N/S															
Seeds	23.96	0.107	c	91.50	6.357	a	3.82 ↗	24.00	0.417	c	1.00	65.67	2.549	b	2.74 ↗
Pod walls	5.15	0.226	b	9.88	1.255	b	1.92	5.67	0.802	b	1.10	19.01	4.376	a	3.69 ↗
Reproductive plant parts	6.00	0.529	b	20.56	3.223	a	3.43 ↗	7.07	0.939	b	1.18	17.97	1.093	a	3.00 ↗
Vegetative plant parts	5.69	0.554	c	23.29	2.453	b	4.09 ↗	5.73	0.332	c	1.01	33.70	4.206	a	5.92 ↗
Roots	5.98	0.637	b	41.40	2.763	a	6.92 ↗	4.62	0.463	b	0.77	45.32	3.635	a	7.58 ↗
C/N															
Seeds	10.03	0.068	a	9.09	0.167	b	0.91 ↘	10.19	0.172	a	1.02	8.65	0.201	b	0.86 ↘
Pod walls	116.78	3.771	a	121.51	15.853	a	1.04	124.34	3.690	a	1.06	58.33	14.867	b	0.50 ↘
Reproductive plant parts	30.49	1.111	a	40.93	2.799	b	1.34 ↗	34.66	2.243	ab	1.14	34.67	2.292	ab	1.14
Vegetative plant parts	26.59	0.493	a	32.44	2.684	a	1.22	28.95	1.059	a	1.09	25.37	3.270	a	0.95
Roots	12.55	0.239	a	12.29	0.453	a	0.98	13.36	0.801	a	1.06	12.20	0.619	a	0.97

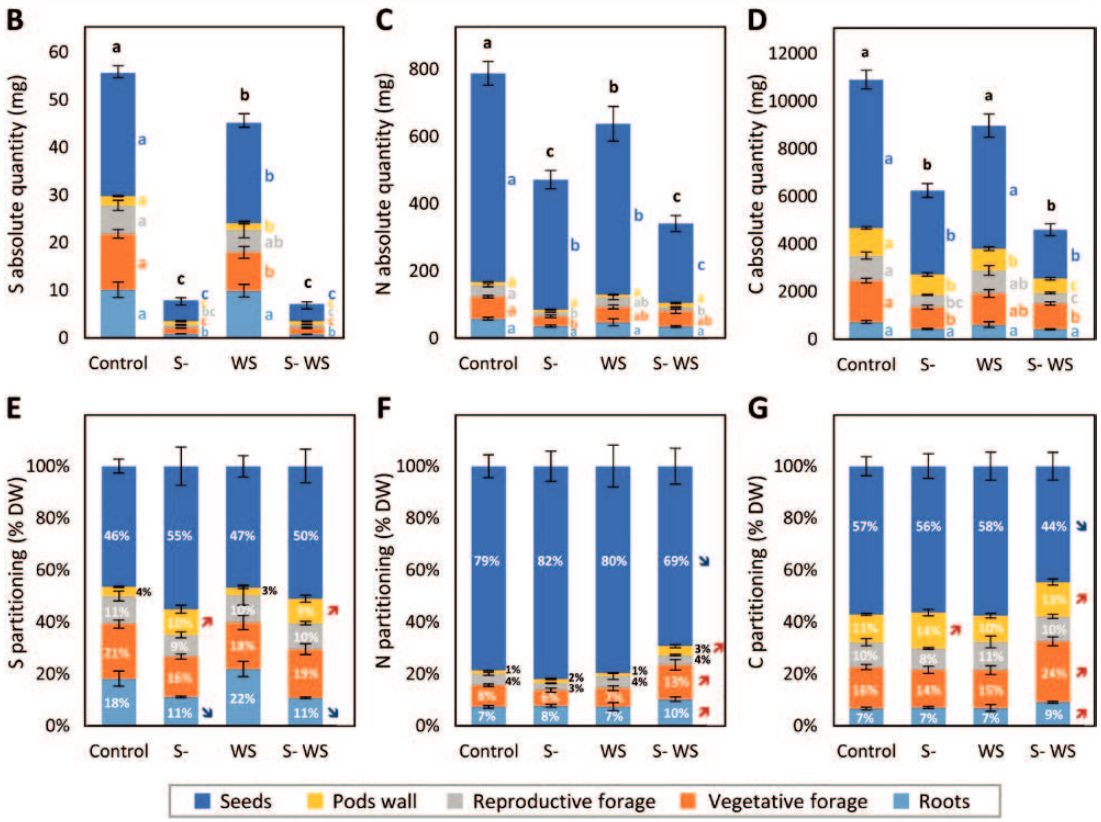
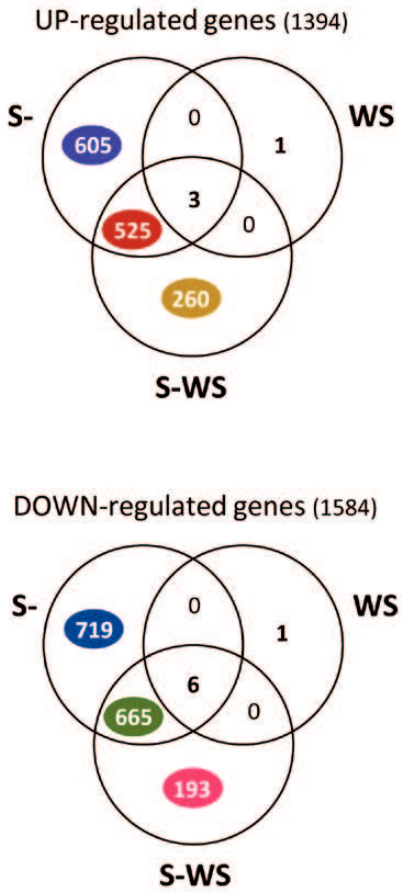


Fig. 3. Effects of water stress combined with S deficiency on the accumulation and distribution of N, C, and S in different pea plant compartments at maturity. (A) Sulfur (S), nitrogen (N), and carbon (C) contents (%), and N/S and C/N ratios, in each compartment. (B–D) Absolute quantity (mg) of S, N, and C in each compartment. (E–G) Proportions of N, C, and S in the dry biomass (% dry weight) of each compartment. Data are means (±SE). Bold and colored values (A), different letters (B–D), or arrows (E–G) indicate significant differences as determined by ANOVA followed by a SNK test (P<0.05, n=4 plants per condition). S–, Sulfur deficiency alone; WS, water stress alone; S–WS, combined stresses. Data are shown for seeds pooled from G1–G4.

protein composition, we have formulated an integrative view of the effects of the interaction between water stress and S deficiency in pea (Fig. 6). Moreover, a transcriptome analysis of developing pea seeds (9 DAP) revealed the molecular processes occurring at the end of the combined stress period. Our results indicated synergistic and mitigating effects of these two

A



B

	GO Term	Description	Annotated	Significant	*P*-value
UP-REGULATED GENES		Response to S- only (605 genes)			
	GO:0055114	Oxidation-reduction process	1913	70	1.3E-06
	GO:0043086	Negative regulation of catalytic activity	144	12	3.1E-05
		Response to S- and S-WS only (525 genes)			
	GO:0043086	Negative regulation of catalytic activity	144	17	1.1E-10
	GO:0006508	Proteolysis	885	37	4.9E-08
	GO:0055114	Oxidation-reduction process	1913	60	4.1E-07
	GO:0009086	Methionine biosynthetic process	10	4	1.1E-05
	GO:0006791	Sulfur utilization	7	3	1.3E-04
	GO:0019419	Sulfate reduction	2	2	2.4E-04
	GO:0008272	Sulfate transport	23	4	4.1E-04
		Response to S-WS only (260 genes)			
	GO:0016925	Protein sumoylation	3	2	1.4E-04
	GO:0006355	Regulation of transcription, DNA-templated	1452	21	8.0E-04
DOWN-REGULATED GENES		Response to S- only (719 genes)			
	GO:0006412	Translation	717	63	6.1E-21
	GO:0006879	Cellular iron ion homeostasis	6	4	3.6E-06
	GO:0006826	Iron ion transport	13	4	1.5E-04
	GO:0009765	Photosynthesis, light harvesting	24	5	1.6E-04
	GO:0015979	Photosynthesis	145	15	3.6E-04
	GO:0042254	Ribosome biogenesis	91	8	9.9E-04
		Response to S- and S-WS only (665 genes)			
	GO:0006412	Translation	717	79	1.1E-24
	GO:0009765	Photosynthesis, light harvesting	24	12	1.2E-13
	GO:0042180	Cellular ketone metabolic process	562	37	1.1E-07
	GO:0044106	Cellular amine metabolic process	394	27	2.8E-06
	GO:0015979	Photosynthesis	145	25	8.7E-06
	GO:0009309	Amine biosynthetic process	183	16	1.7E-05
	GO:0006526	Arginine biosynthetic process	17	5	4.8E-05
	GO:0006414	Translational elongation	54	8	5.8E-05
	GO:0009082	Branched-chain amino acid biosynthesis	20	5	1.1E-04
	GO:0042026	Protein refolding	12	4	1.7E-04
	GO:0015995	Chlorophyll biosynthetic process	26	5	4.2E-04
	GO:0005978	Glycogen biosynthetic process	17	4	7.3E-04
		Response to S-WS only (193 genes)			
	GO:0006801	Superoxide metabolic process	23	3	4.2E-04

Fig. 4. Effect of water stress combined with S deficiency on the transcriptome of pea seeds from group 2 (G2) at 9 d post-pollination. (A) Venn diagrams showing the numbers of up- and down-regulated genes in response to stresses compared to the control. (B) GO term enrichment analysis for the differentially expressed genes in response to stresses. Terms in the 'Biological Process' category are shown for genes up- and down-regulated in response to the different stresses. For each term, the number of genes present in the genome ('annotated'), the number of genes present in the gene lists ('significant'), and the associated *P*-value (Fisher's test, threshold of $P<0.001$) are given. S–, S deficiency alone; WS, water stress alone; S–WS, combined stress.

combined abiotic stresses on seed yield components and seed composition, respectively, and revealed genes involved in the early response of pea seeds to these stresses.

S nutrition helps to maintain yield in pea plants exposed to a moderate water stress episode

Our results showed that short-term and moderate water stress during flowering in pea did not significantly affect seed yield and total plant biomass, although the individual seed weight and the number of reproductive nodes were slightly reduced (Table 1, Fig. 6). A similar weak response was also observed by Ney *et al.* (1994), who reported that a short-term drought in pea of about 6 d (with a leaf water potential between −1.1 MPa and −1.4 MPa) did not lead to major developmental changes except for the number of flowering nodes. In contrast, S deficiency negatively affected yield components and seed composition (Fig. 6), indicating that the amount of S accumulated during the first 3 weeks of plant growth was not sufficient to maintain seed production and quality. The combination of water stress and S deficiency had a synergistic effect on the reduction of seed yield (Table 1, Fig. 6), leading to a decrease of the harvest index, which suggests failure in the allocation of assimilated photosynthates to seeds (Sinclair, 1998). In addition, S-deficient plants experiencing a water-stress period that was 3 d longer did not survive (Supplementary Fig. S1A), which demonstrated the critical need to maintain S nutrition in order for pea to maintain yield when facing water stress, even if the stress is moderate.

The strong response of S-deprived pea plants to water stress may be explained by a reduced adaptation to water stress. This could be in part attributed to the role of S in the production of metabolites that play roles in repair (e.g. the methyl donor *S*-adenosylmethionine), osmoprotection or antioxidation (Chan *et al.*, 2013; Anjum *et al.*, 2015). In particular, detoxification of increased levels of reactive oxygen species (ROS) produced during the drought period (Cruz de Carvalho, 2008) may necessitate an increased production of the ROS-scavenger molecule glutathione, the level of which drastically decreases in plants grown under S deficiency (Nikiforova *et al.*, 2003; Ostaszewska-Bugajska *et al.*, 2015). Alternatively, the response of double-stressed plants may be attributed to the role of

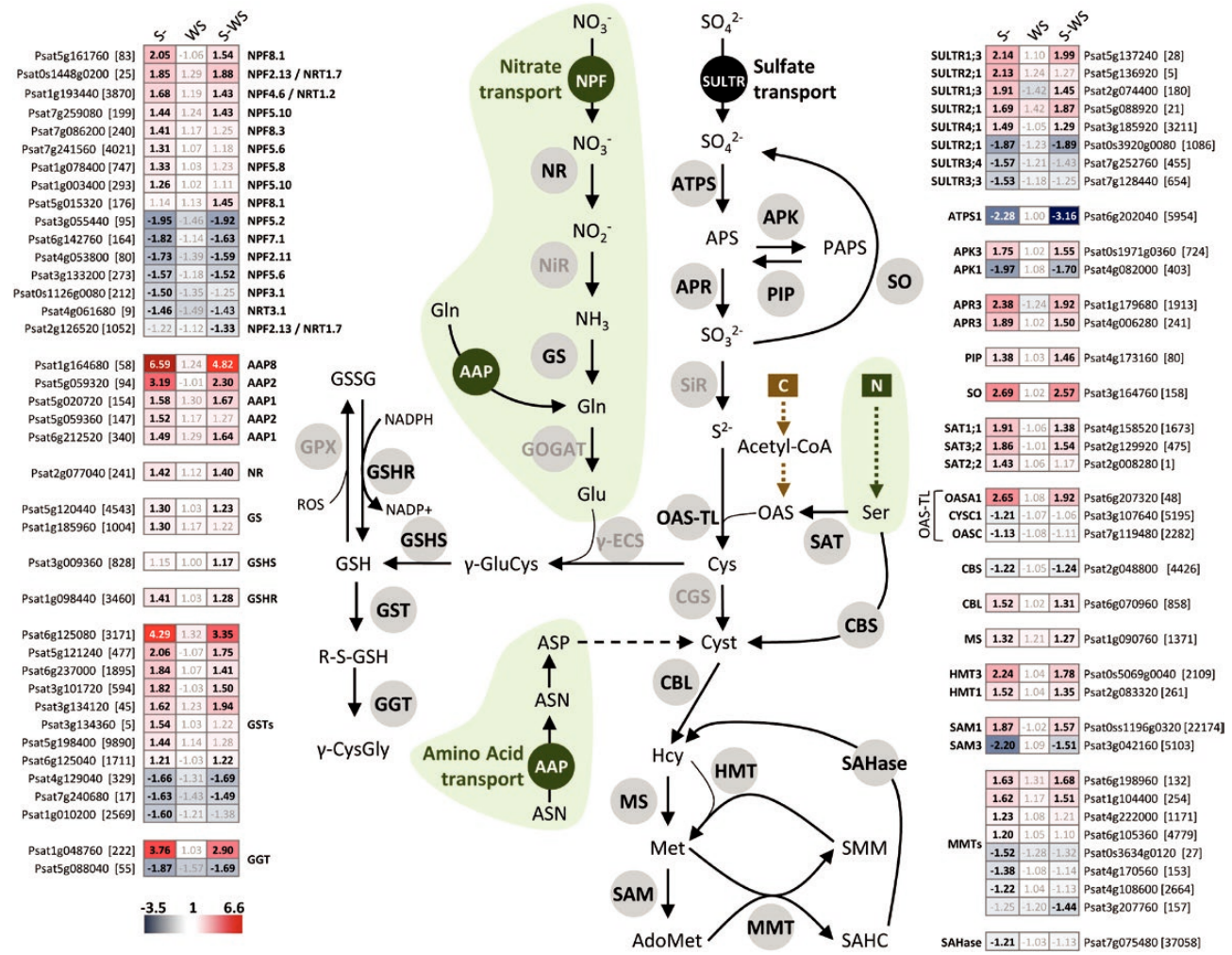


Fig. 5. Effects of water stress combined with S deficiency on the expression of genes related to S and N transport and metabolism in pea. For each gene, the fold-change in expression under stress conditions compared to the control is given. The values are color-coded from low expression in blue to high expression in red, and values and enzymes in bold indicate significant differences between stress and control conditions. S–, S deficiency alone; WS, water stress alone; S–WS, combined stresses. Gene annotation refers to the closest *Arabidopsis thaliana* homolog (Supplementary Table S3). For each gene, the mean expression level under control conditions (expressed in counts) is given in square brackets. Enzymes: APK, APS kinase; APR, APS reductase; ATPS, ATP sulfurylase; CBL, cystathionine β-lyase; CBS, cystathionine β-synthase; CGS, cystathionine γ-synthase; γ-ECS, γ-glutamylcysteine synthase; GGT, γ-glutamyl transferase; GOGAT, glutamate synthase; GS, glutamine synthetase; GSHS, glutathione synthetase; GSHR, glutathione reductase; GPX, glutathione peroxydase; GST, glutathione S-transferase; HMT; homocysteine-*S*-methyltransferase; MMT, S-adenosylmethionine methyltransferase; MS, methionine synthase; NiR, nitrite reductase; NR, nitrate reductase; OAS-TL, OAS thiol-lyase; PIP, 3′(2′),5′-bisphosphate nucleotidase; SAHase, *S*-adenosylhomocysteine hydrolase; SAM, *S*-adenosylmethionine synthetase; SAT, serine acetyltransferase; SiR, sulfite reductase; SO, sulfite oxidase. Metabolites: AAP, amino acid permease; AdoMet, *S*-adenosylmethionine; APS, adenosine 5′-phosphosulfate; ASN, asparagine; ASP, aspartate; Cys, cysteine; Cyst, cystathionine; Gln, glutamine; Glu, glutamate; γ-GluCys, γ-glutamylcysteine; γ-CysGly, γ-cysteinylglycine; GSH, glutathione; GSSG, glutathione disulfide; Hcy, homocysteine; Met, methionine; OAS, O-acetylserine; PAPS, 3′-phoshoadenosine 5′-phosphosulfate; SAHC, *S*-adenosylhomocysteine; SMM, S-methylmethionine. Transporters: AAP, amino acid permeases; NPF, nitrate peptide transporter family; SULTR, sulfate transporters (green shading indicates N transport and metabolism).

sulfate and sulfide in signaling stomatal closure (Ernst *et al.*, 2010; Malcheska *et al.*, 2017; Rajab *et al.*, 2019), possibly by promoting ABA biosynthesis or gating open the anion channel of guard cells (Cao *et al.*, 2014; Malcheska *et al.*, 2017). Plants grown under combined stress conditions could thus be more susceptible to drought due to defects in stomatal closure. In addition, studies in maize have shown that drought can affect the long-distance mobility of sulfate (Ahmad *et al.*, 2016). Such an impairment of the root-to-shoot transport of sulfate might exacerbate the negative effect of S deficiency on the production in double-stressed leaves of sulfate-derived metabolites that are essential for the survival of the plant.

