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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

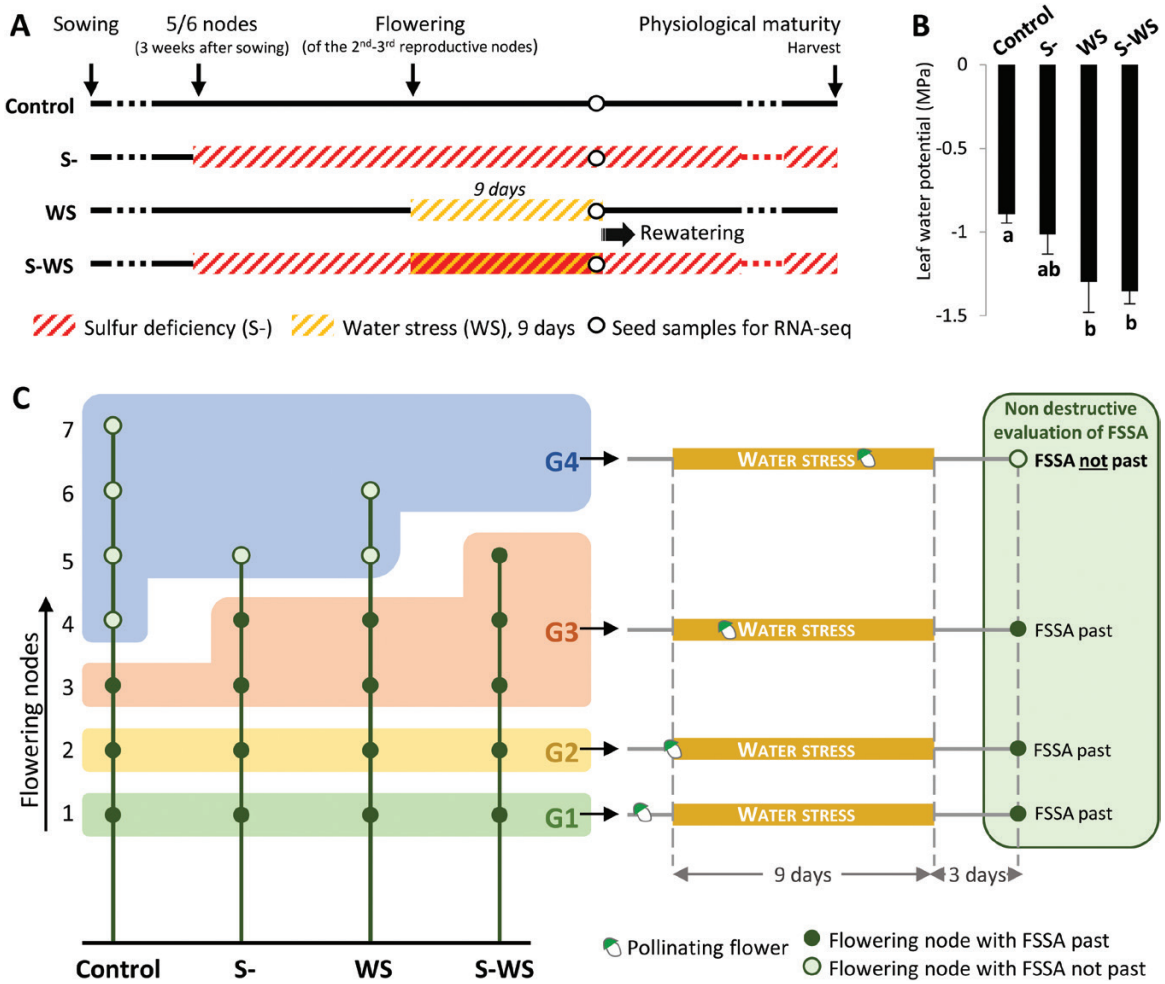


Fig. 1. Experimental design for studying the interaction between water stress and S deficiency in pea (cv. 'Caméor'). (A) Developmental stages at which stresses were imposed. Control, plants were well watered under non-limiting S conditions; S–, plants were deprived of S from the mid-vegetative stage 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 all groups of seeds was

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.

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.

