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RESEARCH PAPER

Leaf status and environmental signals jointly regulate proline metabolism in winter oilseed rape

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Abstract

Proline metabolism is an essential component of plant adaptation to multiple environmental stress conditions that is also known to participate in specific developmental phases, particularly in reproductive organs. Recent evidence suggested a possible role for proline catabolism in *Brassica napus* for nitrogen remobilization processes from source leaves at the vegetative stage. Here, we investigate transcript levels of Δ1-PYRROLINE-5-CARBOXYLATE SYNTHASE (P5CS) and PROLINE DEHYDROGENASE (ProDH) genes at the vegetative stage with respect to net proline biosynthesis and degradation fluxes in leaves having a different sink/source balance. We showed that the underexpression of three P5CS genes in source leaves was accompanied by a reduced commitment of *de novo* assimilated 15N towards proline biosynthesis and an overall depletion of free proline content. We found that the expression of ProDH genes was strongly induced by carbon starvation conditions (dark-induced senescence) compared with early senescing leaves. Our results suggested a role for proline catabolism in *B. napus*, but acting only at a late stage of senescence. In addition, we also identified some P5CS and ProDH genes that were differentially expressed during multiple processes (leaf status, dark to light transition, and stress response).

Keywords: *Brassica napus*, fluxes, osmotic stress, Δ1-PYRROLINE-5-CARBOXYLATE SYNTHASE (P5CS), PROLINE DEHYDROGENASE (ProDH), proline, regulation, senescence.

Introduction

Winter oilseed rape (*Brassica napus* L.) is a major oleaginous crop ranked the third most essential source of plant oil in the world. Considering the agroecological transition and climate change contexts, there is a need to maintain crop seed yield and quality under fluctuating environments and to reduce chemical inputs. However, oilseed rape is highly demanding for mineral nitrogen (N) inputs (150–250 kg N ha⁻¹) and characterized by a poor N use efficiency, with only 50% of the N absorbed by plants being present in seeds (Rossato et al., 2001; Rathke et al., 2005). Therefore, optimization of N management by oilseed rape under reduced N input conditions and/or during abiotic stress is a promising target for future crop improvement (Masclaux-Daubresse et al., 2010; Bouchet et al., 2016).

In plants, N is taken up first from the soil by roots through nitrate and ammonia transporters. Nitrate is preferentially transported in the xylem to be assimilated in source leaves and then further reallocated to sink tissues in the form of amino acids (Tegeder and Masclaux-Daubresse, 2018). Although oilseed
proline is characterized by a strong N uptake efficiency at the vegetative stage, it has a low N use efficiency, mainly due to a low N remobilization efficiency (Malagoli et al., 2005). At the vegetative stage, N remobilization in oilseed rape occurs essentially between leaves according to their specific sink/source balance, through regulated sequential senescence processes, and involves a complex proteolytic degradation system (Girondé et al., 2015). Indeed, a complete metabolic reprogramming is triggered to help protein degradation and successive steps of proteinogenic transamination of amino acids into glutamine for N recycling and reallocation (Chrobok et al., 2016). The specific overexpression of glutamine synthetase in midveins during natural leaf senescence and under N limitation conditions suggests that glutamine is a major vector of N for N remobilization through the phloem in oilseed rape (Orsel et al., 2014). However, others amino acids may also contribute to N remobilization, depending on the physiological leaf status and environmental agroclimatic conditions. In B. napus, previous studies pointed out the importance of proline metabolism for stress response under N and water depletion, as well as a potential role in N remobilization from source tissues at the vegetative stage (Albert et al., 2012; Faës et al., 2015; Zhang and Becker, 2015; Clément et al., 2018).