One of the main findings of our study was that the combined stress impeded reproductive development of pea. Double-stressed plants produced fewer reproductive nodes, fewer and smaller seeds, and FSSA was reached earlier in these plants (Fig. 1, Table 1), reflecting adaptive mechanisms aimed at accelerating seed production to ensure the survival of the species, as previously observed in response to drought (Desclaux and Roumet, 1996) or S deficiency (Hoefgen and Nikiforova, 2008). This was associated with a reduced leaf chlorophyll content during the early reproductive phase (Supplementary Fig. S1B), an indication of senescence, which is a highly regulated process providing nutrients to newly formed organs, including

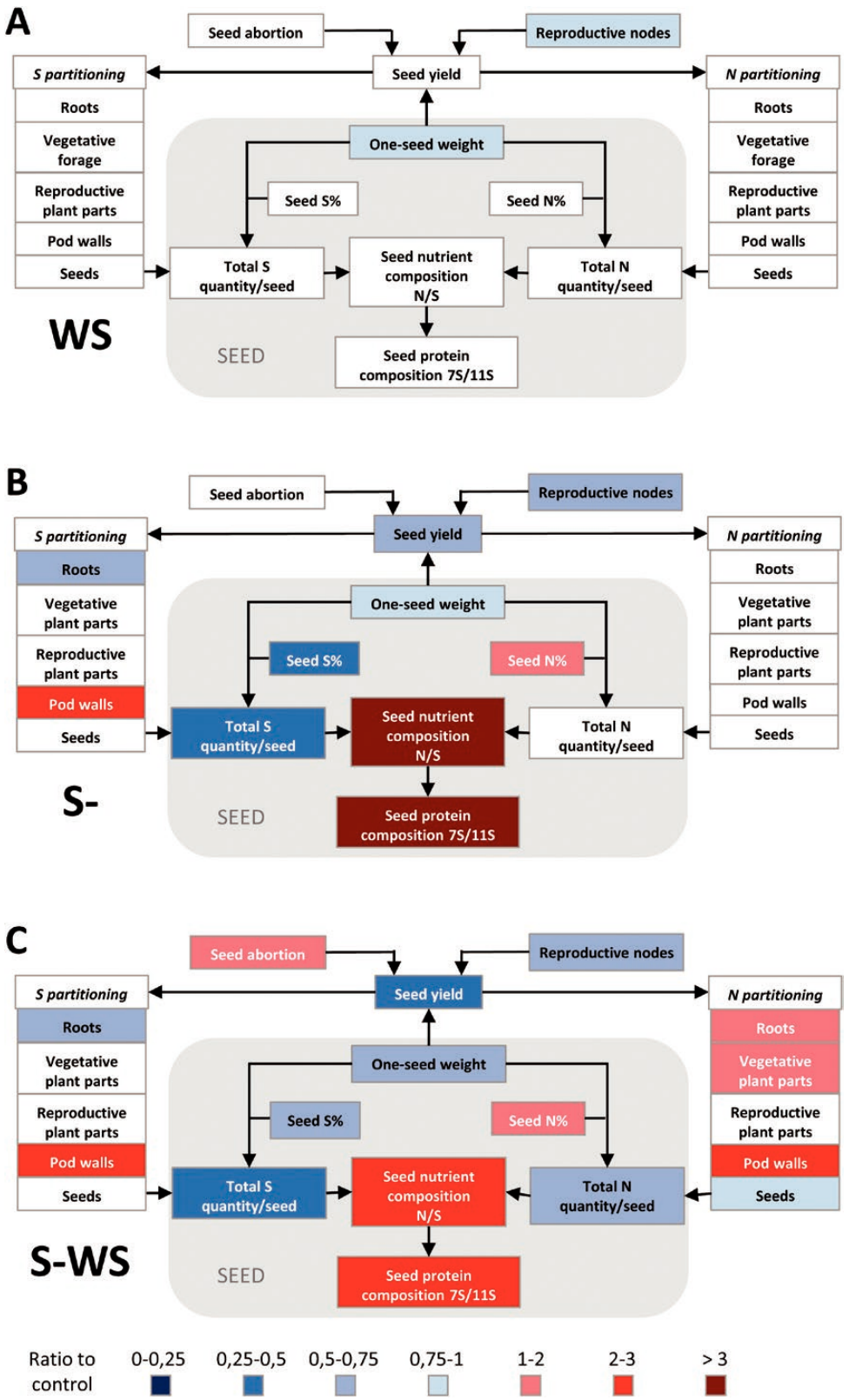


Fig. 6. Summary of the effects of water stress with or without S deficiency on yield components, nutrient partitioning, and seed nutrient and protein composition at harvest in pea. (A) Summary for water-stress conditions. (B) Summary for sulfur-deficient conditions. (C) Summary for combined stress condition. The colors correspond to the ratios of values for stressed plants versus control plants, and indicate whether the ratio was significantly decreased (blue) or increased (red). S–, S deficiency; WS, water stress; S–WS, combined stresses.

developing seeds (Guiboileau *et al.*, 2010). The reduced level of leaf chlorophyll could be attributed to a lower production of sulfate-derived molecules and in particular sulfide, which has been shown to prevent autophagy and senescence in Arabidopsis (Álvarez *et al.*, 2012; Dong *et al.*, 2017). By contrast, chlorophyll content remained higher in leaves of the

double-stressed plants by the end of the reproductive period (Supplementary Fig. S1B), presumably because the seed compartment no longer needed to be supplied with nutrients. This was consistent with the nutrient partitioning between plant organs, especially for C and N, the contents of which remained high in the roots and in the vegetative parts of the plant, and increased in the pod walls, a sign that nutrients could no longer be translocated to seeds (Fig. 3).

Water stress mitigates the effects of S deficiency on the developing seed transcriptome and rebalances the protein composition in mature seeds

S deficiency decreased the relative abundance of S-rich globulins (11S) in pea seeds (Fig. 2), as previously described for this species (Chandler *et al.*, 1984; Evans *et al.*, 1985), and for other crops (e.g. wheat, Bonnot *et al.*, 2017; rapeseed, D'Hooghe *et al.*, 2014). This has been shown to be primarily regulated at the transcriptional level (Chandler *et al.*, 1983) and it is compensated by an increase in the S-poor 7S globulins (Chandler *et al.*, 1984, Fig. 2). Seed N content, which is proportional to seed protein content by a multiplication factor of 5.4 in pea (Mariotti *et al.*, 2008), was significantly higher in seeds from S-deficient plants (Table 2). This was also the case for plants subjected to the combined stresses, and hence the conditions used in this study revealed adaptive processes that kept the seed protein content high through the accumulation of 7S globulins. Interestingly, the seed protein composition was less affected by the combined stresses, reflecting a mitigating effect of water stress (Figs 2, 6). Similar results have been obtained in oilseed rape, where heat stress during seed filling has been shown to mitigate the negative effect of S deficiency on the S-poor/S-rich globulin ratio (Brunel-Muguet *et al.*, 2015). In our experiments, the S quantity per seed was similarly low in the S-deficient and combined-stress plants, while the N and C quantities per seed specifically decreased in response to the combined stress (Supplementary Table S5, Fig. 3, Table 2). Because of the reduced weight of the seeds from the combined-stress plants, we hypothesized that these seeds required less N and C to be filled, enabling a better balance between N and S compared to seeds of plants deprived of S. Accordingly, the N/S ratio was less affected in seeds of the double-stressed plants compared to S deficiency alone (Table 2, Supplementary Table S5). Hence, our data demonstrated a lower seed sink strength for N and C but similar seed S uptake, thus rebalancing the seed protein composition (Fig. 6). We assumed that this would reduce the sensing of S deficiency and mitigate its effect on the developing seed transcriptome. Accordingly, fewer genes were regulated in response to the combined stresses than under S deficiency alone. Moreover, similar biological processes were regulated in response to S deficiency alone and to the combined stresses, but the extent of the transcriptional regulation was greater under S deficiency than under the combined stresses (Fig. 4).

In order to identify candidate genes for rebalancing the N/S ratio in response to the double-stresses, we examined the expression of genes related to S and N metabolism and transport in developing seeds that were at a transition stage towards storage-protein accumulation (9 DAP, Supplementary Table S7). This revealed a tight regulation of these genes by S deficiency with or without water stress (Fig. 5). In fact, the genes of almost all enzymes of S metabolism, from APS reductase to methionine synthase, were up-regulated in response to these stresses. Three up-regulated genes encoded isoforms of serine acetyltransferase, which catalyses the synthesis of O-acetylserine, the precursor of cysteine, from serine and acetyl-CoA, and thus represents a key enzyme connecting S metabolism with the N and C metabolisms. O-acetylserine has been proposed to act as a signal in the transduction pathways sensing S and N availability (Kim *et al.*, 1999): its accumulation in the siliques of Arabidopsis increases in response to low-S and high-N conditions, and exogenous application of O-acetylserine to immature soybean cotyledons regulates seed storage-protein accumulation in a similar way to S deficiency (i.e. decrease of S-poor globulins; Kim *et al.*, 1999). In our present study, the similar regulation of genes related to N and S metabolism (including serine acetyltransferase) in S– and S–WS seeds strongly suggested that the rebalancing of globulin composition in the double-stressed seeds did not involve specific regulation of these pathways, at least within the seed.

The rebalancing of the seed globulin composition in the double-stressed seeds could be controlled at the transcriptional level through the recognition by specific TFs of motifs in storage-protein promoters (Fujiwara and Beachy, 1994). Interestingly, one of the two GO terms with a significantly high occurrence in the list of genes specifically up-regulated in response to the combined stresses in seeds at 9 DAP was 'Regulation of transcription' (Fig. 4B). This list of regulators included six TFs with a known role during seed development (Supplementary Tables S8, S9), such as ABI5 that is necessary for accumulation of 47-kDa vicilin in mature pea seeds (Le Signor *et al.*, 2017). Studies in common bean (*Phaseolus vulgaris*) suggest that ABI5 interacts with the 7S globulin promoter through a G-box motif (Ng and Hall, 2008), which is essential for beta-phaseolin (7S) accumulation (Pandurangan *et al.*, 2016). The up-regulation of ABI5 in response to the combined stresses was intriguing since vicilins did not accumulate in seeds of the double-stressed plants to as high an extent as they did under S deficiency alone (Fig. 2). Hence, the activity of ABI5 might be tightly regulated in these seeds to avoid an over-accumulation of vicilins at the expense of legumins. Interestingly, the second GO term enriched in the list of genes up-regulated in response to the combined stress was 'Protein sumoylation' (Fig. 4B), and the two genes carrying these GO terms were homologous to AT5G60410 (Supplementary Tables S8, S9), a small ubiquitin-related modifier (SUMO) E3 ligase named SIZ1, which has been shown in Arabidopsis to negatively regulate ABI5 activity through sumoylation (Miura *et al.*, 2009). Owing to the co-regulation of *SIZ1* and *ABI5* in early developing seeds in response to the combined stresses, it is possible that SIZ1 plays a role in controlling the activity of ABI5 under these conditions. In future work, it will be interesting to test whether sumoylation of ABI5 occurs in seeds through SIZ1 to prevent a high accumulation of vicilins under S deficiency in order to maintain the N/S ratio as much as possible when the sink strength for N is reduced, as observed for seeds of the double-stressed plants.

Conclusions

This study revealed the importance of S for stabilizing seed yield in pea plants facing short and moderate episodes of water stress, and showed that the adaptive responses of S-deprived plants to water stress are much more complicated than a simple additive response. The combined stresses induced pleiotropic effects that were aimed at accelerating seed production, specifically leading to seed abortion, while rebalancing the seed globulin composition, probably as the result of a lower seed sink strength for N. Moreover, combined stress mitigated the impact of S deficiency on the transcriptome of seeds at 9 DAP. The transcriptomics data clearly advance our knowledge of the molecular responses of developing pea seeds to S deficiency occurring either with or without water stress. Candidate genes for fine-tuning the regulation of globulin synthesis under stressed conditions were identified, and our future work will investigate their relevance for potential applications (e.g. for stabilizing the accumulation of S-rich globulins) by reverse genetics using TILLING (Targeting Induced Local Lesions in Genomes) mutants in pea (Dalmais *et al.*, 2008).

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. Effects of water stress combined with S deficiency plant phenotype and estimated leaf chlorophyll content.

Fig. S2. Correlation between the N/S and 7S/11S ratios in mature seeds from groups G1–G4.

Fig. S3. RT-qPCR validation of the RNA-seq results.

Table S1. List of proteins identified by LC-MS/MS in the 1-DE protein profile of seeds.

Table S2. Summary of RNA-seq mapping results.

Table S3. List of genes differentially expressed in response to stresses compared to the control.

Table S4. Detailed protein composition of the different groups of mature seeds.

Table S5. Effects of stresses on S, C, and N contents and quantity in each group of seeds.

Table S6. Variation in S, C, and N contents and quantity between seed groups for each treatment.

Table S7. Expression of globulin genes in seeds at 9 DAP.

Table S8. GO term enrichment analysis for the differentially expressed genes.

Table S9. List of genes encoding transcription factors and SUMO-ligases specifically up-regulated in response to the combined stresses.

Data deposition

The MS proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Vizcaíno *et al.*, 2014) with the dataset identifier PXD011029. The raw RNA-seq data have been deposited to the NCBI SRA database (http://www.ncbi.nlm.nih.gov/bioproject/) under accession number PRJNA517587.

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Annexe 2

frontiers in Plant Science

ORIGINAL RESEARCH
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Transcriptional Reprogramming of Pea Leaves at Early Reproductive Stages

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Pea (*Pisum sativum* L.) is an important source of dietary proteins. Nutrient recycling from leaves contributes to the accumulation of seed proteins and is a pivotal determinant of protein yields in this grain legume. The aim of this study was to unveil the transcriptional regulations occurring in pea leaves before the sharp decrease in chlorophyll breakdown. As a prelude to this study, a time-series analysis of ^{15}N translocation at the whole plant level was performed, which indicated that nitrogen recycling among organs was highly dynamic during this period and varied depending on nitrate availability. Leaves collected on vegetative and reproductive nodes were further analyzed by transcriptomics. The data revealed extensive transcriptome changes in leaves of reproductive nodes during early seed development (from flowering to 14 days after flowering), including an up-regulation of genes encoding transporters, and particularly of sulfate that might sustain sulfur metabolism in leaves of the reproductive part. This developmental period was also characterized by a down-regulation of cell wall-associated genes in leaves of both reproductive and vegetative nodes, reflecting a shift in cell wall structure. Later on, 27 days after flowering, genes potentially switching the metabolism of leaves toward senescence were pinpointed, some of which are related to ribosomal RNA processing, autophagy, or transport systems. Transcription factors differentially regulated in leaves between stages were identified and a gene co-expression network pointed out some of them as potential regulators of the above-mentioned biological processes. The same approach was conducted in *Medicago truncatula* to identify shared regulations with this wild legume species. Altogether the results give a global view of transcriptional events in leaves of legumes at early reproductive stages and provide a valuable resource of candidate genes that could be targeted by reverse genetics to improve nutrient remobilization and/or delay catabolic processes leading to senescence.

Keywords: legumes, leaves, reproductive period, nitrogen remobilization, transcriptomics, co-expression, transcription factors, transporters

INTRODUCTION

Grain legumes accumulate large amounts of proteins in their seeds, which are widely used for human and animal nutrition. In legumes, symbiotic nitrogen fixation, and nitrate uptake by roots are two complementary modes of nitrogen acquisition that decline during the reproductive period (Salon et al., 2001). Nitrogen stored in plant parts is then remobilized to sustain seed protein accumulation. The contribution of nitrogen remobilization to seed protein yield varies from 45 to 90%, depending on the species and conditions (Warembourg and Fernandez, 1985; Kurdali et al., 1997). In pea (*Pisum sativum* L.), 70% of the amount of nitrogen in mature seeds is derived from remobilization processes (Jensen, 1987; Schiltz et al., 2005). The chloroplast enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase, which plays an essential role in carbon fixation, is one major source of nitrogen in leaves (Jiang et al., 1993). Its degradation starts before leaf senescence, a catabolic process leading to yellowing, chloroplast disassembly, and finally cell death (Kohzuma et al., 2017). Because most leaf nitrogen is stored in the form of proteins with roles in the photosynthetic machinery, nitrogen remobilization may affect photosynthetic activities, which may curtail the reproductive period and limit seed yield. Nutrient deficiencies, high temperature and drought, are environmental factors accelerating leaf senescence, thereby shortening the reproductive period and impacting negatively seed filling (Olsson, 1995; Srivalli and Khanna-Chopra, 1998). Stay-green varieties, where leaf senescence is delayed, are used in some cereal improvement programs since they display a greater grain yield under post-anthesis drought (Borrell et al., 2001). However, stay-green phenotypes are not necessarily associated with higher yields, especially when chlorophyll catabolism is blocked since the active degradation of chlorophyll is a prerequisite for nitrogen remobilization from the pigment-associated proteins (Thomas, 1997; Thomas and Howarth, 2000). Hence, optimizing the balance between nutrient recycling and leaf longevity is necessary to increase and stabilize protein yield. This requires the identification of the underlying molecular determinants that could be targeted in breeding programs for higher and stable protein yields.

The mechanisms controlling nutrient recycling have been mainly studied during senescence associated with leaf yellowing. Genes up-regulated during this process, generally referred to as senescence-associated genes (SAGs) or senescence-enhanced genes, were identified (Buchanan-Wollaston et al., 2005). Several SAGs are related to autophagy, a vesicular trafficking process that regulates nutrient recycling and remobilization by participating in the methodical degradation of the cell constituents (Masclaux-Daubresse et al., 2017). Several lines of evidence indicate that senescence-related transcription factors (TFs) can directly regulate autophagy genes in plants (Garapati et al., 2015). Transcriptomics revealed that a large number of NAC (no apical meristem, transcription activation factors, and cup-shaped cotyledon) TFs are expressed during leaf senescence (Balazadeh et al., 2010; Breeze et al., 2011; Yang et al., 2016). Functional studies in Arabidopsis showed that NACs can act as positive or negative regulators of senescence (Yang et al., 2011; Liang et al., 2014; Garapati et al., 2015; Zhao et al., 2015; Pimenta et al., 2016). However, we are far from a comprehensive understanding of the pathways and regulatory networks influencing nutrient recycling in crops, especially in grain legumes such as pea, a monocarpic species that exhibits different patterns of whole plant senescence compared to Arabidopsis, and in which the production of seeds triggers nutrient remobilization (Noodén and Penney, 2001; Pic et al., 2002). The aim of the present study was to unveil the transcriptional reprogramming of pea leaves at stages preceding the sharp decrease in chlorophyll breakdown. Nitrogen remobilization between tissues was highly dynamic during this period, as shown through a time-series analysis of the translocation of ^{15}N absorbed in the form of nitrate up to flowering. Leaves of the vegetative and reproductive nodes were analyzed by transcriptomics and a gene co-expression approach was used to highlight potential regulators of specific biological processes. The same approach in the fodder legume species *M. truncatula* revealed a number of shared co-expression modules.

MATERIALS AND METHODS

Plant Growth Conditions

Pea (*Pisum sativum* L, genotype "Caméor") and *Medicago truncatula* (*M. truncatula*, Gaertn., A17 genotype) plants were grown in a greenhouse under controlled temperature (at least 18°C during the day and 15°C during the night) and photoperiod (16h/d). *M. truncatula* seeds were scarified and vernalized 4d at 5°C before sowing. Plants were grown in 7L (pea) or 3L (*M. truncatula*) pots containing 40% attapulgite and 60% clay balls. Plants were not inoculated with Rhizobia. Nitrogen nutrition of all plants relied on the absorption of nitrate for the purpose of long-term ^{15}N-labeling. Two nitrogen availability conditions were used. Control plants (N+) were supplied with the nutrient solution previously described (Zuber et al., 2013) until tissue collection. For N– plants, nitrate was depleted at the beginning of flowering using the same solution without KNO_3 and $Ca(NO_3)_2$ (replaced by 1.85 mM KCl and 0.25 mM $CaCl_2$). Leaf chlorophyll content at the first flowering node was measured using a SPAD-502 chlorophyll meter on 12–16 plants per condition and stage (Minolta Camera Co. Ltd., Japan). The plant, pod and seed characteristics in **Table S1** were measured at maturity (63 days after flowering) from eight biological replicates (i.e., individual plants). An analysis of variance was performed to reveal significant effects of nitrogen limitation on these traits (Statistica v7.0 software).

Dynamic of Nitrogen Remobilization at the Whole Plant Level in Pea and *M. truncatula*

For each time point [beginning of flowering, 14, 27, and 63 days after flowering (DAF)], six plants were used per condition (N+, N–): four plants were supplied with the nutrient solutions described above labeled with 3 atom% excess of ^{15}N (as $K^{15}NO_3$) until flowering (i.e., 35 days labeling), and two unlabeled plants served to estimate natural ^{15}N abundance. The pots were organized in a randomized complete-block design. For each time point and condition, leaves of the vegetative nodes (lower leaves), and reproductive nodes (upper leaves), stems, roots, pods (*M.*

truncatula), seeds, and pod wall (pea) were harvested separately. The dry matter of each tissue was determined after oven-drying at 80°C for 48 h. All tissues were ground using the cutting mill SM200 (Retsch, Haan, Germany), then using the ZM 200 grinder (Retsch). Total N and $^{15}N/^{14}N$ ratio were determined from 5 mg powder using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). The calculation of endogenous nitrogen (i.e., stored during the vegetative phase) remobilized across plant tissues between two developmental stages was determined from elemental and isotope amounts in the different organs using the PEF (Plant Elemental Flux) tool developed in visual basic applications (Salon et al., 2014). The quantitative values for nitrogen remobilized (mg) from or to each tissue between two time points were subjected to a *t*-test using Statistica software (v7.0) to reveal significant effects of nitrogen deficiency on the quantity of nitrogen remobilized from each tissue.

Leaf Samples and RNA Extraction

Lower and upper leaves were collected from 6 to 8 individual plants deprived or not of nitrate, at three stages: flowering, 14 and 27 DAF. The absence of nodules on the root system was checked at the time of tissue collection. Lower leaves corresponded to leaves of the two last vegetative nodes and upper leaves corresponded to leaves of nodes carrying flowers at the flowering stage, and to leaves of the third and fourth reproductive nodes at 14 and 27 DAF. The leaf samples were immediately frozen in liquid nitrogen, then stored at −80°C. RNA was extracted from 100 mg of frozen powder using the RNeasy Plant Mini Kit according to manufacturer's protocol (Qiagen, Courtaboeuf, France). RNA quality was checked on agarose gel 1.5%, then using the Agilent 2100 Bioanalyzer.