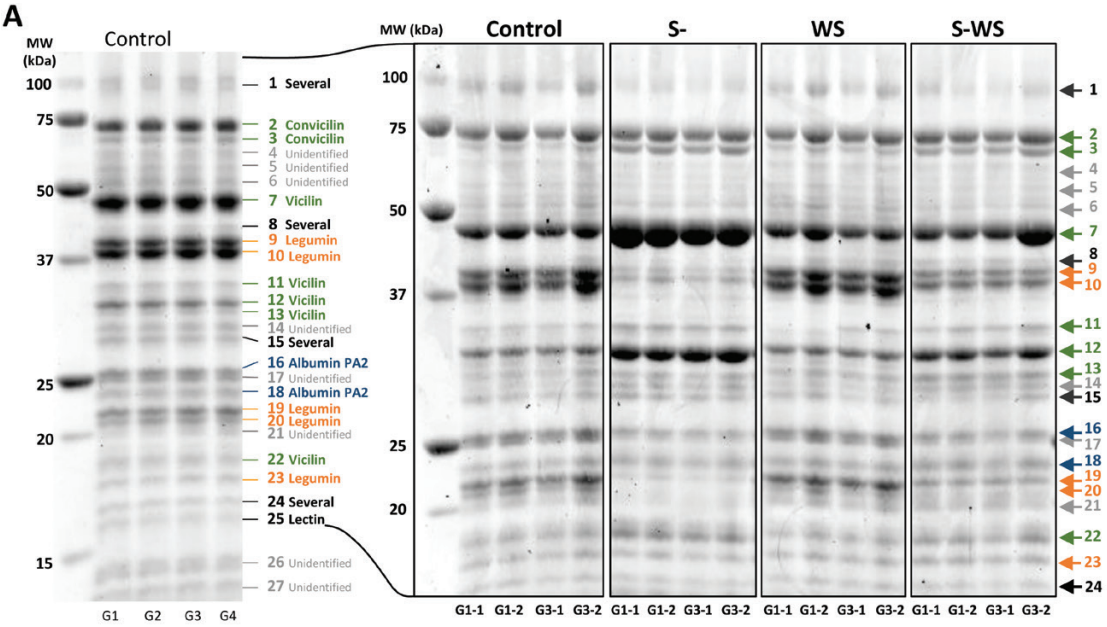
Differences in nutrient allocation between tissues of stressed plants

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 low levels (Supplementary Table S7), indicating that, under

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.

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 protein composition, we have formulated an integrative view

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

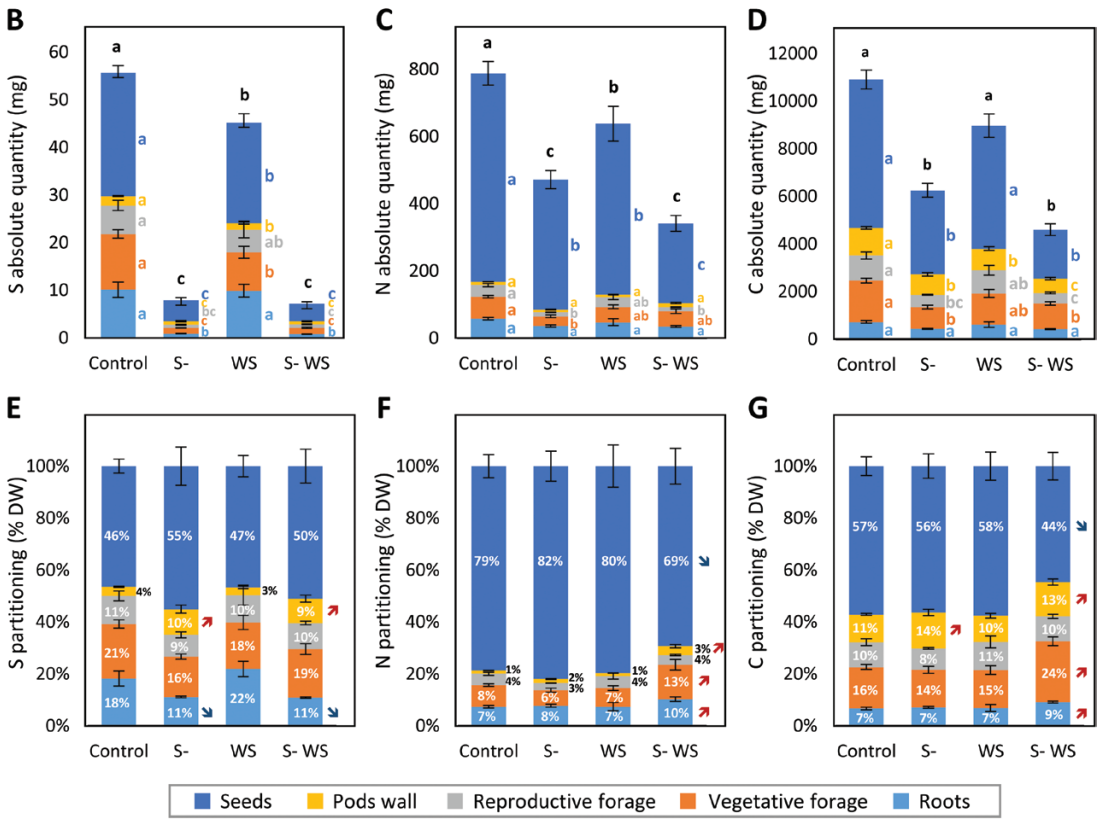
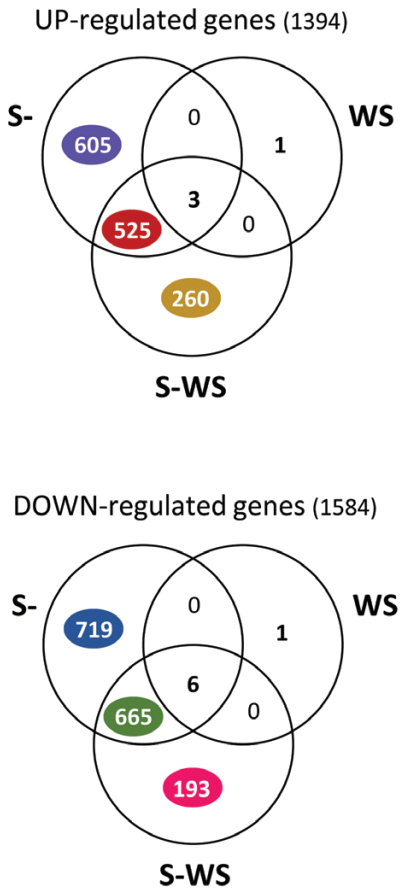