Proline is considered as a compatible osmolyte that accumulates widely in the plant kingdom during varying abiotic stress conditions (i.e. drought, high salinity, high light, heavy metals, and phosphate starvation, and in response to biotic challenges) (Chrobok et al., 2016). The role of proline is initiated first by the NADPH-dependent delta-1-pyrroline-5-carboxylate (P5C) (Szabados and Savoure, 2010). This enzyme produces a glutamate semialdehyde that is spontaneously converted to pyrroline-5-carboxylate (P5C). Then P5C is further reduced to proline by the NADPH-dependent proline dehydrogenase, which convert proline successively to P5CS2 and P5C dehydrogenase, which convert proline successively to glutamate semialdehyde that is spontaneously converted to pyrroline-5-carboxylate (P5C) (Ben Rejeb et al., 2014). In Arabidopsis, two P5CS genes were identified with specific functions: P5CS1 is induced by drought and osmotic stress while P5CS2 is required during seed maturation and expressed in response to biotic stress (Savouré et al., 1995; Yoshida et al., 1995; Fabro et al., 2004; Székely et al., 2008). In B. napus, a total of six P5CS1 genes have been identified (Wang et al., 2014). Previous analysis revealed that a P5CS1 (BnaA05g035760D) and a P5CS2 (BnaA09g35230D) were involved in proline production during water shortage, salt stress, or polyethylene glycol (PEG) stress (Xue et al., 2009; Albert et al., 2012). Proline catabolism occurs in mitochondria via the sequential action of proline dehydrogenase (ProDH) and P5C dehydrogenase, which convert proline successively to P5C then to glutamate (Schertl et al., 2014). Two ProDH genes exist in Arabidopsis, with ProDH1 playing an essential role for proline-dependent oxidation/respiration in Arabidopsis mitochondria (Cabassa-Hourtou et al., 2016). Recently, the response of the single mutants prodh1 and prodh2 and the double mutant prodh1prodh2 to a prolonged darkness treatment was analysed. The results showed that ProDH1 and ProDH2 were both involved in the proline-dependent mitochondrial respiration during dark-induced senescence (Launay et al., 2019). In B. napus, six ProDH1 and two ProDH2 genes were identified by phylogenetic analysis with parental ancestors (B. rapa and B. oleracea) (Faës et al., 2015). BnaA&C.ProDH1a and BnaA&C. ProDH1b genes were very highly expressed in leaves and roots, whereas BnaA&C.ProDH1c genes were almost exclusively expressed in reproductive organs. BnaProDH2 genes were mainly expressed in flowers, roots, and senescent leaves. Analysis of promoter-β-glucuronidase (GUS) lines showed a strong expression of BnaProDH2 genes in vascular tissues, proposing a role for proline degradation, which would provide energy for phloem transport (Faës et al., 2015). Proline biosynthesis can also derive from ornithine through the action of ornithine δ- or α-aminotransferase, but these alternative pathways were shown to be irrelevant for proline biosynthesis during stress conditions (Funck et al., 2008).

Regulation of proline metabolism under optimal or stress conditions is well documented and usually involves the modulation of the P5CS/ProDH balance at the transcriptional level, through either an abscisic acid (ABA)-dependent or an ABA-independent pathway (Zarattini and Forlani, 2017). Under osmotic/salt/drought and multiple other abiotic stress conditions, induction of P5CS gene transcription allows plants to acclimate to the stress by accumulating proline and scavenging reducing power (Yoshida et al., 1995; Signorelli and Monza, 2017). During the post-stress metabolic recovery phase, induction of ProDH gene transcription plays a key role in proline recycling, as it is a readily available source of N and reducing power for mitochondrial metabolism (Sharma and Verslues, 2010; Dobrá et al., 2011). Additionally, light and dark conditions can affect proline metabolism in Arabidopsis. P5CS gene transcription is promoted during the light period and repressed during dark periods, whereas ProDH transcript levels show the opposite behaviour (Hayashi et al., 2000; Abrahám et al., 2003). Prolonged exposure to darkness (dark-induced senescence) was also reported to cause an activation of ProDH transcription and a depletion of proline content, mediated by activation of sucrose-dependent bZIP factors (Dietrich et al., 2011; Launay et al., 2019). At the reproductive stage, decreasing water potential during flower senescence in Rosa hybrida L. can trigger the accumulation of proline through increasing P5CS transcription (Kumar et al., 2008; Zhang and Becker, 2015).