RT-qPCR Using *ELSA* as Indicator of Leaf Senescence

For profiling the expression of the *Early Leaf Senescence Abundant cysteine protease gene (ELSA)* (Pic et al., 2002) by RT-qPCR, leaf samples collected at flowering, 14 and 27 DAF on plants deprived or not of nitrate ($n = 6$–8) were used. RT-qPCR was performed with the iScript cDNA synthesis kit according to manufacturer's protocol (Bio-Rad, Marnes-la-Coquette, France) and the GoTaq qPCR Master Mix (Promega, Charbonnières, France) using 10 ng cDNA and 0.2 μM of each primer in a final volume of 5 μl. Analyzes were performed in triplicates from each biological replicate using the LightCycler 480 system (software v1.5.0, Roche, Meylan, France) as previously described (Zuber et al., 2013). The normalization method was ΔΔct using actine, histone, and EF1 α as reference genes (primers in **Table S2**). Analyses of variance and Student-Newman-Keuls (SNK) tests using the Statistica software (v7.0) revealed significant changes in gene expression between stages and/or in response to nitrate deficiency.

Transcriptomics of Leaves and Validation by RT-qPCR

Three biological replicates of leaves from vegetative and reproductive nodes were subjected to transcriptomics. Pea NimbleGen-microarrays were developed to profile expression of 40795 sequences: 40454 mRNA originating from the PsCameor_Uni_Lowcopy set (Alves-Carvalho et al., 2015), 323 putative precursors of miRNA predicted in the "Test assembly multiple k-mer" contig set (Alves-Carvalho et al., 2015), and 18 controls. Two specific oligonucleotides were used for each mRNA sequence and one oligonucleotide was used per miRNA precursor sequence (forward and reverse). These probes were spotted in triplicates on the GENOPEA array. *M. truncatula* NimbleGen-microarrays (Herrbach et al., 2017) were used in parallel. They represent 83029 probes (spotted in triplicates) corresponding to transcribed regions of the *M. truncatula* genome from the Symbimics program (https://iant.toulouse.inra.fr/symbimics/). The Ambion MessageAmp™ II aRNA Amplification Kit was used to amplify sufficient amounts of copy RNA extracted, as described above, from upper leaves and lower leaves of three biological replicates (independent plants). The Double stranded cDNA synthesis was realized using T7-oligo-dT and the antisense RNA (aRNA) was created by *in vitro* transcription according to manufacturer's protocol (Life technologies SAS, Saint Aubin, France). The labeling with Cy3 or Cy5 was performed by reverse transcription of aRNA using labeled nucleotides (Cy3-dUTP or Cy5-dUTP, Perkin-Elmer-NEN Life Science Products). For each nutritional condition and leaf type, the following co-hybridizations were performed: 14 DAF vs. flowering, 27 DAF vs. 14 DAF. For each comparison, a dye swap was realized. The hybridization of labeled samples on the slides, scanning and data normalization were performed as previously described (Lurin et al., 2004). Differential analysis was based on the $\log_2$ ratios averaged on the dye-swap: the technical replicates were averaged to get one $\log_2$ ratio per biological replicate and these values were used to perform a paired *t*-test. The raw *P*-values were adjusted by the Bonferroni method, which controls the family wise error rate, and probes were considered as differentially expressed when the Bonferroni corrected *P*-value was <0.05. Transcriptome datasets were deposited in the NCBI Gene Expression Omnibus database with the accession numbers GSE109789 for pea and GSE109521 for *M. truncatula*. All pea sequences with "PsCam" accession numbers could be retrieved from the pea RNAseq gene atlas at http://bios.dijon.inra.fr/ (PsUniLowCopy data set).

Twenty genes differentially regulated between two stages were selected for RT-qPCR analyses (as describe above) in leaves from three biological replicates of plants well-supplied with nitrate. For each leaf sample (lower and upper leaves) and developmental period (14 DAF vs. flowering, 27 vs. 14 DAF), Pearson's correlation coefficient (r) between microarray and RT-qPCR expression levels were calculated (**Table S3**, primers in **Table S2**). Hierarchical clustering of transporter and TF genes was performed using the Genesis software (v1.8.1; default parameters) (Sturn et al., 2002). Gene Ontology (GO) term enrichment analysis was performed using topGO (elim method

and Fisher's exact test) in Bioconductor v2.9 implemented in BIOS (Architecture BioInformatique Orientee Services, http://bios.toulouse.inra.fr/). Phylogenetic trees were generated from protein sequences using the Neighbor-joining method of the ClustalW2 program available at https://www.ebi.ac.uk/Tools/phylogeny/. Orthologous genes between pea and *M. truncatula* (v4.02) were identified using OrthoFinder v1.1.8 (MCL clustering algorithm and DIAMOND v0.9.10.111 for the alignment with default parameters). Of the 19055 clusters identified, 15445 were retained for transcriptome comparisons because they were made of a unique gene per species (14980 sequences with probes on the arrays).

Gene Co-expression Network Construction

Log_2 intensity values from each red and green channels were normalized based upon quantiles using the preprocess Core package (v1.34.0) available in R (v3.3.1). Gene variance was calculated using the gene filter R package (Gentleman et al., 2018) (v1.54.2) and only sequences displaying a variance >0.2 were retained for co-expression studies. Gene co-expression networks were built using the Expression Correlation plugin (v1.1.0, http://apps.cytoscape.org/apps/expressioncorrelation) of Cytoscape (v3.5.1) (Cline et al., 2007). We have chosen r cut-off of 0.95 and −0.95 (r^2 >0.9) to build P-REMONET from the pea transcriptome dataset, and of 0.90 and −0.90 (r^2 >0.81) to build M-REMONET from the *M. truncatula* transcriptome dataset. The node degree of the networks followed a power-law distribution. A Prefuse Force Directed layout was used to visualize the entire networks in Cytoscape. For ease of visualization of TF-related modules, the genes connected to the TFs were organized using the Circular Layout algorithm.

RESULTS

Dynamics of Nitrogen Remobilization During the Reproductive Phase in Pea

An overview of nitrogen remobilization between tissues was obtained through a time-series analysis of the translocation of ^{15}N absorbed in the form of nitrate during the vegetative phase (**Figure 1**). From the beginning of flowering to seed filling in the first pods (14 days after flowering, DAF), nitrogen taken up during the vegetative period was remobilized from leaves below the first flowering node (lower leaves; 46.5%, **Figure 1A**) and roots (53.5% of the total amount of remobilized nitrogen). This pool of nitrogen was mainly redistributed toward leaves of the reproductive part (upper leaves) and to pod walls. Then, from 14 DAF until the end of 1st pod seed filling (27 DAF), nitrogen was remobilized from stems (20%), lower, and upper leaves (80%) to seeds, pod wall, and roots. Roots behave as a transient sink of nitrogen during this period, probably because leaves, and stems provide sufficient amounts of nitrogen to fulfill seed nitrogen requirements. At later stages (27–63 DAF), nitrogen was remobilized from all tissues to seeds, which at maturity contained 54% of nitrogen derived from remobilization processes (**Figure 1A**). This shift to systemic remobilization to seeds coincided with the beginning of chlorophyll degradation in leaves (starting 33 DAF, **Figure S1A**). The increased expression of the early senescence marker *ELSA* in lower and upper leaves 27 DAF was indicative of a molecular switch toward proteolysis (**Figure S1B**). The 4-fold higher expression of *ELSA* in upper leaves 27 DAF, compared to lower leaves, suggests higher proteolytic activities in these leaves. Altogether, the data indicate that 27 DAF is a transition stage toward leaf senescence.

Nitrate deficiency during the reproductive phase triggered major changes in the dynamics of nitrogen remobilization (**Figure 1B**). From flowering to 14 DAF, nitrogen remobilization from roots decreased while nitrogen remobilization from lower leaves increased significantly in response to nitrate deficiency. From 14 to 27 DAF, roots became the major source of nitrogen specifically under nitrate deficiency and nitrogen remobilization from other tissues was significantly reduced in that condition, especially from lower leaves that became a transient sink for nitrogen. This may be part of the mechanisms used by plants to avoid precocious senescence in response to nitrogen deficiency. While leaf nitrogen content decreased continuously from flowering to maturity under nitrate-sufficient conditions, it remained unchanged between 14 and 27 DAF in nitrate-deprived plants (**Figure S2**). These data and the lower expression of *PsELSA* in lower and upper leaves of these plants, suggest a lower remobilization rate in response to nitrate deficiency (**Figure S1B**), associated with a maintained chlorophyll content (**Figure S1A**). At later stages (27–63 DAF), nitrogen remobilization from almost all tissues was significantly reduced in response to nitrate deficiency and, at maturity, these plants were characterized by a reduced seed yield and one-seed weight (**Table S1**).

Transcriptome Changes in Pea Leaves at Early Reproductive Stages

The molecular processes regulated in pea leaves at stages characterized by dynamic nitrogen remobilization between tissues, from flowering to 27 DAF, were investigated by transcriptomics. An analysis of transcriptome changes occurring in leaves of the vegetative and reproductive nodes under both nitrate-sufficient and -deficient conditions was carried out. The GENOPEA array representing 40777 pea sequences was used. Quantitative RT-PCR data for 20 genes differentially expressed showed high correlations with array data (Pearson's correlation coefficient r ranging from 0.80 to 0.93, **Table S3**), confirming the robustness of the approach to identify genes differentially regulated in pea leaves. An analysis of gene ontology (GO) terms significantly enriched (Fisher's P-value<0.005) in the lists of genes differentially regulated during the time course provided an overview of the biological processes activated or repressed (**Figure 2**). Major changes occurred in the upper leaf transcriptome from flowering to 14 DAF regardless of nitrate supply. Between 14 and 27 DAF, 2074 and 2193 genes were, respectively, up- and down-regulated in lower leaves specifically under nitrate supply. Many GO terms in **Figure 2** are related to transport processes. Expression patterns and annotations of the 678 transport-related probes differentially regulated between

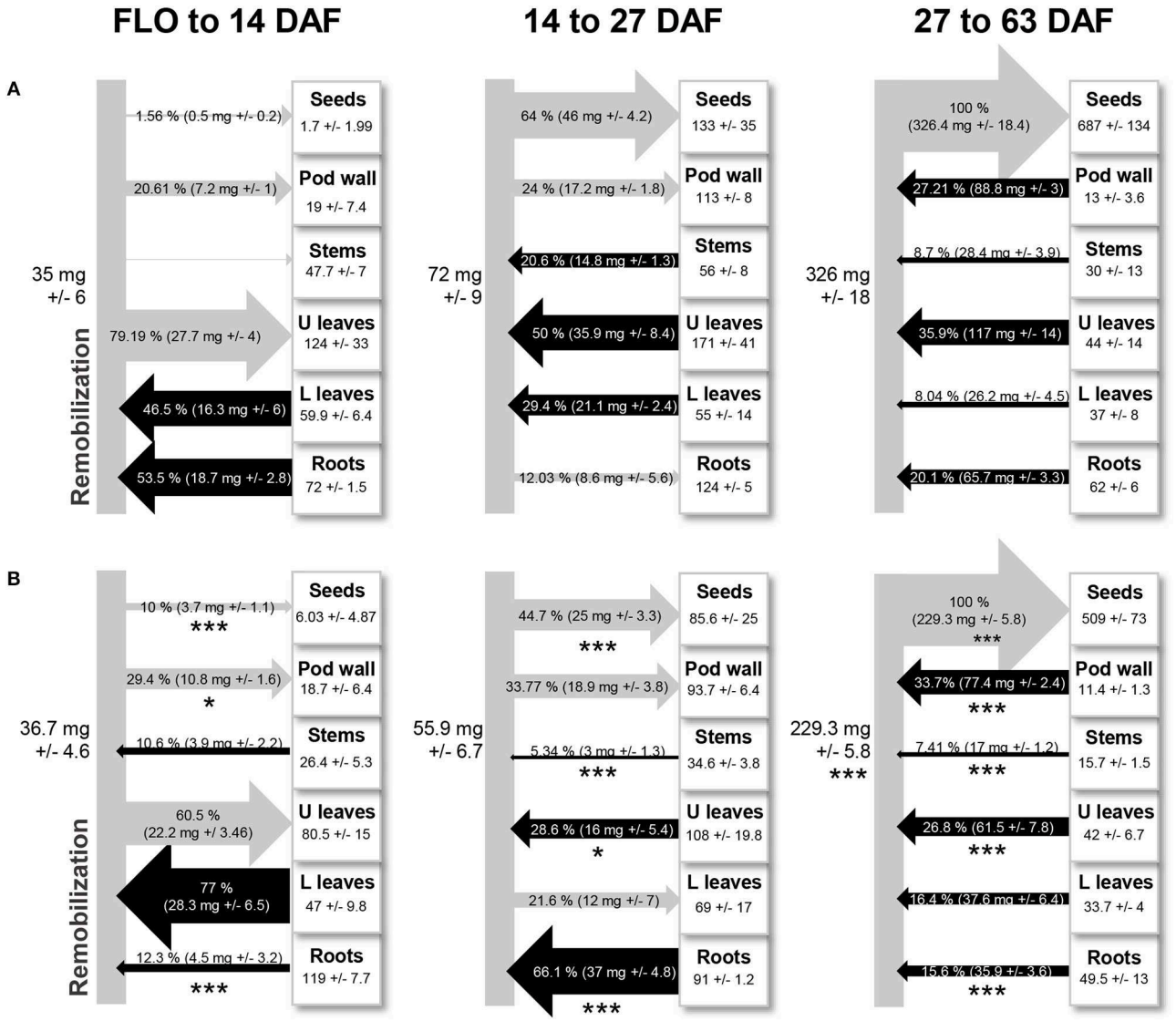

FIGURE 1 | Nitrogen remobilized between plant parts during the reproductive phase in pea, with **(A)** or without **(B)** nitrate supply from flowering. Black arrows indicate that nitrogen is remobilized from a tissue, and gray arrows indicate that nitrogen is redistributed toward a tissue. In squares are mean values of nitrogen quantity in each compartment (roots, leaves of the vegetative nodes = L leaves, leaves of the reproductive nodes = U leaves, stems, pod wall, and seeds) at the last stage (14, 27, and 63 days after flowering). Data are mean values ± standard errors. In each diagram and for each tissue, stars indicate significant variations in the amount of nitrogen remobilized in response to nitrogen deprivation: $^{*}P < 0.1$, $^{**}P < 0.05$, $^{***}P < 0.01$ (*t*-test, data from 4 individual plants).

at least two developmental stages are presented in **Table S4**, thus providing a set of candidate genes for controlling the transfer of nutrients. The most differentially regulated genes (more than 4-fold) are presented in **Figure 3A**. These 88 genes were classified into six main clusters based on hierarchical clustering of their expression patterns. GO analysis revealed an over-representation of genes encoding transporters of sulfate (SULTR), metal ions, and lipids. The previously reported role of sulfate-derived molecules in controlling autophagy and SAGs (Álvarez et al., 2012; Yarmolinsky et al., 2014) prompted us to study the expression and homologies of *SULTR* genes. A phylogenetic tree based on alignments of all SULTRs present in the Pea Gene Atlas (Alves-Carvalho et al., 2015) and a search for the well-characterized Arabidopsis homologs revealed that the differentially regulated genes belong to groups 2 and 3 of low-affinity SULTR (**Figure 3B**). Of the five differentially regulated SULTR genes, four were up-regulated in leaves of the reproductive nodes 14 DAF (**Figure 3C**), suggesting they could contribute to sulfate transport in these leaves.

TF Genes Differentially Regulated in Pea Leaves Between Stages

To identify putative regulators in pea leaves, genes belonging to the categories "TF activity" (GO:0003700) and "regulation

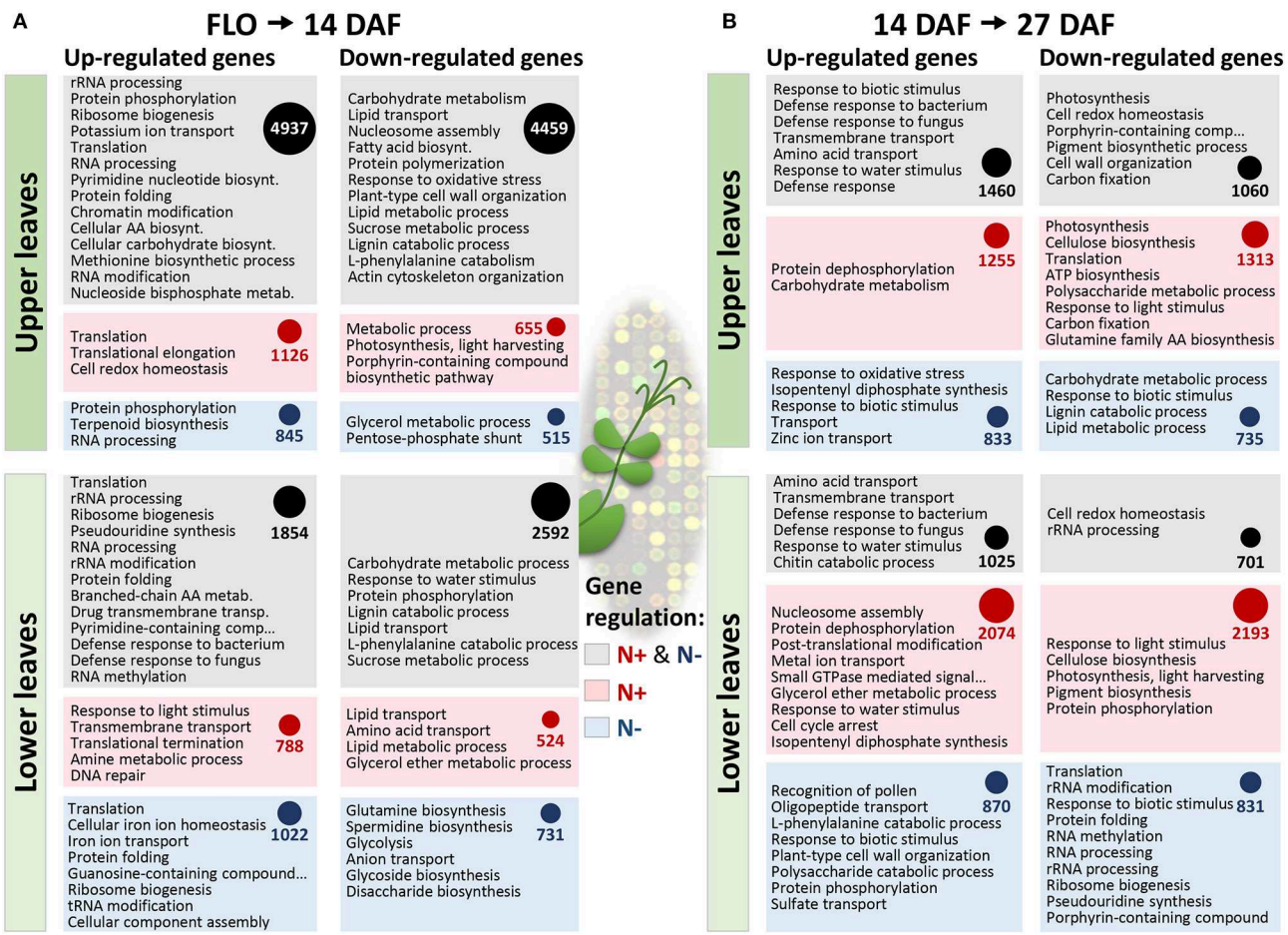

FIGURE 2 | Transcriptome changes in leaves of vegetative (lower leaves) and reproductive (upper leaves) nodes during pea seed development and in response to nitrate availability. The number of genes whose expression varied (Bonferroni-corrected *P*-value < 0.05) between the beginning of flowering and 14 days after flowering (**A**, 14 DAF) and/or between 14 and 27 days after flowering (**B**, 27 DAF). The number of genes whose expression varied regardless of nitrate nutrition are shown in gray boxes, while the number of genes whose expression varied specifically under nitrate-supply (N+) or nitrate-deprivation (N–) are shown in red and blue boxes, respectively. The circles are proportional to the number of genes in each box. GO terms significantly enriched (Fisher *P* values < 0.005) in each gene list are sorted according to *P* values (lowest at the top).

of transcription" (GO:0045449), and significantly regulated between at least two stages, were selected. The annotation and expression patterns of these 625 probes are available in **Table S5**. We subsequently focused on the 78 TF genes displaying more than a 4-fold change in expression. They belonged to various families, the most enriched TF families in this dataset being NAC and ethylene response factor (ERF), followed by myeloblastosis (MYB), nuclear factor Y (NF-Y), and WRKY TFs (**Figure 4A**). These were classified into eight main clusters based on hierarchical clustering of their expression patterns (**Figure 4B**). The regulation of *NAC* and *ERF* genes suggested specialized functions at early or late stages and/or in leaves at specific positions. For example, while *NAC2/PsCam033601* and *NAC100/PsCam038037* were up-regulated in all sample comparisons, *NAC1/PsCam050102* expression only increased in upper leaves 14 DAF. The well-known regulation of NAC transcript abundance by miR164 in Arabidopsis (Guo et al., 2005; Kim et al., 2009) prompted us to examine whether it could also apply to pea. By exploiting an internal miRNA database, we observed that NAC1 and NAC100 are indeed predicted targets of members of the miR164 family in pea (**Table S6**).