Fig. 3. Effects of water stress combined with S deficiency on the accumulation and distribution of N, C, and S in different pea plant compartments at maturity. (A) Sulfur (S), nitrogen (N), and carbon (C) contents (%), and N/S and C/N ratios, in each compartment. (B–D) Absolute quantity (mg) of S, N, 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.

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 combined abiotic stresses on seed yield components and seed

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.

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 sulfate and sulfide in signaling stomatal closure

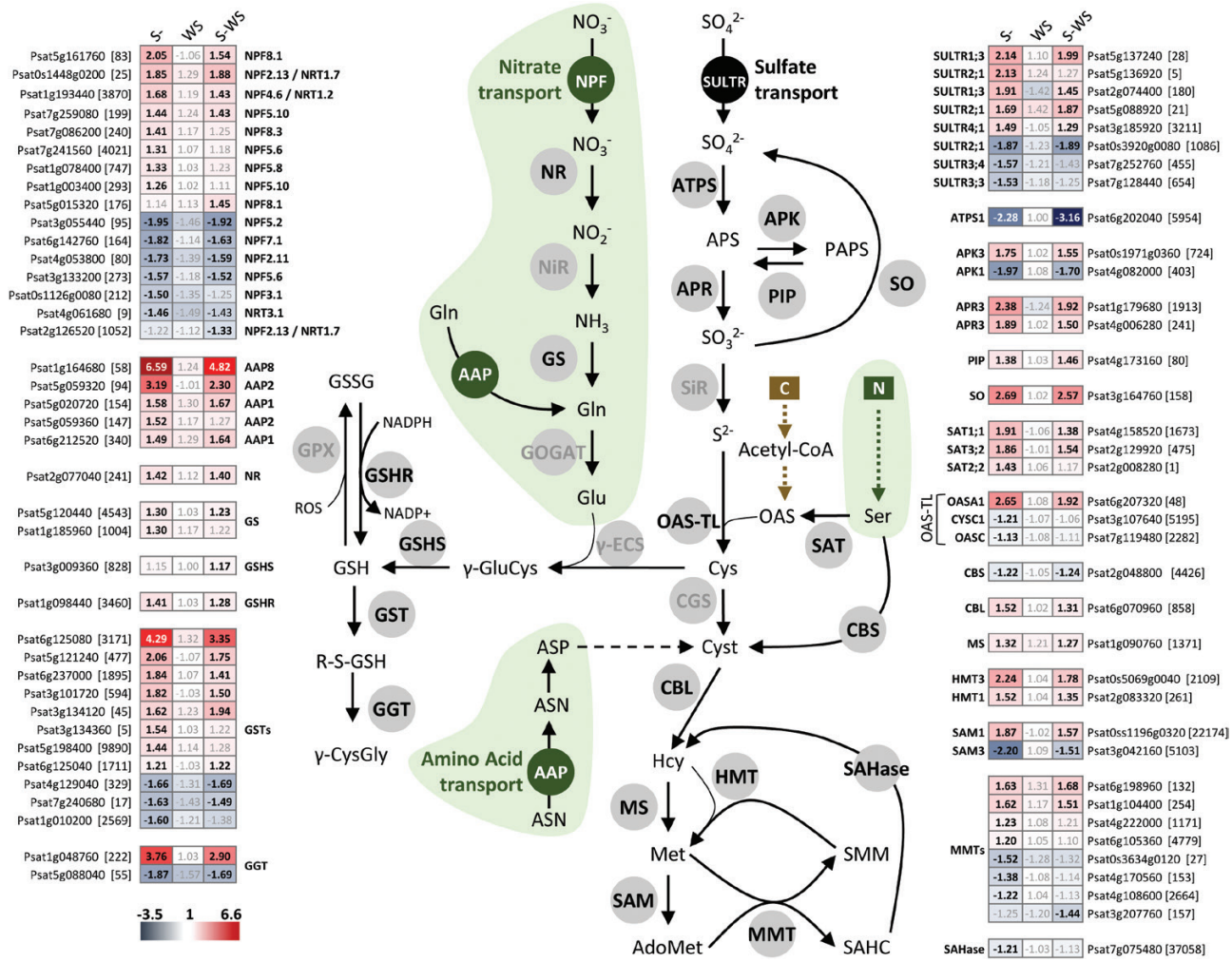


Fig. 5. Effects of water stress combined with S deficiency on the expression of genes related to S and N transport and metabolism in pea. For each gene, the fold-change in expression under stress conditions compared to the control is given. The values are color-coded from low expression in blue to high 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).