Considering the role of ProDH1 genes during dark-induced senescence in Arabidopsis (Launay et al., 2019), and the specific expression of ProDH2 genes in the midveins of source tissues in B. napus (Faës et al., 2015), proline catabolism might contribute to phloem-dependent N remobilization processes in source leaves of B. napus. Such a role would demonstrate that proline metabolism is a novel target for the improvement of N remobilization efficiency in crops. In this work, we addressed the question of the role of proline metabolism in the source leaves at the vegetative stage in the economically important plant crop B. napus. We systematically analysed
the expression of the P5CS and ProDH genes related to the biosynthesis and degradation of proline, and measured the associated net proline fluxes (synthesis vs degradation) using $^{15}$N labelling experiments. Considering that the allopolyploid nature of oilseed rape can confer multiple possibilities of sub-/neofunctionalization of proline metabolism-associated gene copies, we also analysed their expressions during stress conditions (osmotic stress and dark-induced senescence), in order to delimit the regulatory genes of interest. The analysis of the BnaP5CS/BnaProDH transcript balance with respect to the modifications of net proline metabolic fluxes revealed that proline catabolism was weakly activated according to the sink/source balance of the leaves. The depletion of proline content in source leaves was associated with a decrease in the net proline biosynthesis flux and was correlated with decreased expression of five BnaP5CS1 genes. In addition, we identified some BnaP5CS and BnaProDH genes that were differentially expressed during multiple processes (leaf status, dark to light transition, and stress response).

Materials and growth methods

Plant material and growth conditions

Seeds of oilseed rape B. napus genotype Aviso were obtained from our ‘Bracysol’ biological resource centre. After a 3 d germination step on soaked blotting paper, seedlings were transferred in 4 litre pots filled with a non-fertilized commercial substrate (Falentin, reference 9220163F3). Plants were grown in a 6 m$^3$ growth chamber under a 14 h light/10 h dark cycle (22 °C/16 °C) in ambient air (65% and 80% humidity) with a photosynthetically active radiation of 100 µmol photons m$^{-2}$ s$^{-1}$ at the top of the canopy. A commercial fertilized solution (Liquoplant Bleu, 2.5% N, 5% P, 2.5% K) was applied twice a week on plants. All experiments were performed on plants grown for 60 days after sowing (60 DAS), possessing 14 leaf ranks, annotated from the bottom to the top (L3 to L16). The two oldest leaves (L1 and L2) had already fallen off, confirming that the remobilization processes between leaves holding a different sink/source balance were operating.

Chlorophyll and soluble protein contents

Relative chlorophyll quantification in SPAD units was achieved using a non-destructive chlorophyll SPAD-502 meter (Minolta). For protein extraction, samples were freeze-dried for 72 h then ground to a fine powder. Soluble proteins were extracted in a buffer containing 20 mM citrate, 160 mM Na$_2$HPO$_4$ (pH 6.8), and a pinch of polyvinylpyrrolidone powder. Soluble proteins were extracted in a buffer containing 20 mM extraction, samples were freeze-dried for 72 h then ground to a fine powder. Soluble proteins were extracted in a buffer containing 20 mM citrate, 160 mM Na$_2$HPO$_4$ (pH 6.8), and a pinch of polyvinylpyrrolidone. After a 30 min centrifugation step at 4 °C, proteins from the supernatant were quantified using the Bradford reagent with BSA as the standard.

Nitrogen and carbon percentage

N% and C% were determined on 1 mg of lyophilized plant powder and the Dumas combustion method with an NA1500CN Fison instrument (Thermoquest, Runcorn, Cheshire, UK) analyser.