TF-Related Co-expression Modules in Pea Leaves

To predict putative regulations by the TFs, a co-expression network based on high Pearson correlations (r <-0.95 or >0.95) was built from the normalized intensities (Log_2) of the 48 samples hybridized on the arrays. Variables with low overall variance were filtered out to reduce the impact of noise (see Materials and Methods). The filtered dataset (11949 probes), provided in **Table S7**, can be imported in Cytoscape and easily converted into an interaction Network using the Expression Correlation package (Cline et al., 2007). This Pea REMObilization NETwork (P-REMONET) consisted of 4523

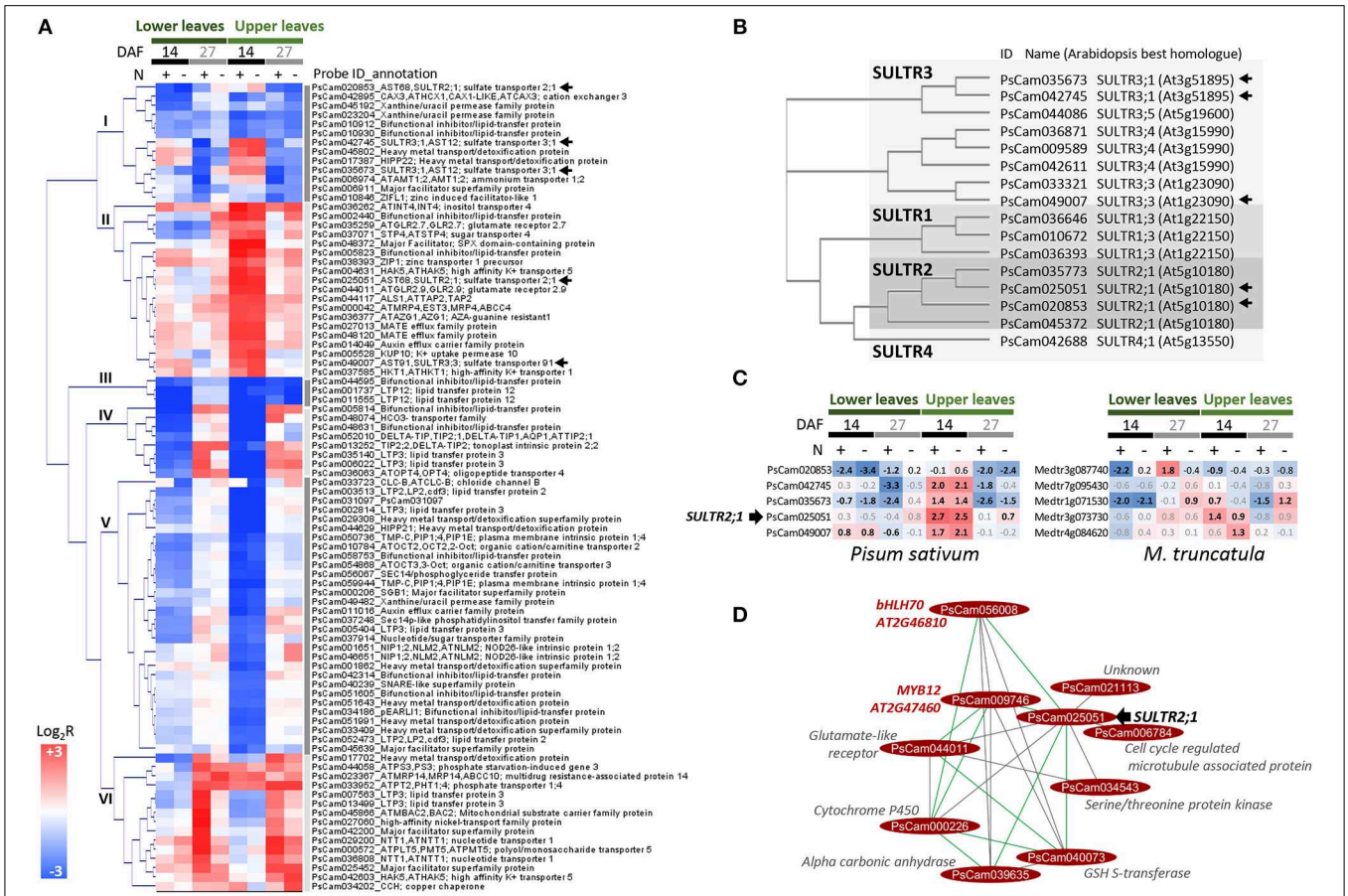

FIGURE 3 | Transporter genes differentially expressed (≥4-fold) in pea leaves between at least two stages. **(A)** Hierarchical clustering of expression profiles in lower and upper leaves. The color scale indicates Log_2R for the comparisons 14 DAF vs. flowering (14), 27 DAF vs. 14 DAF (27), and in response to nitrate-deficiency (N–). Arrows indicate sulfate transporter (SULTR) genes. **(B)** Phylogenetic tree of SULTR sequences identified in the Pea RNAseq Atlas (Alves-Carvalho et al., 2015). **(C)** Expression (array data) of pea SULTR genes marked by an arrow in **(A,B)** and expression profile of the *Medicago truncatula (M. truncatula)* orthologs (In bold, Bonferroni-corrected *P*-value < 0.05). **(D)** Genes connected to PsCam025051/SULTR2;1 in P-REMONET.

nodes (i.e., genes) and 67447 edges (i.e., co-expression links). A total of 436 components were identified in P-REMONET, the largest containing 3225 nodes/genes (**Figure S3A**). Of the TF genes differentially regulated at least 4-fold, 39 were connected to one, two, or many genes. Several TFs were linked together, leading to 30 different TF-related modules (**Table 1**). The list of genes in each module is available in **Table S8** along with the strength (r), type of interaction (i.e., correlation either positive or negative), and expression patterns. Several modules contain TF genes whose regulation depends on nitrogen availability, such as NAC073#2 and NAC043, which were down-regulated 27 DAF specifically under nitrate deficiency (**Table 1** and **Table S8**).

To investigate the robustness of P-REMONET for predicting TF-TF or TF-target interactions, a search for the best Arabidopsis homologs was performed for each gene in the TF-related modules. The P-REMONET predictions showed similarities to interactions validated in Arabidopsis. For example, module M22 consisted of two positively correlated genes, PsCam002187 and PsCam001382, respectively, homologous to MYC2 and JAZ5 (jasmonate-zim-domain protein 5), which interact in yeast two-hybrid assays (Chini et al., 2009). Module M4 was enriched for genes related to cell wall biosynthesis and contains two potential regulators, MYB46 (PsCam038865) and MYB83 (PsCam038898), shown in Arabidopsis to bind to the same secondary wall MYB-responsive element consensus sequence and activate the same set of direct targets involved in secondary wall biosynthesis (Zhong and Ye, 2012). Module M7 for two NAC073 TFs sharing 70% homologies (NAC073#1 and NAC073#2) was enriched in genes for cellulose biosynthesis, including two cellulose synthase genes. Consistently, NAC073 in Arabidopsis was named SND2 for Secondary wall-associated NAC Domain protein 2 and transactivates the cellulose synthase 8 promoter (Hussey et al., 2011). These observations validated P-REMONET as a useful tool to predict relevant regulations.

The largest TF-related modules in P-REMONET contain genes down-regulated during the time course (TFs belonging to cluster VI in **Table 1**). The higher number of connections was

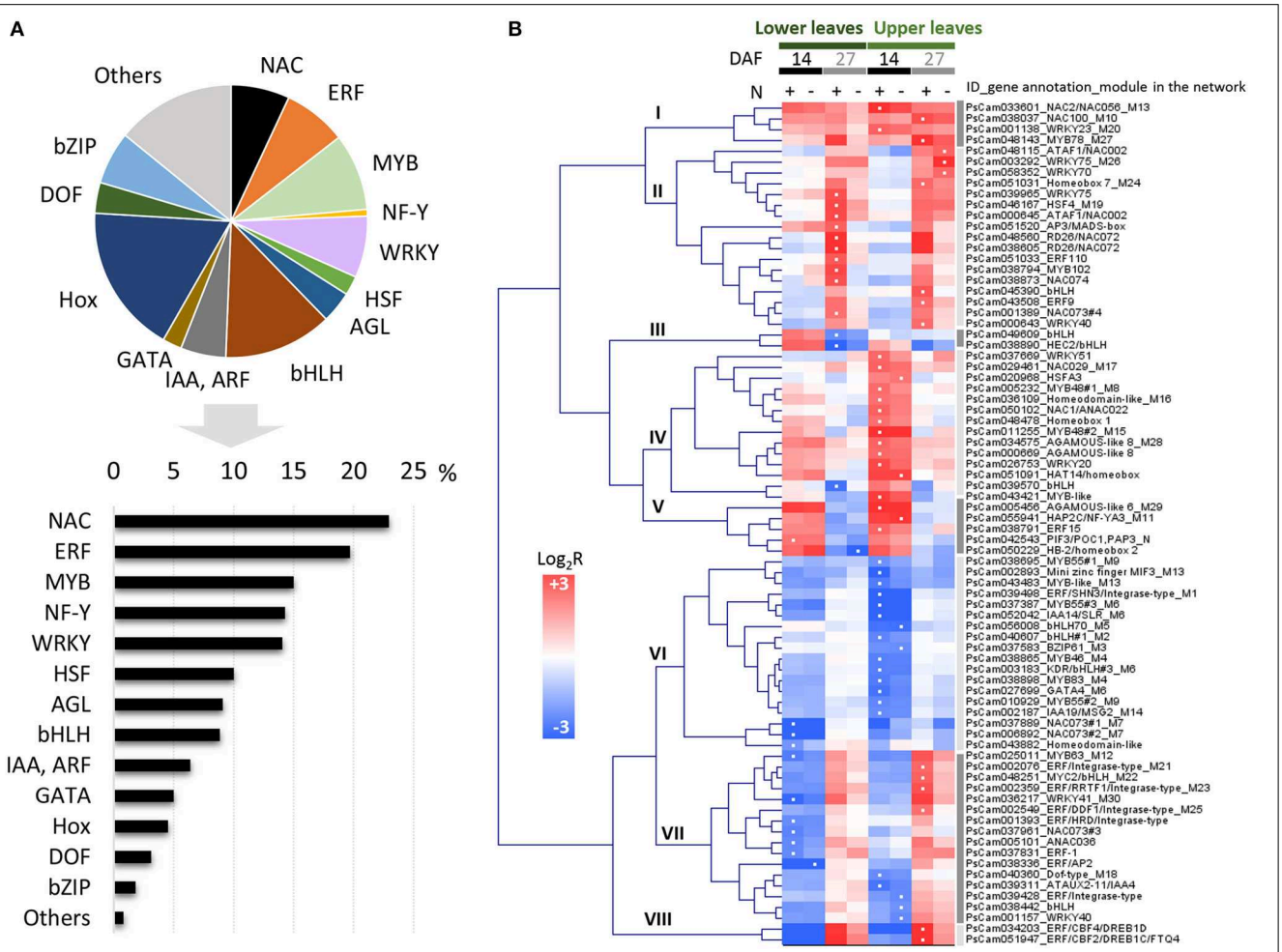

FIGURE 4 | Genes with GO terms related to TF activity (GO:0003700) and regulation of transcription (GO:0045449) differentially expressed at least 4-fold in pea leaves between two developmental stages. **(A)** Number of differentially expressed genes represented as a percentage (bars) of the total number of genes per TF family (pie chart). **(B)** Hierarchical clustering of their expression profiles in lower and upper leaves. The color scale indicates Log_2R for the comparisons 14 DAF vs. flowering (14), 27 DAF vs. 14 DAF (27), and in response to nitrate-deficiency (N–), and white dots in squares indicate the highest values. MYB, myeloblastosis; NF-Y, nuclear factor Y; HSF, heat shock transcription factor; AGL, agamous-like; bHLH, basic helix-loop-helix; IAA, indoleacetic acid-induced protein; ARF, auxin response factor; Hox, Homeodomain/homeobox; DOF, DNA-binding One Zinc Finger; bZIP, Basic Leucine Zipper.

identified for module M1, which contained 197 genes connected to the ethylene response factor/Apetala2 TF (ERF/AP2#1, PsCam039498, **Table 2**), suggesting this TF acts as a hub. Several TFs in these modules could act in concert since they were positively connected: ERF/AP2#1 and a plant AT-rich sequence and zinc-binding protein (PLATZ) in module M1, bZIP61, bHLH#2, and a GATA-type zinc finger TF in module M3, bZIP34, bHLH70, MYB12, and ERF/AP2#3 in module M5 (**Table 1**). An analysis of GO terms for the co-expressed genes predicted biological processes that could be repressed in coordination with the down-regulation of the TFs (**Table S8**).

Other TFs were positively connected with genes up-regulated at 14 or 27 DAF, thus identifying some putative transcriptional activators of processes induced during the time course (**Table 1** and **Table S8**). Two of these modules, M11 and M12, are depicted in **Figures S3B,C** since they contain the higher number of positive links with the TFs. The TF in module M11 (PsCam055941) was homologous to the subunit A3 of the nuclear factor Y (NF-YA3, AT1G72830), which in Arabidopsis stimulates the transcription of various genes by recognizing and binding to a CCAAT motif in promoter regions (Leyva-González et al., 2012). In pea, *NF-YA3* was up-regulated in lower and upper leaves 14 DAF compared to flowering, then down-regulated 27 DAF (cluster V in **Figure 4B**), highlighting important regulations of this gene during the time course investigated. In contrast, the TF gene in module M12 (PsCam025011), homologous to *MYB63* (AT1G79180), was down-regulated 14 DAF, then up-regulated 27 DAF in both vegetative and upper leaves (cluster VII in **Figure 4B**), suggesting a role at the transition stage toward chlorophyll breakdown and

TABLE 1 | TF-related modules in the P-REMONET co-expression network.

TFs in Figure 4B (differentially regulated by at least 4-fold between stages)	Cluster (Figure 4B)	Nodes pos-neg	Edges	% genes regulated by N	Module (ID)	Additional TF sequences in the module (regulated <4-fold between stages)
PsCam039498_ERF/AP2#1†	VI	192-4	7694	25%	M1 (197)	PLATZ (PsCam038319)† ERF/AP2#3 (PsCam039388)
PsCam040607_bHLH#1	VI	69-0	1465	20%	M2 (70)	
PsCam037583_BZIP61	VI	63-0	1338	3%	M3 (64)	GATA-type zinc finger (PsCam020847) bHLH#2 (PsCam052606)
PsCam038898_MYB83†	VI	38-3	264	33%	M4 (89)	PLATZ (PsCam038319)†
PsCam038865_MYB46	VI	58-2	1064	20%		
PsCam056008_bHLH70	VI	44-14	679	27%	M5 (59)	Myb12 (PsCam009746) bZIP34 (PsCam037351) ERF/AP2#3 (PsCam039388)
PsCam037387_MYB55#3	VI	40-0	339	30%	M6 (102)	RING/FYVE/PHD-type znf (PsCam038341)† GATA9 (PsCam039673)
PsCam052042_IAA14, SLR	VI	21-0	122	14%		
PsCam027699_GATA4†	VI	48-0	630	30%		
PsCam003183_KDR/bHLH#3	VI	48-0	726	16%		
PsCam037889_NAC073#1	VI	40-0	413	41%	M7 (50)	NAC043 (PsCam000593)†
PsCam006892_NAC073#2†	VI	38-0	388	49%		
PsCam038695_MYB55#1	VI	4-0	5	25%	M9 (19)	–
PsCam010929_MYB55#2†	VI	15-0	58	44%		
PsCam002187_IAA19, MSG2†	VI	6-0	15	43%	M14 (7)	–
PsCam002893 MIF3	VI	13-2	44	37%	M13 (24)	–
PsCam043483_MYB-like†	VI	9-2	35	67%		
PsCam033601_NAC2, NAC056	I	1-9	33	54%		
PsCam038037_NAC100	I	9-3	31	54%	M10 (13)	–
PsCam001138_WRKY23†	I	0-3	3	75%	M20 (4)	–
PsCam048143_MYB78†	I	0-1	1	50%	M27 (2)	–
PsCam046167_HSF4	II	3-0	3	25%	M19 (4)	–
PsCam051031_Homeobox 7†	II	1-0	1	100%	M24 (2)	–
PsCam003292_WRKY75	II	1-0	1	0%	M26 (2)	–
PsCam005232_MYB48#1†	IV	2-24	306	37%	M8 (27)	–
PsCam011255_MYB48#2†	IV	4-1	8	83%	M15 (6)	–
PsCam036109_Homeodomain-like	IV	4-0	5	20%	M16 (5)	–
PsCam029461_NAC029	IV	3-1	6	80%	M17 (5)	–
PsCam034575_AGAMOUS-like 8†	IV	1-0	1	50%	M28 (2)	–
PsCam005456_AGAMOUS-like 6	V	0-1	1	0%	M29 (2)	–
PsCam055941_NF-YA3†	V	10-0	32	81%	M11 (11)	–
PsCam025011_MYB63†	VII	10-0	40	9%	M12 (11)	Zinc finger-type (PsCam004767)
PsCam002076_ ERF/AP2#2	VII	2-0	2	0%	M21 (3)	ERF/AP2#4 (PsCam039693)
PsCam048251_MYC2†	VII	1-0	1	100%	M22 (2)	JAZ5 (PsCam001382)†
PsCam002359_RRTF1	VII	1-0	1	50%	M23 (2)	–
PsCam002549_Integrase-type†	VII	1-0	1	100%	M25 (2)	Integrase-type DDF1 (PsCam002503)†
PsCam036217_WRKY41	VII	1-0	1	50%	M30 (2)	–
PsCam040360_Dof-type	VII	3-0	4	25%	M18 (4)	–

*The table describes the co-expression modules containing the TFs differentially expressed at least 4-fold in leaves between two developmental stages. The modules were retrieved from P-REMONET (**Figure S3A**). †indicates that gene expression varied significantly in response to nitrate (N) nutrition. The cluster in **Figure 4B** to which belong the TFs is indicated, along with the number of positive (pos) and negative (neg) connections, of edges, proportion of genes regulated by nitrogen availability, module, and number of different IDs/genes in the module. In the last column are additional TFs, regulated <4-fold, in the modules. Details about genes in each module are provided in **Table S8**.*

TABLE 2 | TF-related co-expression modules conserved between pea and *M. truncatula*.

Pea TF ID clusters in Figure 4B modules in Table S8	*M. truncatula* ID	Pea sequence ID of genes connected to the TFs	Best *M. truncatula* homologs also connected to the TFs	Gene annotation (best Arabidopsis homolog)
ERF/AP2#1[†] PsCam039498 Cluster VI, **module M1** (down-reg. 14 DAF in lower and upper leaves)	MT0007_00880	PsCam049838 (+)	MT0011_00523 (+)	Protein kinase (AT3G26700)
		PsCam038319 (+)	MT0031_10256 (+)	**PLATZ transcription factor** (AT1G32700)
		PsCam025580 (+)	MT0003_11081 (+)	Extensin-like; proline-rich cell wall protein (AT4g38770)
		PsCam023684 (+)	MT0003_11081 (+)	Extensin-like; proline-rich cell wall protein (AT4g38770)
		PsCam036606 (+)	MT0040_10268 (+)	SKU5, cell wall modifying enzyme (AT1G76160)
		PsCam012843 (+)	MT0031_00200 (+)	*S*-adenosylmethionine synthetase (AT2G36880)
		PsCam036120 (+)	MT0067_10073 (+)	Invertase/pectin methylesterase inhibitor (AT4G02320)
		PsCam027004 (+)	MT0010_00419 (+)	Glycosyl hydrolase 9B8 (AT2G32990)
MYB83[†] PsCam038898 Cluster VI, **module M4** (down-reg. 14 DAF in lower and upper leaves)	MT0003_10639	PsCam012843 (+)	MT0031_00200 (+)	*S*-adenosylmethionine synthetase (AT2G36880)
		PsCam036606 (+)	MT0040_10268 (+)	SKU5, cell wall modifying enzyme (AT1G76160) **PLATZ transcription factor** (AT1G32700/AT4G17900)
		PsCam038319 (+)	MT0031_10256 (+)	
		PsCam006765 (+)	MT0066_10032 (+)	Laccase 17 (AT5G60020)
		PsCam005256 (+)	MT0012_10060 (+)	Adenine nucleotide alpha hydrolases-like (AT2G03720)
bHLH70 PsCam056008 Cluster VI, **module M5** (down-reg. 14 DAF in upper leaves)	MT0028_10309	PsCam034290 (–)	MT0002_10493 (–)	Weak chloroplast movement under blue light-like protein (DUF827) (AT2G26570)
NAC073#1 PsCam037889 Cluster VI, **module M7** (down-reg. 14 DAF in lower and upper leaves and 27 DAF in upper leaves under nitrate deficiency)	MT0019_00537	PsCam038807 (+)	MT0003_00396 (+)	FASCICLIN-like arabinogalactan-protein 12 (AT5G60490)
		PsCam043546 (+)	MT0001_01114 (+)	Protein of unknown function, DUF538 (AT2G03350)
		PsCam033940 (+)	MT0001_00233 (+)	Cellulose synthase CESA7 (AT5G17420)
		PsCam057773 (+)	MT0010_00304 (+)	TRICHOME BIREFRINGENCE-LIKE 33 (AT2G40320)
		PsCam036683 (+)	MT0040_10197 (+)	Glycosyl hydrolase 9B5 (AT1G19940)
		PsCam000957 (+)	MT0039_00275 (+)	GERMIN-LIKE, GLP10 (Cell wall-related, AT3G62020)
		PsCam023534 (+)	MT0039_00275 (+)	GERMIN-LIKE, GLP10 (Cell wall-related, AT3G62020)
		PsCam013191 (+)	MT0039_00275 (+)	GERMIN-LIKE, GLP10 (Cell wall-related, AT3G62020)

The pea sequence IDs were from the Pea Gene Atlas (http://bios.dijon.inra.fr/) and the M. truncatula IDs are from the Symbimics program (https://iant.toulouse.inra.fr/symbimics/). The best M. truncatula homologs (v4.02) were identified using OrthoFinder v1.1.8. The signs indicate whether the correlation with TF gene expression was positive (+) or negative (–). Genes were annotated by homology with sequences in The Arabidopsis Information Resource (TAIR, https://www.arabidopsis.org/). [†] *indicates that gene expression varied significantly in response to nitrate nutrition.*

senescence. Annotations of the co-expressed genes indicated that MYB63 may activate defense responses. These data were summarized in **Figure 5**, which provides a global view of the TF-related co-expression modules identified in pea leaves, depending on the developmental stages and nitrate availability.