(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

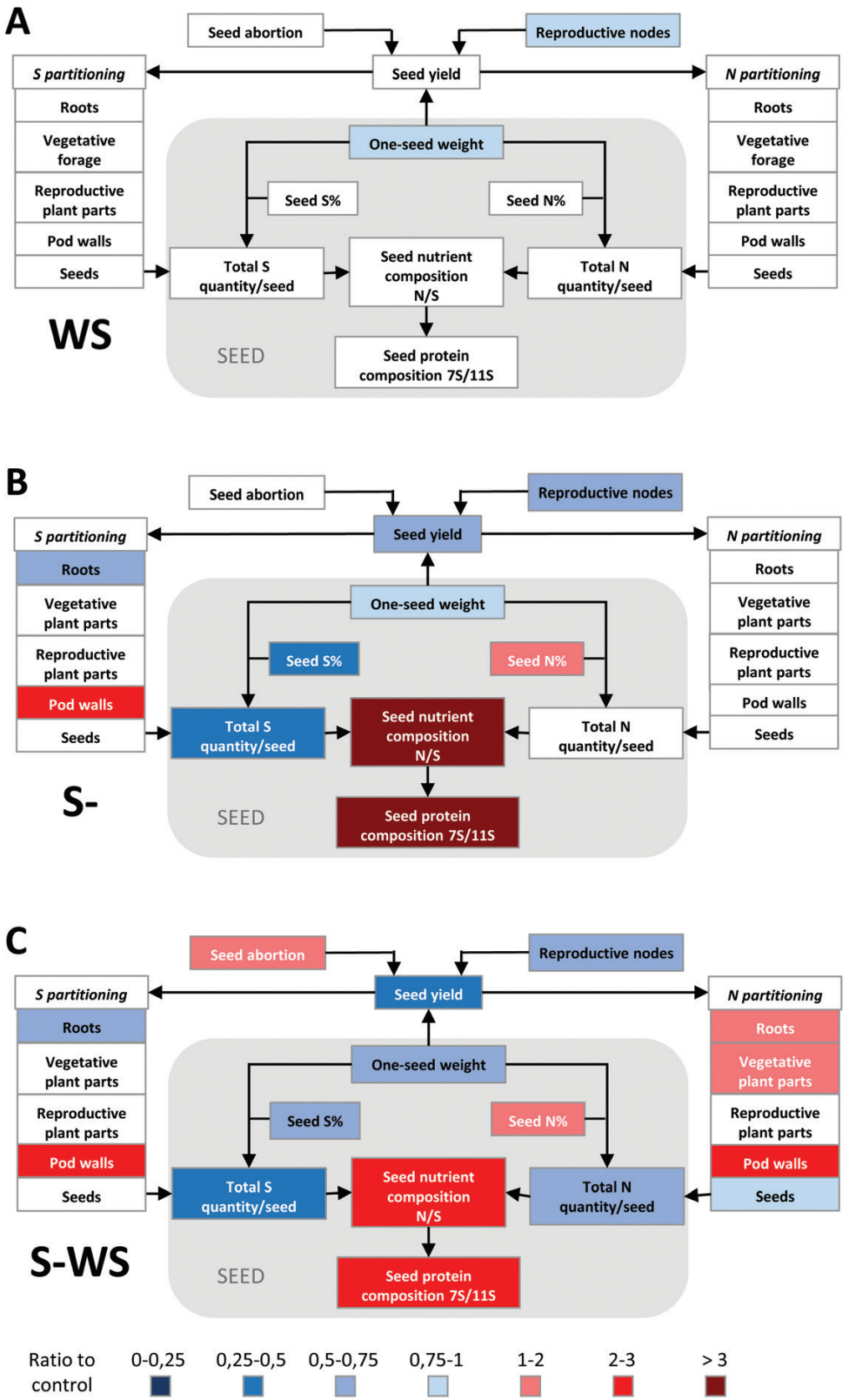


Fig. 6. Summary of the effects of water stress with or without S deficiency on yield components, nutrient partitioning, and seed nutrient and protein composition at harvest in pea. (A) Summary for water-stress conditions. (B) Summary for sulfur-deficient conditions. (C) Summary for combined stress condition. 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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References

Ahmad N, Malagoli M, Wirtz M, Hell R. 2016. Drought stress in maize causes differential acclimation responses of glutathione and sulfur metabolism in leaves and roots. BMC Plant Biology **16**, 247.

Alexa A, Rahnenfuhrer J. 2018. Gene set enrichment analysis with topGO. R package version 2.34.0. doi:10.18129/B9.bioc.topGO.

Álvarez C, García I, Moreno I, Pérez-Pérez ME, Crespo JL, Romero LC, Gotor C. 2012. Cysteine-generated sulfide in the cytosol negatively regulates autophagy and modulates the transcriptional profile in Arabidopsis. The Plant Cell **24**, 4621–4634.

Alves-Carvalho S, Aubert G, Carrère S, *et al*. 2015. Full-length *de novo* assembly of RNA-seq data in pea (*Pisum sativum* L.) provides a gene expression atlas and gives insights into root nodulation in this species. The Plant Journal **84**, 1–19.

Anjum NA, Gill R, Kaushik M, Hasanuzzaman M, Pereira E, Ahmad I, Tuteja N, Gill SS. 2015. ATP-sulfurylase, sulfur-compounds, and plant stress tolerance. Frontiers in Plant Science **6**, 210.