Amino acid content and $^{15}$N labelling

Prior to metabolite extraction, samples were freeze-dried for 72 h, allowing measurement of the dry weight. Metabolites were extracted with a mixture of MTBE/MeOH/H$_2$O, except for n-3-aminobutyric acid (111 µM final concentration in a 0.9 ml MeOH/H$_2$O fraction) which was used as internal standard for amino acid quantification (Salem et al., 2016). A 200 µl aliquot of the remaining MeOH/H$_2$O fraction was evaporated using a SpeedVac concentrator at 35 °C for 2 h. Amino acids were re-suspended in 50 µl of ultrapure water, and 5 µl were derivatized using an AccQTag derivatization kit (Waters) following the manufacturer’s procedures. Separation and UV detection of amino acids was performed on an Acquity UPLC-DAD (Renault et al., 2010). Amino acids were quantified using external calibration curves for each amino acid and normalized with the internal standard. For $^{15}$N experiments, isotopologues (M+0, M+1, M+2, M+3, M+4) from AccQTag-derivatized amino acid were analysed by MS by coupling a triple quadrupole spectrometer (Waters) to the analytical system. The electrospray source was settled in positive mode and each isotopologue was tracked with the Selected Ion Recording function. The settings for the mass spectrometer electrospray source were as follow: 4,4 kV capillary voltage, 35 V cone voltage, 150 °C cone temperature, 450 °C desolvation temperature, and 900 l h$^{-1}$ N flow for desolvation. Derivatization with AccQTag added ~20% of artificial M+1 enrichment for each amino acid, which was corrected for each sample using the M+1 enrichment observed in $^{15}$N-free external standards. Considering an amino acid with n atoms of N, true $^{15}$N labelling (%)$^{15}$N labelling (%) was calculated as:

$$^{15}\text{N labelling} (\%) = \frac{\sum_{x=M}^{M+x} \frac{[M+x]}{[M+x]}}{\sum_{x=M}^{M+x} \frac{[M+x]}{[M+x]} + \sum_{x=M}^{M+x} \frac{[M-x]}{[M-x]}}$$

Net $^{15}$N labelling for each isotopologue was calculated by subtracting the M+x labelling (%) from T0 of the experiment (natural abundancy).

Identification of P5CS genes and phylogenetic analysis

P5CS genes were identified by blasting Arabidopsis thaliana P5CS1 (AT2G39800) and P5CS2 (AT3G35610) coding sequences retrieved from TAIR database (Berardini et al., 2015), respectively, on the available genomes from ENSEMBL or Phytozone database [Arabidopsis lyrata (v.1.0), B. napus var Darmor (AST_PROJE301401assembly 2014), B. rapa (Brapa_1.0), B. oleracea (BOL), Oryza sativa (IRGSP-1.0), Oryza sativa (v1.0), and human (GRCh38.p12)]. Sequence alignments and phylogenetic analysis were performed with MEGA X software (Tamura et al., 2013). The evolutionary phylogenetic gene tree was generated with the maximum likelihood method and the nucleotide substitution model of Tamura–Nei, assuming uniform rates among sites (1000 bootstraps).

Quantitative analysis of transcript levels

Total RNAs were extracted with the NucleoSpin RNA Plus Kit (Machery Nagel) from frozen or lyophilized tissue ground into a fine powder. After RNA extraction, cDNA was synthesized from 1 µg total RNA using the AMV reverse transcriptase kit (Promega). cDNA was then subjected to SYBR Green I qPCR amplification. The qPCR reaction was performed using the 7500 real-time PCR system (Applied Biosystems). mRNA levels were calculated using the ΔΔCt method. Real-time PCR analysis was performed with the following primers:

**Light**:

- **BnaP5CS1**: F: 5'CTTGAATGACTTGGTCGTCATG3', R: 5'AGTTGGCAGGCCTTAATACG3'.
- **BnaP5CS2**: F: 5'ACACCACTTCGGAGTTTAT3', R: 5'GACAGCTAGCTGACTGATG3'.
- **BnaProDH**: F: 5'CTTTGTAGGAGGCACCTTGA3', R: 5'GCTTCAACAGGACACCAATG3'.
- **BnaProDH2**: F: 5'GCCTTAATACG3', R: 5'GACAGCTAGCTGACTGATG3'.