Comparing Nitrogen Remobilization and TF Modules Between Pea and *M. truncatula*

A comparative study in *M. truncatula* was performed by coupling nitrogen remobilization analysis at the whole plant level with a transcriptome analysis of leaf samples collected under the same conditions as were the pea samples. The dynamic of nitrogen remobilization was similar between pea and *M. truncatula* from flowering to 14 DAF (**Figure S4**). Some differences occurred between 14 and 27 DAF: unlike pea, nitrogen was mainly remobilized from lower leaves of *M. truncatula* during this period. For transcriptomics comparisons, we focused on the 14980 orthologous sequences with a unique gene per species. A Pearson's distance correlation matrix was generated to compare transcriptomics data (expressed in $\log_2$ ratio) between pea and *M. truncatula* (**Figure 6A**). The correlations were positive between species ($0.13 \leq r \leq 0.45$) for all pairwise comparisons, indicating transcriptional regulations at least in part conserved between the species.

To identify shared regulators between pea and *M. truncatula*, we focused on the 39 TF genes highly regulated in pea leaves and for which putative targets were identified in P-REMONET. A *M. truncatula* ortholog was found for 31 of these TFs (**Table S9**). After building a gene co-expression network (M-REMONET) from the normalized intensities (Log_2) of the 48 leaf samples hybridized on the *M. truncatula* arrays (21164 probes, 8778 nodes, 108210 edges), a search for co-expression modules containing these TFs was performed. Four TFs (ERF/AP2#1, MYB83, bHLH70, NAC073#1) were closely connected to genes orthologous between the species (**Figure 5**). These putative conserved targets were listed in **Table 2** along with the type of correlation with the TFs (positive or negative). Notably, the

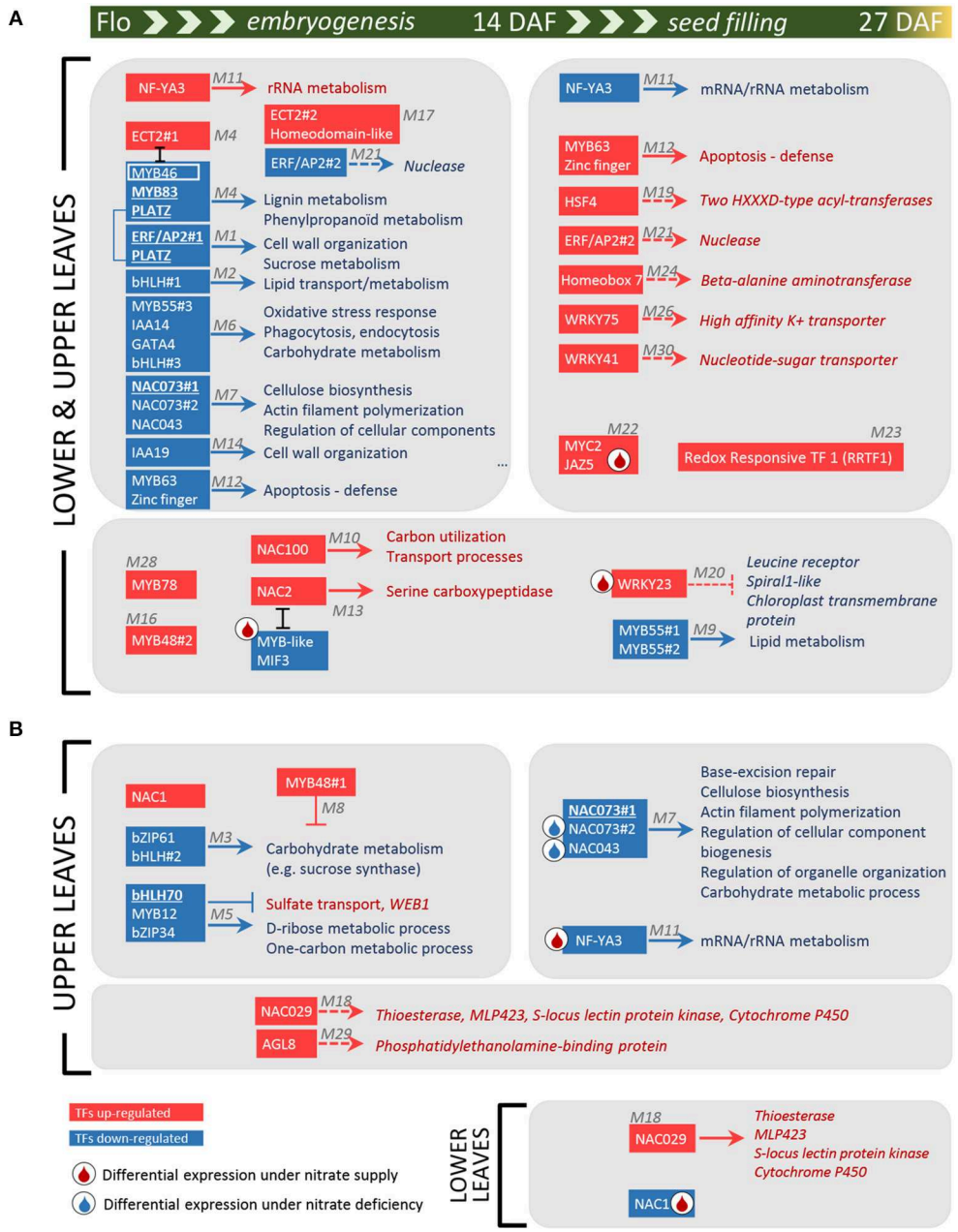

FIGURE 5 | Diagram of TF-related co-expression modules in pea leaves during embryogenesis and seed filling. Red and blue rectangles correspond to TFs up- and down-regulated, respectively, during the time course of nutrient remobilization: from the beginning of flowering to 14 DAF (left panel), from 14 to 27 DAF (right panel) or throughout the time course (large panels). The arrows indicate a co-expression between TFs and biological processes (TopGO annotation, **Table S8**) or genes (in italic) was identified in P-REMONET (corresponding modules in gray). ⊥ and ⊣ indicate negative expression correlations between genes. The colors of the TFs and related processes indicate the genes were up- (red) or down- (blue) regulated during the time course in both lower and upper leaves **(A)** or in specific leaf types **(B)**. TFs in bold and underlined have shared putative targets in the co-expression networks from *M. truncatula* and pea. A droplet indicates significant variations in gene expression specifically in response to nitrate supply (red) or deficiency (blue).

connected genes in modules M1, M4, and M7 (**Table 2**) were related to cell wall metabolism/structure, suggesting important transcriptional regulation of cell wall structure in leaves of both species. In the M7 module depicted in **Figure 6B**, of the eight genes similarly connected to NAC073#1 in both species, seven were related to cell wall metabolism (**Table 2**). Almost all genes in this module were down-regulated in lower and upper leaves 14 DAF (**Table S8**), suggesting major modifications of cell wall structure in these leaves at early reproductive stages.

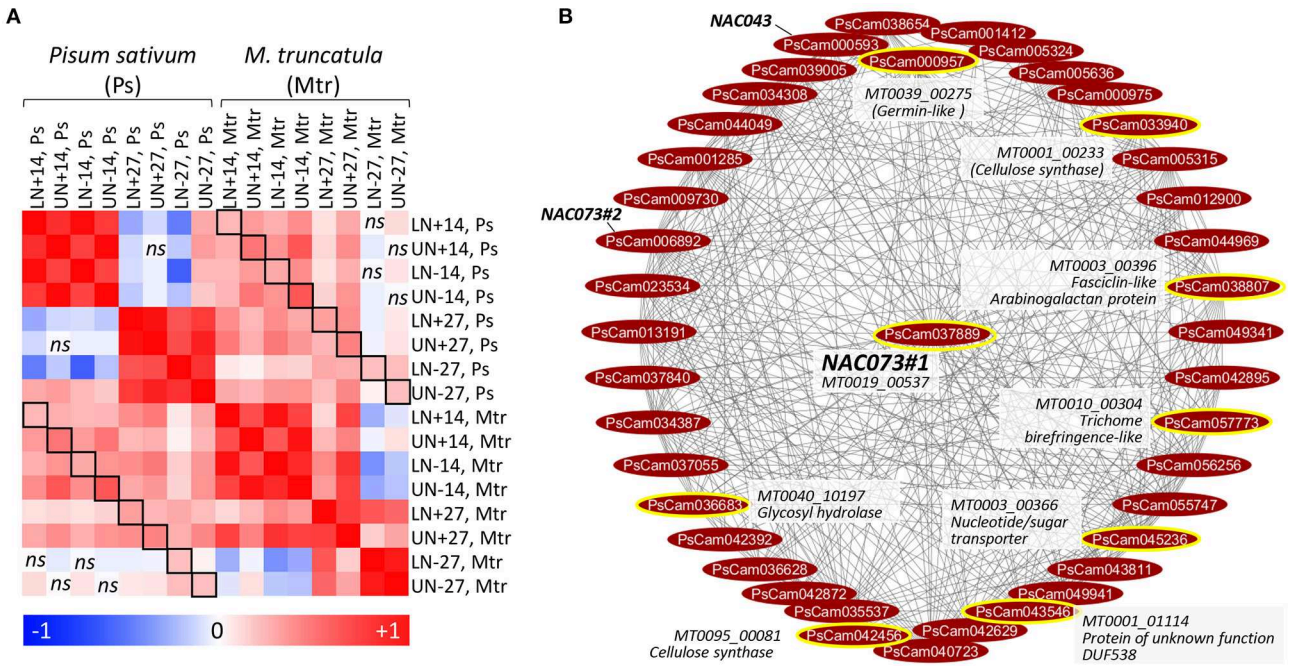

FIGURE 6 | Comparison of leaf transcriptomes and TF-related modules between pea and *M. truncatula*. **(A)** Pair-wise Pearson correlation coefficients calculated from each sample comparison expressed in $\log_2$ ratio: beginning of flowering vs. 14 DAF (14), 14 vs. 27 DAF (27), for lower (L), and upper (U) leaves, with nitrogen (N+), or without nitrogen (N–). The color scale indicates the degree of correlations between transcriptomes. All correlations were significant ($P < 0.05$, $n = 14980$ sequences) except sample comparisons indicated by *ns* (non-significant). Squares indicate the same samples compared between the two species. **(B)** NAC073#1 (PsCam037889)-related network in module M7. The connected genes were organized using the circular layout algorithm from Cytoscape. Orthologous genes connected to the NAC073#1 ortholog (MT0019_00537) in *M. truncatula* (data from **Table 2**) were encircled in yellow and annotated.

DISCUSSION

To provide a first overview of the transcriptional regulations occurring in pea leaves during seed development, we focused on stages of the reproductive phase preceding the sharp decrease in chlorophyll breakdown, up to a transition stage toward senescence (27 DAF, **Figure 1**). A long-term ^{15}N-nitrate-labeling experiment indicated that these stages were associated with dynamic nitrogen recycling and remobilization between tissues, leaves from vegetative and reproductive nodes contributing, respectively, to 29 and 44% of the total amount of nitrogen remobilized during this period (**Figure 1**). The subsequent stages were associated with nitrogen recycling from all tissues, including pod walls, and at maturity, 54% of nitrogen accumulated in pea seeds was derived from remobilization processes (**Figure 1**). Our data demonstrated that leaves are the main source of remobilized nitrogen, followed by pod wall, roots and stems, which is consistent with data previously obtained in a pulse-chase ^{15}N-labeling experiment (Schiltz et al., 2005). A transcriptome analysis of leaves from vegetative and reproductive nodes from flowering to 27 DAF showed that most of the well-known SAG, such as the cysteine protease gene *SAG12*, were not significantly up-regulated in our leaf samples. By contrast, genes that might contribute to promote nutrient recycling while maintaining leaves in a healthy metabolic state, i.e., with limited protein degradation, were identified. Complemented by a gene co-expression approach targeted on the most regulated TFs, this study provides a repertoire of regulatory predictions, some of which were conserved in the forage legume species *M. truncatula* (**Table 2**), that can broadly serve as a backdrop for studying the role of individual genes in legumes.

Molecular Features of Leaves From the Reproductive Nodes During Seed Embryogenesis

Sulfur Transport and Metabolism

From the beginning of flowering to 1st node seed filling (14 DAF), seeds progress through embryogenesis on the reproductive nodes. This period was associated with deep transcriptional changes in leaves of the reproductive nodes regardless of nitrate availability (**Figure 2A**), as exemplified by transporter gene expression (**Figure 3A** and **Table S4**). *SULTR* genes were among the most up-regulated transporter genes in upper pea leaves 14 DAF as compared to flowering (**Figure 3A**). The most up-regulated was homologous to SULTR2;1 (PsCam025051, **Figure 3C**), which has been shown in Arabidopsis to be expressed in vascular tissues and proposed to regulate internal translocation and distribution of sulfate (Takahashi et al., 2000). The over-representation of genes related to methionine metabolism in the set of genes up-regulated 14 DAF in leaves of the reproductive nodes suggests sulfate can be used for methionine metabolism

in these leaves (**Figure 2A**). Sulfate transport in upper pea leaves can also contribute to avoid precocious senescence owing to the role of sulfate-derived compounds in preventing autophagy and senescence in Arabidopsis and tomato (Álvarez et al., 2012; Yarmolinsky et al., 2014).

The gene co-expression approach enabled us to deduce some possible regulators of SULTR2;1/PsCam025051. The transporter was positively connected to five genes in P-REMONET, one of which encodes a Ser/Thr kinase (PsCam034543, **Figure 3D**). In the green alga *Chlamydomonas reinhardtii*, a Snf1-like Ser/Thr kinase positively regulates sulfate transporters (Davies et al., 1999), and in Arabidopsis all the substitutions at the phosphorylation site Thr-587 of a SULTR led to a complete loss of sulfate transport (Rouached et al., 2005). Hence, the Ser/Thr kinase may be a promising candidate for investigating the signal transduction system regulating sulfate homeostasis in upper leaves. In addition, SULTR2;1/PsCam025051 was negatively connected to two TF genes homologous to *bHLH70* and *MYB12* (module M5 and **Figure 3D**). Many MYB/bHLH complexes have been described in plants (Pireyre and Burow, 2015) and MYB factors have been shown to regulate genes related to sulfate assimilation (Koprivova and Kopriva, 2014), reinforcing the interest of further studies on the interplay of these genes.

Other TF Candidates for Maintaining Leaf Metabolism or Preventing Senescence

The above-mentioned *bHLH70* gene was among the most down-regulated TFs 14 DAF (cluster VI in **Figure 4B**). It was connected to genes with different functions in module M5, suggesting pleiotropic roles. In particular, *bHLH70* was negatively connected to *WEB1* (weak chloroplast movement under blue light 1) in both P- and M-REMONETs (**Table 2**), pointing out bHLH70 as a putative repressor of *WEB1* expression in leaves of both forage and grain legume species. WEB proteins maintain the velocity of chloroplast movements *via* chloroplast-actin filaments in response to ambient light conditions (Kodama et al., 2010). By controlling chloroplast redistribution, they prevent the dismantling of the photosynthetic apparatus by excess light. The increased *WEB1* expression in upper leaves at 14 DAF may be part of the mechanisms by which photosynthesis is maintained before senescence initiation. Our data suggest bHLHL70 to be a good candidate for investigating the regulation of these mechanisms. Another TF candidate up-regulated in upper pea leaves at 14 DAF is *NAC1/PsCam050102* (**Figure 4B**). Overexpression of a NAC1-type TF in wheat delayed leaf senescence, leading to a stay-green phenotype (Zhao et al., 2015). Therefore, the up-regulation of *NAC1* in upper leaves could contribute to prevent senescence, even when nitrate absorption by roots becomes limiting (**Figure 1**). The mRNA abundance of NACs, including NAC1, is controlled by miR164 in Arabidopsis[25]. It was therefore interesting to observe that all four predicted targets of miR164 in pea belong to the NAC family (**Table S6**), of which one corresponds to NAC1. This reinforces the possible regulation of NAC1 transcript abundance by miR164 in pea leaves.

The Early Reproductive Phase Is Accompanied by a Reprogramming of Cell Wall-Related Genes in Leaves of Both Vegetative and Reproductive Nodes

Genes of lignin catabolism and cell wall organization were enriched in the list of genes down-regulated 14 DAF in lower and upper pea leaves (**Figure 2A**), reflecting a shift in cell wall structure at early reproductive stages. Interestingly, three TF-related modules conserved between pea and *M. truncatula* contained genes of cell wall metabolism/organization (**Table 2**). These conserved modules, described in **Table 2**, were identified for:

(*i*) ERF/AP2#1, which shares homologies with Arabidopsis AP2 TFs that have roles in plant protective layers such as the cuticle (Aharoni et al., 2004). In pea and *M. truncatula*, *ERF/AP2#1* was positively linked to five genes related to cell wall organization and to a PLATZ TF responsible for A/T-rich sequence-mediated transcriptional repression (Nagano et al., 2001). The identification of PLATZ and ERF/AP2 in the cell wall network built from a co-expression analysis in rice (Hirano et al., 2013) reinforces their possible coordinated function in controlling cell wall structure.

(*ii*) MYB83, which was similarly connected to the PLATZ TF and co-expressed with a gene encoding an oxidative enzyme (laccase, *LAC17*, **Table 2**) proposed to determine the pattern of cell wall lignification (Schuetz et al., 2014). The role of MYB83 in secondary wall biosynthesis has been demonstrated in Arabidopsis, where its overexpression induced the expression of secondary wall biosynthetic genes and resulted in an ectopic deposition of secondary wall components. In P-REMONET, MYB83 was linked to a third TF, MYB46 (module M4 in **Table S8**), shown in Arabidopsis to act redundantly with MYB83 in regulating secondary cell wall biosynthesis (McCarthy et al., 2009). The authors have shown that simultaneous RNAi inhibition of MYB83 and MYB46 reduced secondary wall thickness in fibers and vessels. Other authors demonstrated that MYB46 was sufficient to induce the entire secondary wall biosynthetic program (Zhong et al., 2007).

(*iii*) NAC073#1, which was positively linked to eight genes orthologous between pea and *M. truncatula*, of which five may have roles in cell wall formation/organization (2 cellulose synthases, a trichome birefringence-like protein, a glycosyl hydrolase and a Fasciclin-like Arabinogalactan protein, **Figure 6B**). In pea, NAC073#1 was positively connected to two additional NAC TFs: NAC073#2 and NAC043 (also named NST1 for Secondary Wall Thickening Promoting Factor1) (**Figure 6B**). Evidence is accumulating to suggest that a subset of closely related NACs act as master transcriptional switches governing secondary wall biosynthesis and fiber development (Zhong et al., 2008). In Arabidopsis, NAC073 and NAC043/NST1 contribute to the formation of secondary cell wall, and their repression resulted in a remarkable reduction in the secondary wall thickening (Zhong et al., 2008).