Bastianelli D, Grosjean F, Peyronnet C, Duparque M, Régnier JM. 1998. Feeding value of pea (*Pisum sativum*, L.) 1. Chemical composition of different categories of pea. Animal Science **67**, 609–619.

Benamar A, Tallon C, Macherel D. 2003. Membrane integrity and oxidative properties of mitochondria isolated from imbibing pea seeds after priming or accelerated ageing. Seed Science Research **13**, 35–45.

Blagrove RJ, Gillespie JM, Randall PJ. 1976. Effect of sulphur supply on the seed globulin composition of *Lupinus angustifolius*. Functional Plant Biology **3**, 173–184.

Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics **30**, 2114–2120.

Bonnot T, Bancel E, Alvarez D, Davanture M, Boudet J, Pailloux M, Zivy M, Ravel C, Martre P. 2017. Grain subproteome responses to nitrogen and sulfur supply in diploid wheat *Triticum monococcum* ssp. *monococcum*. The Plant Journal **91**, 894–910.

Boulter D, Croy RRD. 1997. The structure and biosynthesis of legume seed storage proteins: a biological solution to the storage of nitrogen in seeds. Advances in Botanical Research **27**, 1–92.

Bourgeois M, Jacquin F, Savois V, Sommerer N, Labas V, Henry C, Burstin J. 2009. Dissecting the proteome of pea mature seeds reveals the phenotypic plasticity of seed protein composition. Proteomics **9**, 254–271.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry **72**, 248–254.

Brunel-Muguet S, D'Hooghe P, Bataillé MP, Larré C, Kim TH, Trouverie J, Avice JC, Etienne P, Dürr C. 2015. Heat stress during seed filling interferes with sulfur restriction on grain composition and seed germination in oilseed rape (*Brassica napus* L.). Frontiers in Plant Science **6**, 213.