**Dark**:

- **BnaP5CS1**: F: 5'CTTGAATGACTTGGTCGTCATG3', R: 5'AGTTGGCAGGCCTTAATACG3'.
- **BnaP5CS2**: F: 5'ACACCACTTCGGAGTTTAT3', R: 5'GACAGCTAGCTGACTGATG3'.
- **BnaProDH**: F: 5'CTTTGTAGGAGGCACCTTGA3', R: 5'GCTTCAACAGGACACCAATG3'.
- **BnaProDH2**: F: 5'GCCTTAATACG3', R: 5'GACAGCTAGCTGACTGATG3'.

**Leaf discs assays**

Leaf discs (0.8 cm$^2$) were randomly punched with a cork-borer in both laminas of the leaves. For $^{15}$N labelling experiments, leaf discs were floated for 4 h in a 6-well microplate filled with a buffer containing 10 mM MES-KOH (pH 6.5), 10 mM $^{15}$NH$_4$Cl (99%), or $^{15}$N-l-proline (98%). From each leaf, two time points were sampled (0 and 4 h) in order to calculate $^{15}$N incorporation into amino acids before and after the experiment. For hyperosmotic experiments, leaf discs were floated on a buffer containing 5 mM HEPES (pH 6), 1.5 mM CaCl$_2$, and 10 mM KCl supplemented or not with 400 g of PEG 6000 per kg of H$_2$O. This PEG concentration was too low to be detected in our experiments.
powder, following the manufacturer's instructions. Potential DNA traces were removed with DNase using the RQ1 RNase-free DNase (Promega) according to the manufacturer's protocol. The absence of DNA contamination in samples was controlled by PCR amplification of the BnaEF1α reference gene. RNA integrity was verified by running samples on 1.2% agarose gels. A 1 μg aliquot of total RNA treated with the DNase was reverse-transcribed using oligo(dT)₆₈ primer and Gscrypt reverse transcriptase (Promega) according to the manufacturer's instructions. For quantitative PCR (qPCR) experiments, 2 μl of diluted cDNA were mixed with the LightCycler 480 SYBR Green I Master mix (Roche) and primers pairs (see Supplementary Table S2 at JXB online). qPCRs were achieved on a Light Cycler LC480 (Roche) under the following conditions: an initial step at 95 °C for 5 min, then 45 cycles of 95 °C for 15 s and 60 °C for 40 s. Two reference genes [BnaC. UBQ11 (BnaC04g091500D) and BnaC.RibS3 (BnaC04g130400D)] were selected for their expression stability in our samples harvested from different conditions (leaf ranks, light/dark conditions, and stress conditions) using the GeNorm method (Vandesompele et al., 2002). Gene expression was normalized using the geometrical mean expression of two reference genes and the efficiency of qPCR primers (Supplementary Table S2).

The efficiency of qPCR primers was calculated from a serial dilution of mixed samples from our experiments. Fold change (FC) values were calculated as the ratio of the relative gene expression between two consecutive time points for treated (FC treatment) or control (FC control) leaf discs (T0 and PEG, PEG and PEG+rehydration for osmotic stress; T2d and T4d for dark-induced senescence). Log2 (FC treatment/FC control) corresponded to the log2 value of the ratio (FC treatment/FC control).

Statistical analysis

The variability of the results is expressed as the mean ± SD of n independent biological replicates (n=3–6). Means of different leaf ranks were compared together with one-way ANOVA followed by a post-hoc Tukey's HSD test for multiple pairwise comparison. Means of two treatments (light versus dark; control versus dark-induced senescence; control versus osmotic stress) for each leaf rank were compared using a Student t-test (bilateral distribution, equal variance). For statistical analysis of qPCR experiments, relative expression values were log-transformed to obtain normally distributed values. For each test, a P-value <0.05 was applied. All statistical analyses were achieved with "R" software.