Taken altogether, the data indicate that the transcriptional regulation of cell wall organization and metabolism in leaves of legumes occurs at early reproductive stages and may involve

seven transcription factors pinpointed here for the first time in pea: ERF/AP2#1, a PLATZ TF, MYB83, MYB46, NAC073#1, NAC073#2, and NAC043. The expression of *NAC073#2* and *NAC043* decreased 27 DAF under nitrate-deficiency only (**Figure 5B**), indicating that the intricate control of cell wall metabolism in pea leaves may rely on nitrate-dependent regulations. Although data accumulate in the literature on the role of NAC TFs in regulating cell wall metabolism (Zhong et al., 2007, 2008; McCarthy et al., 2009; Hirano et al., 2013; Schuetz et al., 2014), the full list of their targets remains to be established. The present study highlighted some putative targets for further investigations (**Figure 6B**).

Transcriptional Reprogramming of Leaves at a Transition Stage Toward Senescence

Transcriptome changes in pea leaves 27 DAF, which marks the switch toward senescence-associated yellowing (**Figure S1**), contributed to our understanding of molecular events underlying this transition.

Autophagy-Related Processes

GO enrichment analysis of genes up-regulated in leaves 27 DAF revealed an over-representation of genes involved in defense responses, such as disease resistance proteins (R proteins, **Figure 2B**). Accordingly, several defense-related genes known to be induced by pathogens were found to be expressed during Arabidopsis leaf senescence in a pathogen-independent manner (Quirino et al., 1999). Seven R protein genes up-regulated 27 DAF were in the MYB63-related module (M12 in **Figure S3C**). All contain an NB-ARC domain (Nucleotide-Binding adaptor shared by Apoptotic protease-activating factor-1, R proteins, and *Caenorhabditis elegans* death-4 protein) essential for protein activity (van Ooijen et al., 2008; **Table S10**). Interestingly, in rice, an R protein with NB-ARC domain has been named RLS1 (Rapid Leaf Senescence 1) because the disruption of the gene accelerated leaf senescence due to a rapid loss of chlorophyll (Jiao et al., 2012). The authors showed that *RLS1* is involved in the autophagy-like programmed cell death and partial degradation of chloroplast. The R proteins in module M12 could play a similar role in the autophagy-mediated programmed cell death to promote nutrient remobilization while avoiding rapid senescence. By activating the autophagy process, reactive oxygen species (ROS) are key players in the regulation of programmed cell death (Pérez-Pérez et al., 2012). Therefore, the increased expression 27 DAF of *RRTF1* (**Figure 5A**, module M23 in **Table S8**), encoding the Redox-Responsive TF1 that controls positively the accumulation of ROS in Arabidopsis shoots and roots (Matsuo et al., 2015), might contribute to orchestrate autophagy-mediated programmed cell death. Because autophagy allows the remobilization of nutrients while preserving cell longevity, identifying autophagy regulators is of particular interest. In module M12, all R proteins were positively connected to *MYB63*, which plays a dual function in regulating secondary cell wall formation and genes involved in disease resistance in Arabidopsis (Zhou et al., 2009). MYB63 and three R proteins were also positively connected to a Zinc finger-type TF whose closest Arabidopsis homolog (AT2G40140) was ROS-responsive (Gadjev et al., 2006). All these features indicate these two TFs may regulate autophagy, possibly through ROS perception.

Transporters

In the quest to identify transporters contributing to the recycling of nutrients at the transition toward senescence, the list of transporter genes differentially regulated during the developmental period was examined (**Table S4**). The most up-regulated nitrogen transporters were high-affinity transporters of basic amino acids (e.g., CAT5) and nitrate (NRT2.5). In Arabidopsis, NRT2.5 plays a role in nitrate loading into the phloem during remobilization processes under nitrogen starvation (Lezhneva et al., 2014). The *NRT2.5* homolog in pea was up-regulated 27 DAF in lower and upper leaves whatever nitrate supply (**Table S4**), suggesting a contribution to nitrogen recycling not restricted to low nitrate environments in pea. Although a role for CAT5 in leaf nitrogen remobilization has not yet been demonstrated, one *CAT5* gene (At2g34960) was up-regulated in senescing Arabidopsis leaves (van der Graaff et al., 2006). The up-regulation of *CAT5* in both leaf types 14 DAF and specifically in response to nitrate-deficiency 27 DAF (**Table S4**) suggests this gene could contribute to the recycling of amino acids in pea leaves, notably under nitrate-deficiency at later stages. However, nitrogen/amino acid transporters were not among the most regulated genes at 27 DAF, contrarily to genes encoding transporters of nucleotides, sugars, lipids, phosphate, potassium, nickel, and copper, which were up-regulated at least 4-fold at this stage (**Table S4**). Although these genes have not been reported to play a role in preventing rapid senescence, potassium homeostasis is known to play an essential role in stress-induced senescence (Anschütz et al., 2014), and a recent study highlighted the need to maintain potassium levels in leaves during nitrate starvation to prevent senescence (Meng et al., 2016). The potassium transporter identified (PsCam042603) was homologous to the high affinity K+ transporter gene *HAK5*. In P-REMONET, HAK5 was connected to a TF gene homologous to *WRKY75* (**Figure 6A**), which has been shown to be induced during potassium starvation in Arabidopsis (Devaiah et al., 2007), highlighting the interest to investigate the relationship between this TF and the regulation of potassium transport in leaves.

Translation-Associated Processes

By influencing ribosome structure and function, ribosomal RNA (rRNA) processing, and modifications play key roles in protein synthesis, and thereby control metabolic activities (Bohne, 2014). Interestingly, genes of rRNA processing and modifications, and of translation were among the most represented in the list of genes down-regulated 27 DAF, compared to 14 DAF, in lower leaves, especially under nitrate-deficient conditions (**Figure 2B**). This suggests reduced translational activities in these leaves at the transition toward senescence. Genes related to these functional categories were among the most over-represented in the set of genes up-regulated 14 DAF, compared to flowering, in lower and upper pea leaves (**Figure 2A**), emphasizing the importance of these processes at early reproductive stages. The relationship between these genes and the progression toward senescence

in leaves has not yet been established. However, perturbations of rRNA biogenesis are closely related to cell senescence in human cells (Yuan et al., 2017). Importantly, six of these genes were positively connected to the *NF-YA3* TF (**Figure S3B** and **Table S8**), making it a good candidate for controlling metabolic activities in leaves. In Arabidopsis, overexpression of *NF-YA* members resulted in dwarf late-senescing plants (Leyva-González et al., 2012). Furthermore, overexpression of the soybean gene *NF-YA3* in Arabidopsis enhanced drought resistance (Ni et al., 2013), indicating this nuclear factor subunit may be associated with protective roles in plants, but the targets potentially co-regulated by the NF-Y complex are yet to be identified. Our results pinpoint genes in module M11 (**Table S8**) as attractive candidates for a deeper study of NF-YA3 function in leaves.

Overall, our results provided new information in understanding the complexity of the transcriptional regulations governing leaf metabolism during seed development in pea up to the transition toward senescence. These findings could serve future in depth investigations on specific genes or TF-related modules.

DATA AVAILABILITY

The datasets generated for this study can be found in NCBI Gene Expression Omnibus database, GSE109789 for pea and GSE109521 for M. truncatula.

AUTHOR CONTRIBUTIONS

JB conceived the project. JB, GA, and SB conceived the pea 40 k-arrays. KG, AK, CS, and AL designed the nitrogen remobilization experiment, and the overall research plan with JB. AB contributed to all experiments with GA and MS (molecular aspects), AK and CL (phenotyping and greenhouse experiments), SP and SB (microarray analyses). KG developed the gene networks with contribution of CH and built the nitrogen remobilization diagrams with contributions of JT and J-CA. MT provided the miR164 data and performed the othology search between species. KG analyzed all the data, with contribution of AB for phenotypic characteristics, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01014/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Annexe 3

Detailed protein composition of the different groups of mature pea seeds.

G1 seeds

			Well-watered							Water-stressed						
			Sulfur supply			Sulfur deficiency				Sulfur supply				Sulfur deficiency			
			Control			S-				WS				S-WS			
Bande number	MW (KDa)	Protein annotation	Mean	SE	SNK test	Mean	SE	SNK test	Ratio to Control	Mean	SE	SNK test	Ratio to Control	Mean	SE	SNK test	Ratio to Control
1	96.9	Several proteins	1.84	0.259	a	2.02	0.743	a	1.10	1.82	0.276	a	0.99	1.12	0.156	a	0.61
2	70.1	Convicilin 7S	9.45	0.108	a	9.13	0.758	a	0.97	8.73	0.225	a	0.92	9.29	0.150	a	0.98
3	64.4	Convicilin 7S	2.16	0.074	b	4.08	0.402	a	1.89 ↗	1.79	0.130	b	0.83	4.08	0.125	a	1.89 ↗
4	59.4	unidentified	0.62	0.148	a	0.32	0.020	a	0.51	0.49	0.067	a	0.80	0.68	0.098	a	1.10
5	54.6	unidentified	0.96	0.348	a	0.31	0.050	a	0.32	0.60	0.131	a	0.63	0.45	0.075	a	0.47
6	51.5	unidentified	2.49	0.258	a	0.83	0.093	b	0.33 ↘	2.45	0.390	a	0.98	0.84	0.171	b	0.34 ↘
7	47.8	Vicilin 7S	18.31	0.496	b	27.52	3.015	a	1.50 ↗	15.20	0.729	b	0.83	17.46	1.861	b	0.95
8	42.7	Several proteins	1.47	0.432	a	0.20	0.134	b	0.14 ↘	1.73	0.289	a	1.17	1.89	0.263	a	1.28
9	39.5	Legumin 11S	9.16	0.441	a	1.26	0.354	c	0.14 ↘	8.98	0.718	a	0.98	6.14	0.381	b	0.67 ↘
10	38.1	Legumin 11S	14.29	1.290	a	3.14	0.400	c	0.22 ↘	13.76	0.666	a	0.96	8.52	0.540	b	0.60 ↘
11	33.6	Vicilin 7S	1.59	0.185	bc	2.72	0.078	a	1.71 ↗	1.21	0.330	c	0.76	2.18	0.179	ab	1.37
12	31.8	Vicilin 7S	5.62	0.115	b	14.00	0.803	a	2.49 ↗	6.35	0.485	b	1.13	9.88	0.188	b	1.76
13	30.9	Vicilin 7S	1.09	0.196	a	2.42	0.411	a	2.22	1.62	0.335	a	1.48	1.97	0.190	a	1.80
14	29.7	unidentified	1.48	0.086	b	2.04	0.463	ab	1.38	2.22	0.435	ab	1.50	3.19	0.202	a	2.16 ↗
15	28.9	Several proteins	2.04	0.293	a	2.53	0.182	a	1.24	2.09	0.148	a	1.02	2.62	0.160	a	1.28
16	26.2	PA2 Albumin	3.87	0.226	a	2.16	0.160	b	0.56 ↘	4.14	0.323	a	1.07	1.74	0.305	b	0.45 ↘
17	25.6	unidentified	2.44	0.185	a	1.23	0.083	b	0.50	2.21	0.304	a	0.90	1.98	0.237	a	0.81
18	24.7	PA2 Albumin	2.46	0.427	b	3.45	0.388	b	1.40	3.11	0.230	b	1.27	4.51	0.181	a	1.84 ↗
19	22.8	Legumin 11S	5.31	0.868	ab	1.22	0.180	c	0.23 ↘	6.52	0.408	a	1.23	4.31	0.284	b	0.81
20	21.8	Legumin 11S	2.80	0.469	a	0.45	0.305	c	0.16 ↘	2.88	0.290	ab	1.03	1.70	0.201	b	0.60 ↘
21	20.9	unidentified	1.18	0.292	a	0.40	0.242	b	0.34 ↘	1.39	0.132	a	1.18	1.67	0.049	a	1.41
22	18.8	Several proteins	2.11	0.264	b	5.40	0.311	a	2.57 ↗	2.40	0.185	b	1.14	2.90	0.278	b	1.38
23	17.8	Legumin 11S	1.75	0.034	b	2.10	0.301	ab	1.20	2.04	0.112	ab	1.17	2.53	0.098	a	1.45 ↗
24	16.8	Several proteins	1.20	0.197	b	1.40	0.130	b	1.16	1.31	0.087	b	1.09	2.12	0.231	a	1.76 ↗
25	16.0	Lectin	1.25	0.256	c	5.00	0.410	a	4.02 ↗	1.81	0.070	bc	1.45	2.34	0.213	b	1.88 ↗
26	13.8	unidentified	2.35	0.261	a	5.15	0.202	b	2.19 ↗	2.59	0.095	a	1.10	3.26	0.149	c	1.39 ↗
27	12.8	unidentified	0.95	0.097	a	0.31	0.045	b	0.33 ↘	1.15	0.020	a	1.21	0.80	0.155	a	0.84
		11S	33.30	2.295	a	7.63	1.629	c	0.23 ↘	34.18	1.466	a	1.03	23.19	1.274	b	0.70 ↘
		7S	38.22	0.693	c	59.88	2.770	a	1.57 ↗	34.90	0.560	c	0.91	44.86	1.457	b	1.17 ↗
		Globulins	71.52	1.677	a	67.51	1.252	a	0.94	69.08	0.939	a	0.97	68.05	0.890	a	0.95
		Albumins	6.33	0.513	a	5.61	0.510	a	0.89	7.25	0.193	a	1.15	6.25	0.396	a	0.99
		7S/11S	1.16	0.098	b	9.07	1.977	a	7.81 ↗	1.03	0.065	b	0.89	1.96	0.147	b	1.69

G2 seeds

			Well-watered							Water-stressed						
			Sulfur supply			Sulfur deficiency				Sulfur supply				Sulfur deficiency			
			Control			S-				WS				S-WS			
Bande number	MW (KDa)	Protein annotation	Mean	SE	SNK test	Mean	SE	SNK test	Ratio to Control	Mean	SE	SNK test	Ratio to Control	Mean	SE	SNK test	Ratio to Control
1	96.9	Several proteins	2.01	0.259	ab	1.12	0.224	ab	0.56	2.28	0.582	a	1.13	0.76	0.151	b	0.38 ↘
2	70.1	Convicilin 7S	8.90	0.245	a	8.54	0.804	a	0.96	8.47	0.614	a	0.95	8.99	0.263	a	1.01
3	64.4	Convicilin 7S	1.82	0.133	b	3.87	0.355	a	2.13 ↗	1.62	0.219	b	0.89	3.31	0.329	a	1.82 ↗
4	59.4	unidentified	0.60	0.160	a	0.37	0.093	a	0.62	0.62	0.106	a	1.03	0.54	0.107	a	0.90
5	54.6	unidentified	0.99	0.209	a	0.41	0.081	a	0.42	0.92	0.170	a	0.93	0.50	0.217	a	0.51
6	51.5	unidentified	2.71	0.350	a	1.06	0.268	b	0.39 ↘	2.96	0.294	a	1.09	1.21	0.296	b	0.45 ↘
7	47.8	Vicilin 7S	16.90	0.930	b	29.30	1.496	a	1.73 ↗	14.42	1.125	b	0.85	16.57	0.693	b	0.98
8	42.7	Several proteins	1.49	0.167	a	0.20	0.072	b	0.13 ↘	1.80	0.431	a	1.21	1.81	0.340	a	1.22
9	39.5	Legumin 11S	9.03	0.388	a	1.33	0.252	c	0.15 ↘	8.82	0.337	a	0.98	6.17	0.574	b	0.68 ↘
10	38.1	Legumin 11S	13.36	0.488	a	3.60	0.504	c	0.27 ↘	13.04	0.889	a	0.98	9.19	1.251	b	0.69 ↘
11	33.6	Vicilin 7S	1.77	0.061	ab	2.80	0.426	a	1.58	1.48	0.275	b	0.84	2.28	0.255	ab	1.29
12	31.8	Vicilin 7S	5.89	0.165	c	13.36	0.958	a	2.27 ↗	6.49	0.502	c	1.10	9.09	0.629	b	1.54 ↗
13	30.9	Vicilin 7S	1.66	0.277	a	2.37	0.217	a	1.43	1.95	0.416	a	1.18	2.75	0.405	a	1.66
14	29.7	unidentified	2.40	0.323	ab	1.90	0.251	b	0.79	2.38	0.348	ab	0.99	3.14	0.274	a	1.31
15	28.9	Several proteins	2.23	0.070	a	2.74	0.250	a	1.23	2.23	0.247	a	1.00	2.65	0.336	a	1.19
16	26.2	PA2 Albumin	4.26	0.116	a	2.22	0.244	b	0.52 ↘	3.43	0.334	a	0.81	1.57	0.436	b	0.37 ↘
17	25.6	unidentified	2.09	0.222	ab	1.46	0.188	a	0.70	2.85	0.500	b	1.37	1.77	0.257	ab	0.85
18	24.7	PA2 Albumin	3.20	0.433	a	3.19	0.386	a	1.00	3.40	0.307	a	1.06	4.28	0.385	a	1.33
19	22.8	Legumin 11S	5.57	0.705	b	0.97	0.207	c	0.17 ↘	7.30	0.601	a	1.31	4.37	0.645	b	0.78
20	21.8	Legumin 11S	3.42	0.397	a	0.16	0.029	c	0.05 ↘	2.56	0.395	ab	0.75	1.92	0.309	b	0.56 ↘
21	20.9	unidentified	0.96	0.198	b	0.26	0.089	c	0.27 ↘	1.13	0.112	b	1.18	1.69	0.150	a	1.77 ↗
22	18.8	Several proteins	2.20	0.060	b	5.25	0.356	a	2.39 ↗	2.41	0.178	b	1.09	2.83	0.392	b	1.29
23	17.8	Legumin 11S	1.78	0.056	b	1.91	0.110	b	1.08	1.93	0.085	b	1.09	2.66	0.057	a	1.50 ↗
24	16.8	Several proteins	1.25	0.062	b	1.70	0.089	a	1.36 ↗	1.29	0.068	b	1.03	1.80	0.150	a	1.44 ↗
25	16.0	Lectin	1.44	0.036	c	4.78	0.314	a	3.31 ↗	1.46	0.178	c	1.01	3.23	0.137	b	2.24 ↗
26	13.8	unidentified	2.38	0.112	a	4.89	0.197	c	2.05 ↗	2.20	0.137	a	0.92	3.73	0.221	b	1.57 ↗
27	12.8	unidentified	0.89	0.097	a	0.39	0.051	b	0.44 ↘	1.14	0.123	a	1.28	1.23	0.379	a	1.38
		11S	33.16	0.520	a	7.98	0.843	c	0.24 ↘	33.65	0.654	a	1.01	24.30	2.661	b	0.73 ↘
		7S	36.94	0.790	c	60.25	1.258	a	1.63 ↗	34.43	0.906	c	0.93	42.98	1.718	b	1.16 ↗
		Globulins	70.10	0.414	a	68.22	0.631	a	0.97	68.07	0.424	a	0.97	67.28	0.965	a	0.96
		Albumins	7.46	0.405	a	5.41	0.605	b	0.72 ↘	6.83	0.301	ab	0.91	5.85	0.659	ab	0.78
		7S/11S	1.12	0.041	b	8.02	1.130	a	7.57 ↗	1.03	0.046	b	0.92	1.85	0.265	b	1.66