Burstin J, Gallardo K, Mir RR, Varshney RK, Duc G. 2011. Improving protein content and nutrition quality. In: Pratap A, Kumar J. eds. Biology and breeding of food legumes. Wallingford, UK: CAB International, 314–328.

Cao MJ, Wang Z, Zhao Q, Mao JL, Speiser A, Wirtz M, Hell R, Zhu JK, Xiang CB. 2014. Sulfate availability affects ABA levels and germination response to ABA and salt stress in *Arabidopsis thaliana*. The Plant Journal **77**, 604–615.

Chan KX, Wirtz M, Phua SY, Estavillo GM, Pogson BJ. 2013. Balancing metabolites in drought: the sulfur assimilation conundrum. Trends in Plant Science **18**, 18–29.

Chandler PM, Higgins TJ, Randall PJ, Spencer D. 1983. Regulation of legumin levels in developing pea *Pisum sativum* seeds under conditions of sulfur deficiency: rates of legumin synthesis and levels of legumin mRNA. Plant Physiology **71**, 47–54.

Chandler PM, Spencer D, Randall PJ, Higgins TJ. 1984. Influence of sulfur nutrition on developmental patterns of some major pea seed proteins and their mRNAs. Plant Physiology **75**, 651–657.

Cruz de Carvalho MH. 2008. Drought stress and reactive oxygen species: production, scavenging and signaling. Plant Signaling & Behavior **3**, 156–165.

Dagorn-Scaviner C, Gueguen J, Lefebvre J. 1986. A comparison of interfacial behaviours of pea (*Pisum sativum* L.) legumin and vicilin at air/water interface. Molecular Nutrition & Food Research **30**, 337–347.

Dalmais M, Schmidt J, Le Signor C, *et al*. 2008. UTILLdb, a *Pisum sativum in silico* forward and reverse genetics tool. Genome Biology **9**, R43.

Desclaux D, Roumet P. 1996. Impact of drought stress on the phenology of two soybean (*Glycine max* L. Merr) cultivars. Field Crops Research **46**, 61–70.

D'Hooghe P, Dubousset L, Gallardo K, Kopriva S, Avice JC, Trouverie J. 2014. Evidence for proteomic and metabolic adaptations associated with alterations of seed yield and quality in sulfur-limited *Brassica napus* L. Molecular & Cellular Proteomics **13**, 1165–1183.

Dong Y, Silbermann M, Speiser A, *et al*. 2017. Sulfur availability regulates plant growth via glucose-TOR signaling. Nature Communications **8**, 1174.

Ernst L, Goodger JQ, Alvarez S, Marsh EL, Berla B, Lockhart E, Jung J, Li P, Bohnert HJ, Schachtman DP. 2010. Sulphate as a xylem-borne chemical signal precedes the expression of ABA biosynthetic genes in maize roots. Journal of Experimental Botany **61**, 3395–3405.

Etienne P, Sorin E, Maillard A, Gallardo K, Arkoun M, Guerrand J, Cruz F, Yvin J-C, Ourry A. 2018. Assessment of sulfur deficiency under field conditions by single measurements of sulfur, chloride and phosphorus in mature leaves. Plants **7**, 37.

Evans IM, Gatehouse JA, Boulter D. 1985. Regulation of storage-protein synthesis in pea (*Pisum sativum* L.) cotyledons under conditions of sulphur deficiency. The Biochemical Journal **232**, 261–265.

Fougereux J-A, Doré T, Ladonne F, Fleury A. 1997. Water stress during reproductive stages affects seed quality and yield of pea (*Pisum sativum* L). Crop Science **37**, 1247–1252.

Fujiwara T, Beachy RN. 1994. Tissue-specific and temporal regulation of a beta-conglycinin gene: roles of the RY repeat and other *cis*-acting elements. Plant Molecular Biology **24**, 261–272.

Gallardo K, Firnhaber C, Zuber H, Héricher D, Belghazi M, Henry C, Küster H, Thompson R. 2007. A combined proteome and transcriptome analysis of developing *Medicago truncatula* seeds: evidence for metabolic specialization of maternal and filial tissues. Molecular & Cellular Proteomics **6**, 2165–2179.

Guiboileau A, Sormani R, Meyer C, Masclaux-Daubresse C. 2010. Senescence and death of plant organs: nutrient recycling and developmental regulation. Comptes Rendus Biologies **333**, 382–391.