G3 seeds

				Well-watered								Water-stressed							
				Sulfur supply			Sulfur deficiency					Sulfur supply				Sulfur deficiency			
				Control			S-					WS				S-WS			
Bande number	MW (KDa)	Protein annotation		Mean	SE	SNK test	Mean	SE	SNK test	Ratio to Control		Mean	SE	SNK test	Ratio to Control	Mean	SE	SNK test	Ratio to Control	
1	96.9	Several proteins		2.54	0.023	a	1.36	0.304	a	0.54		2.79	0.487	a	1.10	1.23	0.313	a	0.48	
2	70.1	Convicilin	7S	9.11	0.160	a	9.17	0.375	a	1.01		9.14	0.528	a	1.00	9.00	0.119	a	0.99	
3	64.4	Convicilin	7S	1.73	0.187	b	4.61	0.341	a	2.67	↗	1.82	0.165	b	1.06	4.14	0.199	a	2.40	↗
4	59.4	unidentified		0.32	0.053	a	0.43	0.040	a	1.37		0.90	0.402	a	2.84	0.71	0.096	a	2.23	
5	54.6	unidentified		0.49	0.031	a	0.25	0.016	a	0.51		1.09	0.364	a	2.25	0.75	0.278	a	1.54	
6	51.5	unidentified		2.04	0.109	ab	0.74	0.176	c	0.37	↘	2.94	0.478	a	1.44	1.49	0.210	bc	0.73	
7	47.8	Vicilin	7S	15.81	0.672	b	25.84	1.643	a	1.63	↗	13.97	1.113	b	0.88	21.92	1.729	a	1.39	↗
8	42.7	Several proteins		1.86	0.359	a	0.33	0.102	c	0.18	↘	2.07	0.510	ab	1.11	0.87	0.061	bc	0.47	↘
9	39.5	Legumin	11S	8.76	0.281	a	1.54	0.291	c	0.18	↘	8.98	0.595	a	1.02	3.77	0.338	b	0.43	↘
10	38.1	Legumin	11S	13.27	0.859	a	4.15	0.520	b	0.31	↘	11.86	1.080	a	0.89	6.29	0.470	b	0.47	↘
11	33.6	Vicilin	7S	1.33	0.176	b	2.95	0.272	a	2.21	↗	1.10	0.321	b	0.82	2.63	0.021	a	1.98	↗
12	31.8	Vicilin	7S	5.68	0.210	c	13.83	0.391	a	2.43	↗	5.84	0.371	c	1.03	10.12	0.739	b	1.78	↗
13	30.9	Vicilin	7S	1.88	0.052	a	2.77	0.561	a	1.47		2.28	0.376	a	1.21	2.53	0.418	a	1.34	
14	29.7	unidentified		2.96	0.080	a	2.13	0.269	a	0.72		2.07	0.454	a	0.70	2.95	0.270	a	1.00	
15	28.9	Several proteins		2.38	0.111	a	2.98	0.320	a	1.25		2.21	0.260	a	0.93	2.93	0.214	a	1.23	
16	26.2	PA2 Albumin		4.26	0.244	a	2.21	0.128	b	0.52	↘	4.23	0.452	a	0.99	2.23	0.341	b	0.52	↘
17	25.6	unidentified		2.05	0.086	a	1.43	0.071	b	0.70	↘	2.43	0.226	a	1.18	1.88	0.124	ab	0.91	
18	24.7	PA2 Albumin		3.72	0.214	a	3.32	0.369	a	0.89		3.21	0.454	a	0.86	4.38	0.250	a	1.18	
19	22.8	Legumin	11S	6.30	0.543	a	1.24	0.217	c	0.20	↘	6.54	0.346	a	1.04	2.58	0.175	b	0.41	↘
20	21.8	Legumin	11S	3.83	0.308	a	0.35	0.144	b	0.09	↘	3.06	0.363	a	0.80	0.98	0.048	b	0.26	↘
21	20.9	unidentified		0.98	0.202	a	0.40	0.160	b	0.41	↘	1.26	0.108	a	1.29	1.04	0.142	ab	1.06	
22	18.8	Several proteins		1.99	0.236	b	4.85	0.173	a	2.44	↗	2.39	0.387	b	1.21	4.10	0.176	a	2.07	↗
23	17.8	Legumin	11S	1.71	0.055	b	2.04	0.122	b	1.19		1.96	0.056	b	1.14	2.51	0.102	a	1.47	↗
24	16.8	Several proteins		1.29	0.030	a	1.62	0.196	a	1.26		1.22	0.078	a	0.95	1.68	0.094	a	1.31	
25	16.0	Lectin		1.37	0.055	c	4.47	0.314	a	3.27	↗	1.45	0.098	c	1.06	3.02	0.186	b	2.22	↗
26	13.8	unidentified		2.22	0.104	a	4.63	0.204	c	2.09	↗	2.48	0.150	a	1.12	3.54	0.174	b	1.59	↗
27	12.8	unidentified		0.92	0.161	a	0.46	0.040	b	0.50	↘	1.19	0.020	a	1.29	0.75	0.295	a	0.82	
		11S		33.88	1.179	a	9.32	0.933	c	0.28	↘	32.39	1.840	a	0.96	16.13	1.006	b	0.48	↘
		7S		35.55	0.774	c	59.16	1.413	a	1.66	↗	34.15	0.766	c	0.96	50.33	1.875	b	1.42	↗
		Globulins		69.43	0.756	a	68.48	0.535	a	0.99		66.54	1.594	a	0.96	66.46	0.885	a	0.96	
		Albumins		7.97	0.128	a	5.53	0.312	b	0.69	↘	7.45	0.244	ab	0.93	6.61	0.398	ab	0.83	
		7S/11S		1.05	0.056	b	6.60	0.831	a	6.25	↗	1.07	0.077	b	1.01	3.17	0.282	b	3.01	

G4 seeds

				Well-watered								Water-stressed			
				Sulfur supply			Sulfur deficiency					Sulfur supply			
				Control			S-					WS			
Bande number	MW (KDa)	Protein annotation		Mean	SE	SNK test	Mean	SE	SNK test	Ratio to Control		Mean	SE	SNK test	Ratio to Control
1	100.0	Several proteins		2.28	0.128	a	1.15	n/a	a	0.50		2.03	0.282	a	0.89
2	70.1	Convicilin	7S	9.53	0.404	a	9.26	n/a	a	0.97		9.29	0.379	a	0.97
3	64.4	Convicilin	7S	1.90	0.083	b	4.83	n/a	a	2.54	↗	2.02	0.270	b	1.06
4	59.4	unidentified		0.44	0.041	a	0.35	n/a	a	0.79		0.55	0.118	a	1.26
5	54.6	unidentified		0.54	0.061	a	0.19	n/a	a	0.36		0.39	0.065	a	0.73
6	51.5	unidentified		1.79	0.095	a	0.40	n/a	b	0.22	↘	1.85	0.184	a	1.03
7	47.8	Vicilin	7S	16.50	0.772	b	29.13	n/a	a	1.77	↗	13.50	1.202	b	0.82
8	42.7	Several proteins		1.98	0.457	a	0.40	n/a	a	0.20		2.81	0.658	a	1.42
9	39.5	Legumin	11S	8.58	0.415	a	1.67	n/a	b	0.19	↘	9.89	0.221	a	1.15
10	38.1	Legumin	11S	14.26	0.236	a	3.90	n/a	b	0.27	↘	14.09	1.512	a	0.99
11	33.6	Vicilin	7S	1.47	0.189	a	2.40	n/a	a	1.63		0.87	0.382	a	0.59
12	31.8	Vicilin	7S	5.52	0.417	b	13.82	n/a	a	2.50	↗	5.31	0.508	b	0.96
13	30.9	Vicilin	7S	1.40	0.253	a	2.01	n/a	a	1.44		2.11	0.936	a	1.51
14	29.7	unidentified		2.68	0.368	a	1.50	n/a	a	0.56		2.37	0.502	a	0.89
15	28.9	Several proteins		2.17	0.112	a	2.75	n/a	a	1.27		1.84	0.196	a	0.85
16	26.2	PA2 Albumin		3.26	0.330	a	2.14	n/a	a	0.66		3.39	0.280	a	1.04
17	25.6	unidentified		2.76	0.166	a	1.67	n/a	a	0.61		2.03	0.355	a	0.73
18	24.7	PA2 Albumin		3.46	0.114	a	2.61	n/a	a	0.75		3.68	0.365	a	1.07
19	22.8	Legumin	11S	6.74	0.444	a	0.71	n/a	b	0.11	↘	7.68	0.213	a	1.14
20	21.8	Legumin	11S	2.98	0.372	a	0.23	n/a	b	0.08	↘	3.54	0.613	a	1.19
21	20.9	unidentified		1.17	0.171	a	0.27	n/a	a	0.23		1.36	0.226	a	1.16
22	18.8	Several proteins		1.86	0.189	b	5.32	n/a	a	2.86	↗	1.65	0.240	b	0.89
23	17.8	Vicilin	11S	1.62	0.102	a	1.66	n/a	a	1.03		1.88	0.074	a	1.16
24	16.8	Legumin		1.24	0.037	b	2.23	n/a	a	1.80	↗	1.25	0.028	b	1.01
25	16.0	Lectin		1.36	0.064	b	4.34	n/a	a	3.20	↗	1.67	0.165	b	1.23
26	13.8	unidentified		2.30	0.162	a	4.83	n/a	b	2.10	↗	2.07	0.206	a	0.90
27	12.8	unidentified		0.95	0.098	a	0.24	n/a	b	0.25	↘	1.11	0.019	a	1.17
		11S		34.17	0.703	a	8.17	n/a	b	0.24	↘	37.08	1.572	a	1.09
		7S		36.32	0.460	b	61.45	n/a	a	1.69	↗	33.09	0.255	b	0.91
		Globulins		70.49	0.634	a	69.62	n/a	a	0.99		70.18	1.447	a	1.00
		Albumins		6.72	0.267	ab	4.74	n/a	a	0.71		7.08	0.198	b	1.05
		7S/11S		1.06	0.031	b	7.52	n/a	a	7.06	↗	0.90	0.043	b	0.84

No G4 seeds under combined stress conditons (Figure 1C) and only one replicate with G4 seeds under S- condition. n/a, not applicable.

Annexe 4

Effects of stresses on SCN content and quantity in each group of seeds.

	Well-watered							Water-stressed							
	Sulfur supply			Sulfur deficiency				Sulfur supply				Sulfur deficiency			
	Control			S-				WS				S-WS			
	Mean	SE	SNK test	Mean	SE	SNK test	Ratio to Control	Mean	SE	SNK test	Ratio to Control	Mean	SE	SNK test	Ratio to Control
SULFUR															
Content (%)															
G1	0.17	0.002	a	0.05	0.004	c	0.31 ↘	0.18	0.006	a	1.05	0.10	0.004	b	0.58 ↘
G2	0.18	0.003	a	0.06	0.003	d	0.30 ↘	0.17	0.004	b	0.92 ↘	0.09	0.005	c	0.50 ↘
G3	0.19	0.004	a	0.06	0.003	c	0.31 ↘	0.19	0.002	a	0.99	0.08	0.003	b	0.40 ↘
G4	0.20	0.003	a	0.06	n/a	b	0.30 ↘	0.22	0.008	a	1.08	n/a	n/a	n/a	n/a
Absolute quantity in one seed (mg)															
G1	0.41	0.016	a	0.12	0.011	b	0.29 ↘	0.37	0.014	a	0.89	0.13	0.020	b	0.31 ↘
G2	0.48	0.022	a	0.13	0.007	c	0.27 ↘	0.38	0.021	b	0.78 ↘	0.11	0.023	c	0.22 ↘
G3	0.51	0.014	a	0.14	0.008	c	0.28 ↘	0.46	0.025	b	0.89 ↘	0.14	0.012	c	0.28 ↘
G4	0.56	0.016	a	0.14	n/a	b	0.25 ↘	0.54	0.015	a	0.97	n/a	n/a	n/a	n/a
NITROGEN															
Content (%)															
G1	4.01	0.041	c	4.85	0.063	b	1.21 ↗	4.17	0.064	c	1.04	5.31	0.198	a	1.32 ↗
G2	4.26	0.086	b	4.94	0.118	a	1.16 ↗	4.09	0.088	b	0.96	5.25	0.194	a	1.23 ↗
G3	4.55	0.105	b	5.09	0.113	a	1.12 ↗	4.56	0.037	b	1.00	5.19	0.121	a	1.14 ↗
G4	4.86	0.082	a	5.21	n/a	a	1.07	4.99	0.094	a	1.03	n/a	n/a	n/a	n/a
Absolute quantity in one seed (mg)															
G1	9.96	0.451	ab	11.61	0.387	b	1.17	8.82	0.458	a	0.89	6.95	0.669	c	0.70 ↘
G2	11.34	0.575	ab	11.87	0.282	b	1.05	9.28	0.594	a	0.82	5.82	0.751	c	0.51 ↘
G3	12.22	0.336	a	12.15	0.452	a	0.99	10.97	0.535	ab	0.90	9.67	0.602	b	0.79 ↘
G4	13.54	0.443	a	12.11	n/a	a	0.89	12.50	0.691	a	0.92	n/a	n/a	n/a	n/a
CARBON															
Content (%)															
G1	45.30	0.271	ab	45.21	0.147	ab	1.00	45.54	0.072	a	1.01	44.88	0.052	b	0.99
G2	45.46	0.104	a	45.07	0.141	a	0.99	45.58	0.079	a	1.00	45.33	0.266	a	1.00
G3	45.65	0.094	a	45.00	0.064	b	0.99 ↘	45.63	0.054	a	1.00	44.92	0.140	b	0.98 ↘
G4	45.55	0.068	a	44.89	n/a	b	0.99 ↘	45.63	0.034	a	1.00	n/a	n/a	n/a	n/a
Absolute quantity in one seed (mg)															
G1	112.34	4.504	a	108.09	2.270	a	0.96	96.46	5.640	a	0.86	59.53	7.699	b	0.53 ↘
G2	120.95	4.094	a	108.30	1.132	a	0.90	103.28	5.910	a	0.85	50.94	8.142	b	0.42 ↘
G3	122.48	1.106	a	107.48	3.646	a	0.88	109.78	5.339	a	0.90	83.72	4.968	b	0.68 ↘
G4	126.78	2.222	a	104.35	n/a	a	0.82	114.13	4.427	a	0.90	n/a	n/a	n/a	n/a
N/S															
G1	24.04	0.414	c	97.00	8.149	a	4.04 ↗	23.86	0.832	c	0.99	55.30	3.617	b	2.30 ↗
G2	23.43	0.135	c	91.64	7.132	a	3.91 ↗	24.61	0.839	c	1.05	58.33	4.731	b	2.49 ↗
G3	24.03	0.134	c	86.75	5.297	a	3.61 ↗	24.12	0.320	c	1.00	68.75	2.439	b	2.86 ↗
G4	24.02	0.303	c	87.57	n/a	a	3.65 ↗	23.00	1.207	c	0.96	n/a	n/a	n/a	n/a
C/N															
G1	11.29	0.057	a	9.32	0.129	b	0.83 ↘	10.92	0.185	a	0.97	8.48	0.314	c	0.75 ↘
G2	10.69	0.193	a	9.14	0.258	b	0.85 ↘	11.15	0.254	a	1.04	8.67	0.308	b	0.81 ↘
G3	10.04	0.220	a	8.86	0.213	b	0.88 ↘	10.01	0.078	a	1.00	8.67	0.185	b	0.86 ↘
G4	9.38	0.152	a	8.62	n/a	a	0.92	9.15	0.181	a	0.98	n/a	n/a	n/a	n/a

No G4 seeds under combined stress conditons (Figure 1C) and only one replicate with G4 seeds under S- condition. n/a, not applicable.

Annexe 5

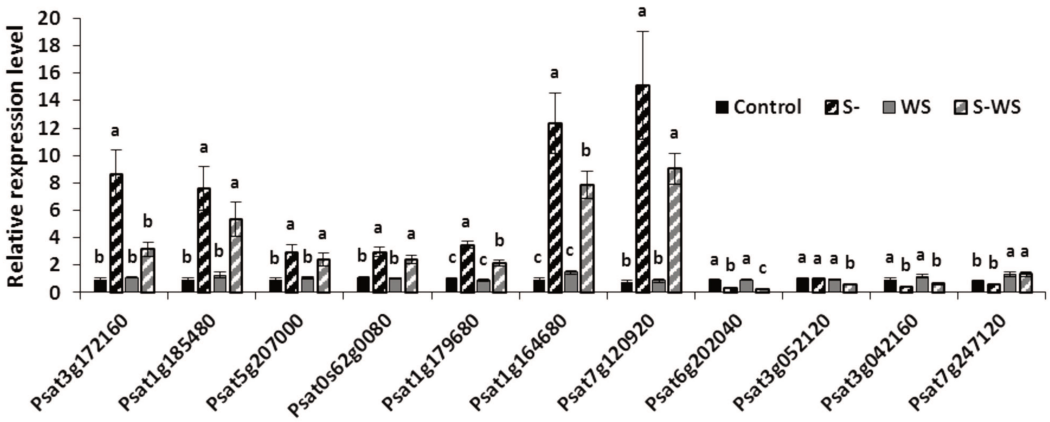
Variation in SCN content and quantity between seed groups for each condition

	G1			G2				G3				G4			
	Mean	SE	SNK test	Mean	SE	SNK test	Ratio to G1	Mean	SE	SNK test	Ratio to G1	Mean	SE	SNK test	Ratio to G1
SULFUR															
Content (%)															
Control	0.17	0.002	c	0.18	0.003	b	1.09 ↗	0.19	0.004	b	1.14 ↗	0.20	0.003	a	1.21 ↗
S-	0.05	0.004	a	0.06	0.003	a	1.08	0.06	0.003	a	1.16	0.06	n/a	a	1.18
WS	0.18	0.006	bc	0.17	0.004	c	0.95	0.19	0.002	b	1.08	0.22	0.008	a	1.25 ↗
S-WS	0.10	0.004	a	0.09	0.005	a	0.94	0.08	0.003	b	0.78 ↘	n/a	n/a	n/a	n/a
Absolute quantity in one seed (mg)															
Control	0.41	0.016	c	0.48	0.022	b	1.17	0.51	0.014	ab	1.23 ↗	0.56	0.016	a	1.36 ↗
S-	0.12	0.011	a	0.13	0.007	a	1.07	0.14	0.008	a	1.16	0.14	n.a.	a	1.13
WS	0.37	0.014	c	0.38	0.021	c	1.02	0.46	0.025	b	1.23 ↗	0.54	0.015	a	1.47 ↗
S-WS	0.13	0.020	a	0.11	0.023	a	0.81	0.14	0.012	a	1.10	n/a	n/a	n/a	n/a
NITROGEN															
Content (%)															
Control	4.01	0.041	c	4.26	0.086	c	1.06	4.55	0.105	b	1.13 ↗	4.86	0.082	a	1.21 ↗
S-	4.85	0.063	a	4.94	0.118	a	1.02	5.09	0.113	a	1.05	5.21	n/a	a	1.07
WS	4.17	0.064	c	4.09	0.088	c	0.98	4.56	0.037	b	1.09 ↗	4.99	0.094	a	1.20 ↗
S-WS	5.31	0.198	a	5.25	0.194	a	0.99	5.19	0.121	a	0.98	n/a	n/a	n/a	n/a
Absolute quantity in one seed (mg)															
Control	9.96	0.451	c	11.34	0.575	bc	1.14	12.22	0.336	ab	1.23 ↗	13.54	0.443	a	1.36 ↗
S-	11.61	0.387	a	11.87	0.282	a	1.02	12.15	0.452	a	1.05	12.11	n/a	a	1.04
WS	8.82	0.458	b	9.28	0.594	b	1.05	10.97	0.535	ab	1.24	12.50	0.691	a	1.42 ↗
S-WS	6.95	0.669	b	5.82	0.751	b	0.84	9.67	0.602	a	1.39 ↗	n/a	n/a	n/a	n/a
CARBON															
Content (%)															
Control	45.30	0.271	a	45.46	0.104	a	1.00	45.65	0.094	a	1.01	45.55	0.068	a	1.01
S-	45.21	0.147	a	45.07	0.141	a	1.00	45.00	0.064	a	1.00	44.89	n/a	a	0.99
WS	45.54	0.072	a	45.58	0.079	a	1.00	45.63	0.054	a	1.00	45.63	0.034	a	1.00
S-WS	44.88	0.052	a	45.33	0.266	a	1.01	44.92	0.140	a	1.00	n/a	n/a	n/a	n/a
Absolute quantity in one seed (mg)															
Control	112.34	4.504	b	120.95	4.094	ab	1.08	122.48	1.106	ab	1.09	126.78	2.222	a	1.13 ↗
S-	108.09	2.270	a	108.30	1.132	a	1.00	107.48	3.646	a	0.99	104.35	n/a	a	0.97
WS	96.46	5.640	a	103.28	5.910	a	1.07	109.78	5.339	a	1.14	114.13	4.427	a	1.18
S-WS	59.53	7.699	b	50.94	8.142	b	0.86	83.72	4.968	a	1.41 ↗	n/a	n/a	n/a	n/a
N/S															
Control	24.04	0.414	a	23.43	0.135	a	0.97	24.03	0.134	a	1.00	24.02	0.303	a	1.00
S-	97.00	8.149	a	91.64	7.132	a	0.94	86.75	5.297	a	0.89	87.57	n/a	a	0.90
WS	23.86	0.832	a	24.61	0.839	a	1.03	24.12	0.320	a	1.01	23.00	1.207	a	0.96
S-WS	55.30	3.617	a	58.33	4.731	a	1.05	68.75	2.439	a	1.24	n/a	n/a	n/a	n/a
C/N															
Control	11.29	0.057	a	10.69	0.193	b	0.95 ↘	10.04	0.220	c	0.89 ↘	9.38	0.152	d	0.83 ↘
S-	9.32	0.129	a	9.14	0.258	a	0.98	8.86	0.213	a	0.95	8.62	n/a	a	0.92
WS	10.92	0.185	a	11.15	0.254	a	1.02	10.01	0.078	b	0.92 ↘	9.15	0.181	c	0.84 ↘
S-WS	8.48	0.314	a	8.67	0.308	a	1.02	8.67	0.185	a	1.02	n/a	n/a	n/a	n/a

No G4 seeds under combined stress conditons (Figure 1C) and only one replicate with G4 seeds under S- condition. n.a., not applicable.