Guilioni L, Wery J, Jeremie L. 2003. High temperature and water deficit may reduce seed number in field pea purely by decreasing plant growth rate. Functional Plant Biology **30**, 1151–1164.

Hoefgen R, Nikiforova VJ. 2008. Metabolomics integrated with transcriptomics: assessing systems response to sulfur-deficiency stress. Physiologia Plantarum **132**, 190–198.

Jones P, Binns D, Chang HY, *et al*. 2014. InterProScan 5: genome-scale protein function classification. Bioinformatics **30**, 1236–1240.

Karjalainen R, and Kortet S. 1987. Environmental and genetic variation in protein content of peas under northern growing conditions and breeding implications. Agricultural and Food Science **59**, 1–9.

Keller A, Nesvizhskii AI, Kolker E, Aebersold R. 2002. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. Analytical Chemistry **74**, 5383–5392.

Kim D, Langmead B, Salzberg SL. 2015. HISAT: a fast spliced aligner with low memory requirements. Nature Methods **12**, 357–360.

Kim H, Hirai MY, Hayashi H, Chino M, Naito S, Fujiwara T. 1999. Role of O-acetyl-l-serine in the coordinated regulation of the expression of a soybean seed storage-protein gene by sulfur and nitrogen nutrition. Planta **209**, 282–289.

Labas V, Grasseau I, Cahier K, Gargaros A, Harichaux G, Teixeira-Gomes AP, Alves S, Bourin M, Gérard N, Blesbois E. 2015. Qualitative and quantitative peptidomic and proteomic approaches to phenotyping chicken semen. Journal of Proteomics **112**, 313–335.

Le Signor C, Aimé D, Bordat A, *et al*. 2017. Genome-wide association studies with proteomics data reveal genes important for synthesis, transport and packaging of globulins in legume seeds. New Phytologist **214**, 1597–1613.

Lee BR, Zaman R, Avice JC, Ourry A, Kim TH. 2016. Sulfur use efficiency is a significant determinant of drought stress tolerance in relation to photosynthetic activity in *Brassica napus* cultivars. Frontiers in Plant Science **7**, 459.

Liao Y, Smyth GK, Shi W. 2014. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics **30**, 923–930.

Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology **15**, 550.

MacManes MD. 2015. An opinionated guide to the proper care and feeding of your transcriptome. bioRxiv, 035642. doi:10.1101/035642. [Preprint].

Malcheska F, Ahmad A, Batool S, *et al*. 2017. Drought-enhanced xylem sap sulfate closes stomata by affecting ALMT12 and guard cell ABA synthesis. Plant Physiology **174**, 798–814.

Mariotti F, Tomé D, Mirand PP. 2008. Converting nitrogen into protein–beyond 6.25 and Jones' factors. Critical Reviews in Food Science and Nutrition **48**, 177–184.

Mcgrath S, Zhao F-J, Blake-Kalff MM. 2003. History and outlook for sulphur fertilizers in Europe. Fertilizers Fertilization **2**, 5–27.

Miura K, Lee J, Jin JB, Yoo CY, Miura T, Hasegawa PM. 2009. Sumoylation of ABI5 by the Arabidopsis SUMO E3 ligase SIZ1 negatively regulates abscisic acid signaling. Proceedings of the National Academy of Sciences, USA **106**, 5418–5423.

Mujoo R, Trinh DT, Ng PKW. 2003. Characterization of storage protein in different soybean varieties and their relationship to tofu yield and texture. Food Chemistry **82**, 265–273.

Munier-Jolain N, Biarnes V, Chaillet I, Lecoeur J. 2010. Physiology of the pea crop. Boca Raton, USA: CRC Press.

Ney B, Duthion C, Turc O. 1994. Phenological response of pea to water stress during reproductive development. Crop Science **34**, 141–146.

Ng DW-K, Hall TC. 2008. PvALF and FUS3 activate expression from the phaseolin promoter by different mechanisms. Plant Molecular Biology **66**, 233–244.

Nikiforova V, Freitag J, Kempa S, Adamik M, Hesse H, Hoefgen R. 2003. Transcriptome analysis of sulfur depletion in *Arabidopsis thaliana*: interlacing of biosynthetic pathways provides response specificity. The Plant Journal **33**, 633–650.

Noguero M, Le Signor C, Vernoud V, *et al*. 2015. DASH transcription factor impacts *Medicago truncatula* seed size by its action on embryo morphogenesis and auxin homeostasis. The Plant Journal **81**, 453–466.

Ostaszewska-Bugajska M, Rychter AM, Juszczuk IM. 2015. Antioxidative and proteolytic systems protect mitochondria from oxidative damage in S-deficient *Arabidopsis thaliana*. Journal of Plant Physiology **186–187**, 25–38.

Pandey P, Ramegowda V, Senthil-Kumar M. 2015. Shared and unique responses of plants to multiple individual stresses and stress combinations: physiological and molecular mechanisms. Frontiers in Plant Science **6**, 723.

Pandurangan S, Diapari M, Yin F, *et al*. 2016. Genomic analysis of storage protein deficiency in genetically related lines of common bean (*Phaseolus vulgaris*). Frontiers in Plant Science **7**, 389.

Pertea M, Kim D, Pertea GM, Leek JT, Salzberg SL. 2016. Transcript-level expression analysis of RNA-seq experiments with HISAT, StringTie and Ballgown. Nature Protocols **11**, 1650–1667.

Pertea M, Pertea GM, Antonescu CM, Chang TC, Mendell JT, Salzberg SL. 2015. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. Nature Biotechnology **33**, 290–295.

Prudent M, Vernoud V, Girodet S, Salon C. 2015. How nitrogen fixation is modulated in response to different water availability levels and during recovery: a structural and functional study at the whole plant level. Plant and Soil **399**, 1–12.

Quinlan AR, Hall IM. 2010. BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics **26**, 841–842.

Rajab H, Khan MS, Malagoli M, Hell R, Wirtz M. 2019. Sulfate-induced stomata closure requires the canonical ABA signal transduction machinery. Plants **8**, 21.

Rangel A, Domont GB, Pedrosa C, Ferreira ST. 2003. Functional properties of purified vicilins from cowpea (*Vigna unguiculata*) and pea (*Pisum sativum*) and cowpea protein isolate. Journal of Agricultural and Food Chemistry **51**, 5792–5797.

Scherer HW, Lange A. 1996. N_2 fixation and growth of legumes as affected by sulphur fertilization. Biology and Fertility of Soils **23**, 449–453.

Schmidt R, Stransky H, Koch W. 2007. The amino acid permease AAP8 is important for early seed development in *Arabidopsis thaliana*. Planta **226**, 805–813.

Schmittgen TD, Livak KJ. 2008. Analyzing real-time PCR data by the comparative C_T method. Nature Protocols **3**, 1101–1108.

Sieh D, Watanabe M, Devers EA, Brueckner F, Hoefgen R, Krajinski F. 2013. The arbuscular mycorrhizal symbiosis influences sulfur starvation responses of *Medicago truncatula*. New Phytologist **197**, 606–616.

Sinclair TR. 1998. Historical changes in harvest index and crop nitrogen accumulation. Crop Science **38**, 638–643.

Song L, Florea L. 2015. Rcorrector: efficient and accurate error correction for Illumina RNA-seq reads. GigaScience **4**, 48.

Spencer D, Rerie W, Randall P, Higgins T. 1990. The regulation of pea seed storage protein genes by sulfur stress. Australian Journal of Plant Physiology **17**, 355–363.

Tang H, Krishnakumar V, Li J. 2015. jcvi: JCVI utility libraries. Zenodo. doi:10.5281/zenodo.31631.

Terry N. 1976. Effects of sulfur on the photosynthesis of intact leaves and isolated chloroplasts of sugar beets. Plant Physiology **57**, 477–479.

Varin S, Cliquet JB, Personeni E, Avice JC, Lemauviel-Lavenant S. 2010. How does sulphur availability modify N acquisition of white clover (*Trifolium repens* L.)? Journal of Experimental Botany **61**, 225–234.

Vizcaíno JA, Deutsch EW, Wang R, *et al*. 2014. ProteomeXchange provides globally coordinated proteomics data submission and dissemination. Nature Biotechnology **32**, 223–226.

Wucher V, Legeai F, Hédan B, *et al*. 2017. FEELnc: a tool for long non-coding RNA annotation and its application to the dog transcriptome. Nucleic Acids Research **45**, e57.

Zhang H, Sonnewald U. 2017. Differences and commonalities of plant responses to single and combined stresses. The Plant Journal **90**, 839–855.

Zhao FJ, Wood AP, McGrath SP. 1999. Effects of sulphur nutrition on growth and nitrogen fixation of pea (*Pisum sativum* L.). Plant and Soil **212**, 207–217.

Zuber H, Poignavent G, Le Signor C, *et al*. 2013. Legume adaptation to sulfur deficiency revealed by comparing nutrient allocation and seed traits in *Medicago truncatula*. The Plant Journal **76**, 982–996.