Annexe 6

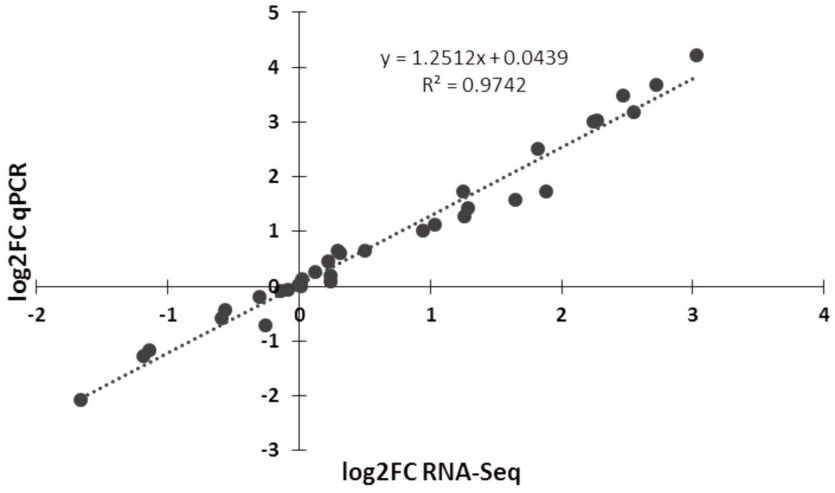
A



B

Gene ID	Description	S-		WS		S-WS	
		log2FC RNA-Seq	log2FC qPCR	log2FC RNA-Seq	log2FC qPCR	log2FC RNA-Seq	log2FC qPCR
Psat3g172160	Zinc-binding dehydrogenase	**2.552**	3.177	0.243	0.196	**1.884**	1.732
Psat1g185480	SelR domain	**2.245**	3.013	0.223	0.457	**1.816**	2.503
Psat5g207000	Eukaryotic protein of unknown function (DUF953)	**1.644**	1.590	0.239	0.086	**1.255**	1.286
Psat0s62g0080	Ribose/Galactose Isomerase	**1.288**	1.425	-0.081	-0.066	**1.033**	1.118
Psat1g179680	Phosphoadenosine phosphosulfate reductase family	**1.254**	1.733	-0.305	-0.191	**0.944**	1.020
Psat1g164680	Transmembrane amino acid transporter protein	**2.721**	3.685	0.309	0.615	**2.269**	3.028
Psat7g120920	2OG-Fe(II) oxygenase superfamily	**3.026**	4.225	0.026	0.136	**2.471**	3.482
Psat6g202040	ATP-sulfurylase	**-1.187**	-1.276	0.000	0.031	**-1.662**	-2.064
Psat3g052120	Alcohol dehydrogenase GroES-like domain	0.010	0.000	-0.143	-0.087	**-0.258**	-0.718
Psat3g042160	S-adenosylmethionine synthetase + C-terminal domain	**-1.139**	-1.155	0.126	0.256	**-0.593**	-0.570
Psat7g247120	Ferritin-like domain	**-0.561**	-0.432	0.293	0.646	**0.506**	0.657

C



Annex 6. RT-qPCR validation of RNA-seq results, 11 genes were selected for DE confirmation in the same RNA samples used for RNA-Seq. A, Relative expression level of the 11 genes in qPCR. Different letters indicate significant differences (P<0.05, ANOVA followed by a SNK test, n = 3 or 4 samples). S-: Sulfur-deficiency alone; WS: water stress alone; S-WS: combined stress. **B,** Description of the 11 genes tested with the result of both RNA-seq and qPCR log2FoldChange compared to the control condition. Bold values for the log2FC RNA-Seq were significantly different compared to control plants (P<0.05, n=3 or 4 samples). **C,** Correlation analysis between RNA-seq and RT-qPCR log2FoldChange results from the same RNA sample.

Annexe 7

Expression of the globulin genes in 9 DAP seeds

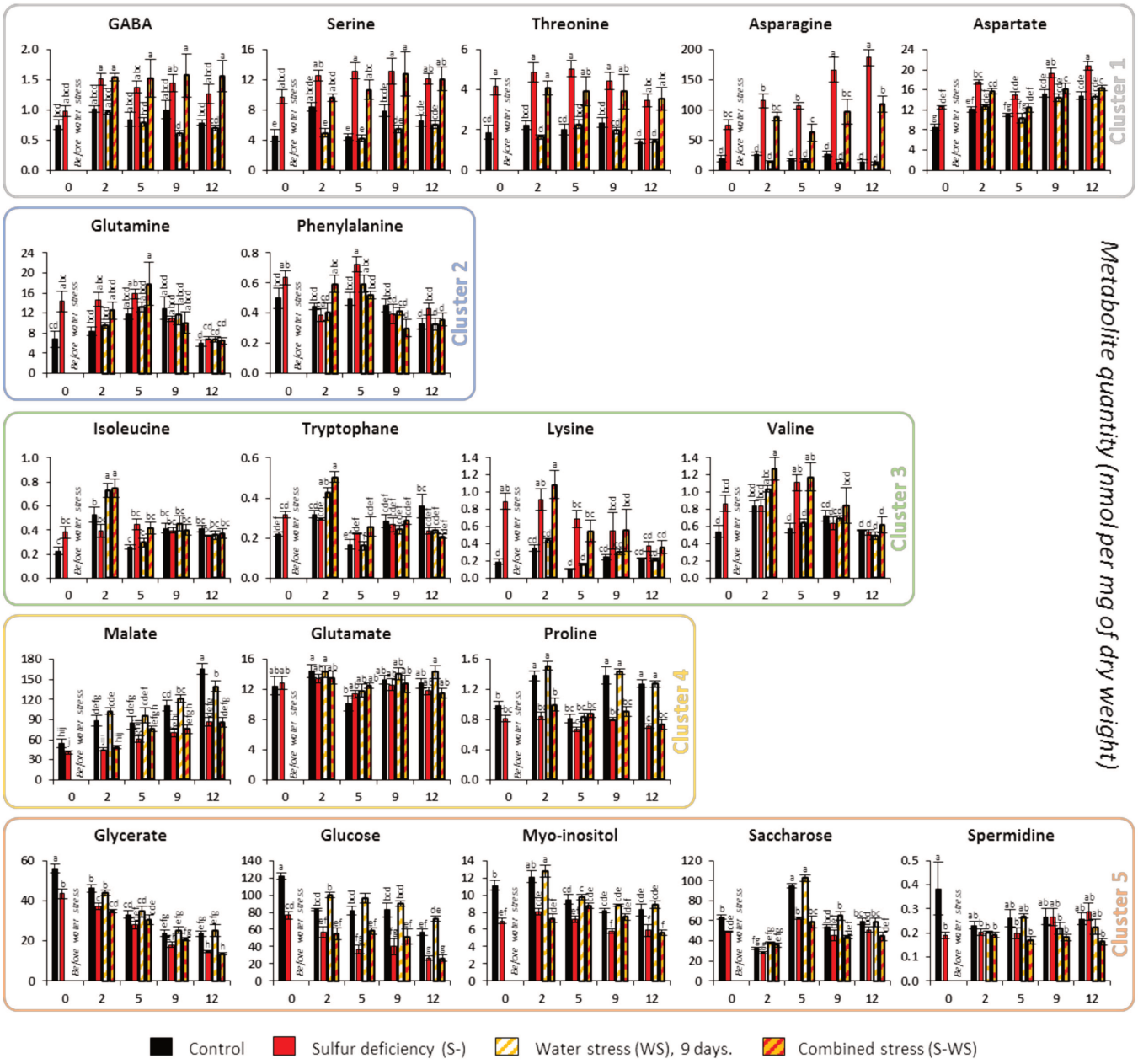
[a] Pea genome ID (https://urgi.versailles.inra.fr/Species/Pisum/Pea-Genome-project)

[b] Expressed in mean normalized counts.

[c] Significant differentces values are in blue and bold (*P*-adjust<0.05).

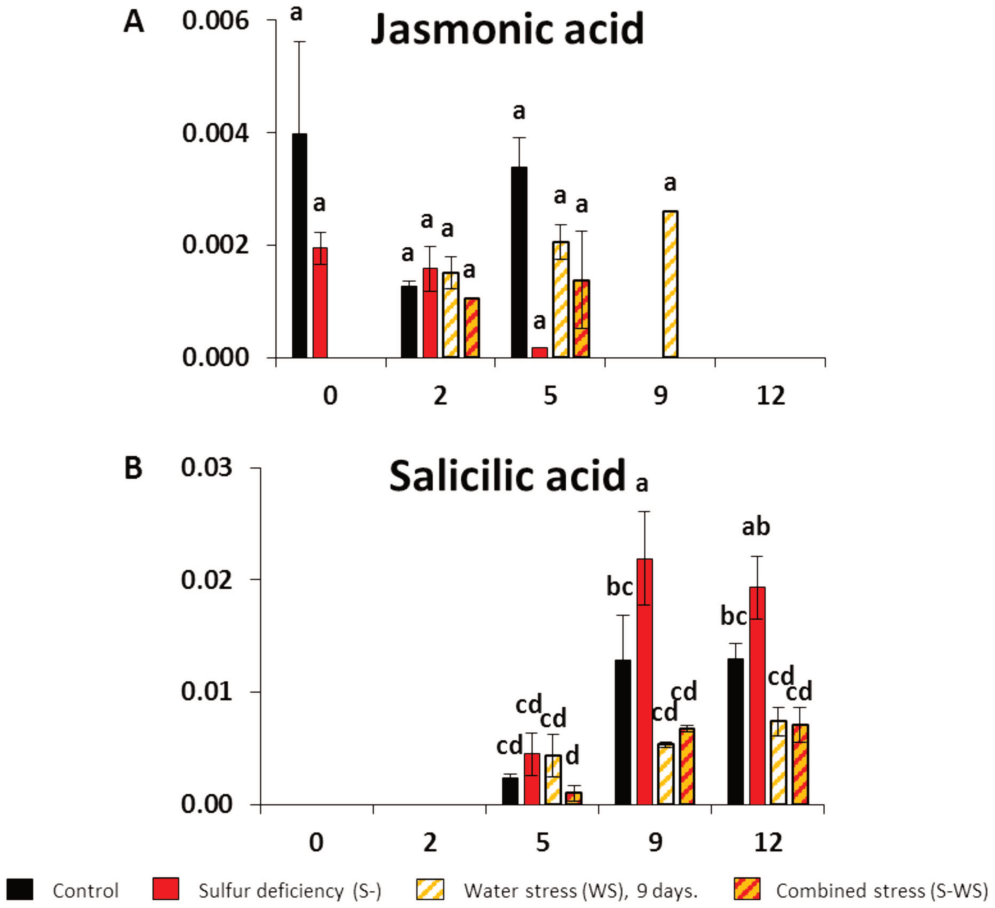
Pea Gene Id[a]	*Medicago truncatula (V4)*		Expression level	log2(Fold change)[c]		
	Gene ID	Annotation	in Control[b]	S-	WS	S-WS
7S GLOBULINS						
Psat5g216000	Medtr7g079730	vicilin 47 kDa protein	284.4	**-0.413**	-0.295	**-0.439**
Psat6g206200	Medtr1g103400	vicilin 47 kDa protein	45.9	-0.466	-0.256	-0.263
Psat0s3779g0080	Medtr7g079740	vicilin 47 kDa protein	385.5	**-0.332**	-0.286	-0.314
Psat0s4055g0080	Medtr7g079730	vicilin 47 kDa protein	533.6	-0.332	-0.389	-0.404
Psat6g206160	Medtr1g103400	vicilin 47 kDa protein	289.4	-0.367	-0.276	-0.364
Psat5g199920	Medtr7g079770	vicilin 47 kDa protein	388.5	-0.286	-0.216	-0.284
Psat0s5g0160	Medtr7g079770	vicilin 47 kDa protein	304.6	-0.284	-0.190	-0.274
Psat0s4851g0160	Medtr7g079770	vicilin 47 kDa protein	1389.2	-0.265	-0.191	-0.249
Psat0s3959g0040	Medtr7g079820	vicilin 47 kDa protein	4.7	-0.235	-0.189	-0.247
Psat0s7016g0040	Medtr7g079740	vicilin 47 kDa protein	473.8	-0.236	-0.176	-0.222
Psat3g104920	Medtr7g079770	vicilin 47 kDa protein	1.8	-0.084	-0.058	-0.106
11S GLOBULINS						
Psat0s1923g0200	Medtr7g097000	legumin A2	33.6	**-1.094**	**-0.917**	**-1.070**
Psat6g054880	Medtr1g072630	legumin storage protein	156.5	**-0.955**	-0.658	**-0.961**
Psat6g055080	Medtr1g072630	legumin storage protein	45.7	**-0.931**	**-0.867**	**-0.956**
Psat3g058800	Medtr7g097000	legumin A2	42.5	**-0.765**	-0.546	**-0.731**
Psat6g125840	Medtr1g072630	legumin storage protein	604.6	**-0.426**	-0.277	-0.391
Psat6g138040	Medtr1g072630	legumin storage protein	976.1	**-0.353**	-0.255	-0.344
Psat6g054960	Medtr1g072630	legumin storage protein	158.1	-0.698	-0.461	-0.669
Psat3g055960	Medtr7g097000	legumin A2	6.2	-0.291	-0.059	-0.249
Psat3g056000	Medtr7g096970	glycinin G4	3.1	-0.003	-0.171	-0.090
Psat6g055000	Medtr1g072630	legumin storage protein	2.1	-0.051	-0.038	0.018

Annexe 8



Annexe 8. Metabolites quantity in nmol per mg of leaf dry weight (n=4 samples).
Values are means ± standard errors. Different letters above each bar represent significant differences (P<0.05, ANOVA followed by a SNK test). S-, S-deficiency alone; WS, water stress alone at 2,5 and 9 days, re-watering at 12 days; S-WS, combined stress.

Annexe 9



Annexe 9. Impact of sulfur deficiency combined or not with water stress on Jasmonic acid and salicylic acid in the leaves during the reproductive phase in pea. (A) Jasmonic acid quantity in ng per mg of leaf dry weight (n=4 samples). **(B)** Salicilic acid quantity in ng per mg of leaf dry weight (n=4 samples).
Values are means ± standard errors. Different letters above each bar represent significant differences (P<0.05, ANOVA followed by a SNK test). S-, S-deficiency alone; WS, water stress alone at 2,5 and 9 days, re-watering at 12 days; S-WS, combined stress.

Abstract

Pea (*Pisum sativum* L.) is a grain legume crop that produces seeds rich in proteins for food and feed and, thanks to its symbiosis with nitrogen-fixing bacteria, enriches the soil with nitrogen. The wider development of pea cultivation is therefore a major agroecological challenge. Water stress combined with sulfur deficiencies in soils (S is a macroelement necessary for plant defense reactions) are two abiotic stresses that interact in the current context of climate change. However, the molecular mechanisms underlying pea adaptation to water stress and their modulation by sulfur nutrition remain to be elucidated. The objectives of this thesis were, first, to describe the impact of an interaction between these two abiotic stresses, water stress and sulfur deficiency, during the reproductive phase on the yield and quality of pea seeds and, second, to characterize using -omics approaches the underlying molecular mechanisms by focusing on developing seeds and leaves, sources of nutrients for developing seeds.

Thus, pea plants (cv. 'Caméor') deprived of sulfate two weeks before flowering were subjected to a period of nine days of moderate water stress from the beginning of flowering. Each stress was also applied individually in parallel with a non-stressed condition. The analysis of mature plants showed a synergistic effect of the two single stresses on yield and yield components. On the other hand, the protein composition of the seed, characterized by the 7S/11S globulin ratio, was less affected in response to the double stress than in response to sulfur deficiency alone, and did not vary in response to water stress. These results show that water stress mitigated the negative effect of sulfur deficiency on seed protein composition. Similar results were obtained for the seed nitrogen/sulfur ratio (correlated to the 7S/11S ratio), suggesting that it is a good indicator of seed protein composition in pea.

In order to determine the mechanisms by which seeds and leaves adapt their metabolism under these multiple stress conditions, developing seeds and leaves of the first two reproductive nodes were collected prior to the application of water stress, during the combined stress period, and during re-watering. These tissues were subjected to omics analyses (proteomics, transcriptomics, ionomics, metabolomics) offering a global view of the response of seeds and leaves to the single and double stresses. The analysis of seeds revealed a mitigated effect of sulfur deficiency combined with water stress on the seed transcriptome and proteome compared to sulfur deficiency alone. The responses of seeds to the single or combined stresses involved a few proteins but these were involved in the removal of reactive oxygen species or in the maintenance of the redox balance. In leaves, transcriptional reprogramming in response to the double stress occurred at the first and last developmental stages studied. This reprogramming involves genes with roles in post-translational modification and protein transport processes. Changes in the leaf proteome occurred at the end of the double stress and during re-watering, highlighting proteins that play a key role in detoxification processes, including metal accumulation.

This new knowledge has allowed the identification of candidate proteins for controlling the mechanisms of seed development or detoxification of cells during abiotic stress. They offer prospects for improving and stabilizing pea yields and seed nutritional quality.

Résumé

Le pois (*Pisum sativum* L.) est une légumineuse qui produit des graines riches en protéines pour l'alimentation humaine et animale et qui, de par sa symbiose avec des bactéries fixatrices d'azote de l'air, enrichit les sols en azote. Le développement plus large de la culture du pois est donc un enjeu agroécologique majeur. Le déficit hydrique combiné à l'appauvrissement des sols en soufre, un macroélément nécessaire aux réactions de défense des plantes, sont deux stress abiotiques qui interagissent dans le contexte actuel de changement climatique. Pourtant, les mécanismes moléculaires sous-jacents à l'adaptation du pois au stress hydrique et leur modulation par la nutrition soufrée restent à élucider. Les objectifs de cette thèse étaient de décrire l'impact d'une interaction entre ces deux stress abiotiques que sont le stress hydrique et la carence en soufre au cours de la phase reproductive sur le rendement et la qualité des graines de pois, puis de caractériser au moyen d'approches omiques les mécanismes moléculaires sous-jacents en se focalisant sur les graines en cours de développement et les feuilles, sources de nutriments pour les graines.

Ainsi, des plantes de pois (cv. 'Caméor') privées de sulfate deux semaines avant floraison ont été soumises à une période de stress hydrique modéré de neuf jours à partir du début de la floraison. Chaque stress a aussi été appliqué individuellement en parallèle d'une condition standard. L'analyse des plantes à maturité montrent un effet synergique des deux stress simples sur le rendement et les composantes du rendement. En revanche, la composition protéique de la graine, caractérisée par le ratio de globulines 7S/11S, est moins affectée en réponse au double stress qu'en condition de carence en soufre seule, et ne varie pas en réponse au stress hydrique. Ces résultats montrent que le stress hydrique atténue l'effet négatif de la carence en soufre sur la composition protéique des graines. Des résultats identiques ont été obtenus pour le ratio azote/soufre des graines (corrélé au ratio 7S/11S), suggérant qu'il soit un bon indicateur de la composition protéique des graines de pois.

Afin de déterminer les mécanismes par lesquels les graines et les feuilles adaptent leur métabolisme dans ces conditions de stress multiples, des graines en développement et les feuilles des deux premiers nœuds reproducteurs ont été prélevées avant l'application du stress hydrique, pendant la période de double stress, et lors du ré-arrosage. Ces tissus ont été soumis à des analyses omiques (protéomique, transcriptomique, ionomique, métabolomique) offrant une vision globale de la réponse des graines et des feuilles aux simples et double stress. Les analyses des graines ont mis en évidence un effet atténué de la carence en soufre combinée au stress hydrique sur le transcriptome et le protéome comparé à une carence en soufre seule. La réponse des graines aux stress simples ou combinés met en jeu un faible nombre de protéines impliquées dans l'élimination des espèces réactives de l'oxygène ou dans le maintien de l'équilibre redox. Dans les feuilles, une reprogrammation transcriptionnelle en réponse au double stress a lieu aux premiers et derniers stades étudiés. Cette reprogrammation fait intervenir des gènes impliqués dans les processus de modifications post-traductionnelles et de transport des protéines. Des changements dans le protéome des feuilles ont lieu à la fin du double stress et lors du ré-arrosage, mettant en lumière des protéines qui jouent un rôle clé dans les processus de détoxification, notamment liés à l'accumulation des métaux.

Ces nouvelles connaissances ont permis l'identification de protéines candidates susceptibles de contrôler les mécanismes de développement des graines ou la détoxification des cellules lors de stress abiotiques. Ils offrent des perspectives d'amélioration et de stabilisation des rendements et de la qualité nutritionnelle des graines chez le pois.