Results

Source status of B. napus leaves is associated with a decrease of proline content

In order to obtain B. napus plants with a gradient of leaf ranks having different sink/source balance, plants were grown for 2 months at the vegetative stage in growth chambers to reach the 16-leaf stage (the two oldest leaves had already fallen off). From this stage, we decided to work on four leaf ranks: a young growing leaf (L15), an immature leaf (L11), a post-mature leaf (L7), and a senescent leaf (L3), essentially functioning as a source tissue (Albert et al., 2012). The status of the four different leaf ranks was checked by quantifying different parameters: chlorophyll and soluble protein contents, total carbon (C) and N contents, the expression of the Cab/SAG12 reporter genes, and free amino acid contents (Gombert et al., 2006). First, dry weight per leaf area was decreased according to the sink/source balance of the leaves whereas the fresh weight per leaf area was relatively maintained, leading to an increase of the FW/DW ratio between the different leaf ranks (Supplementary Fig. S2). Therefore, all results were expressed on a dry weight basis to compare the different leaf ranks, except for chlorophyll content which was measured per leaf area. Nevertheless, the observed decreased of chlorophyll content per leaf area for the different leaf ranks might still be relevant by normalizing with dry weight (Fig. 1A; Supplementary Fig. S1). Along the leaf nodal ranks, we observed a significant decrease of the content of total soluble proteins and the total C and N contents (Fig. 1B–D). Since amino acids (and therefore proteins) have a high C/N ratio, the decrease of C and N% per dry weight seemed to be attributed to an activation of protein degradation. These results suggest that both C and N remobilization are strongly induced from the L11 leaf. Those observations were also confirmed by the expression levels of Cab and SAG12 genes. Cab gene expression was significantly and gradually repressed according to the source status of the different leaf ranks, whereas the SAG12 gene was significantly expressed in the L3 leaf, mirroring its actual senescent status (Fig. 1E, F). From the overall free amino acid profiles, glutamine content showed a significant decrease, except for L11 and L7 (Fig. 1G; Supplementary Table S1). Such variations of glutamine content between the different leaf ranks is often attributed to the activation of the remobilization processes (proteolytic degradation and amino acids exported as glutamine) (Orsel et al., 2014; Tegeder and Masclaux-Daubresse, 2018). Consequently, the L15 leaf may have a higher ‘sink’ demand compared with its ‘source’ export. Interestingly, although at a lower amplitude, proline content showed statistically significant variations between the different leaf ranks, similarly to glutamine content variations, thereby suggesting a potential role for proline metabolism in nutrient recycling processes in the sources leaves of B. napus (Fig. 1H). To summarize, we have identified four leaf ranks with gradually increased levels of source status and gradually decreased levels of proline content from the top to the bottom of the plant axis.

Decrease of BnaP5CS1 gene expression in source leaves correlates with a decrease of net proline biosynthesis flux

Next, we decided to analyse the P5CS and ProDH transcript balance according to the sink/source balance of the leaves and during a dark to light transition. These different conditions may solicit proline metabolism for N reallocation, respiration feeding, and/or redox status adjustment. In order to design gene-specific primers for each copy of BnaP5CS (Supplementary Table S2), we performed a genome-wide analysis of P5CS2 genes in B. napus and its progenitors B. rapa and B. oleracea, using known P5CS2 coding sequences from the Arabidopsis genome as a query. Our analysis identified four AtP5CS2 (Bna.P5CS2) orthologues, corresponding to two P5CS2 orthologues in each progenitor genome (B. rapa and B. oleracea) (Supplementary Fig. S1). We also performed similar work for P5CS1 in order to localize the genomic region of the six P5CS1 genes previously identified (Wang et al., 2014). The identified P5CS1 and P5CS2 genes from B. napus, B. rapa, and B. oleracea were correspondingly renamed in this study based on the genome to which they belonged or inherited (A for B. rapa and C for B. oleracea), their chromosome location, and their phylogenetic relationship (Supplementary Fig. S2).
Considering ProDH genes, a previous work already identified six BnaProDH1 and two BnaProDH2 genes and designed specific primers for each gene (Faës et al., 2015).

First, P5CS transcript levels were quantified relative to the reference genes in the four leaf ranks sampled 3 h before or 3 h after the beginning of the illumination period, in order to evaluate the possibly combined effects of the sink/source balance of the leaves and the natural dark to light transition. Transcript levels for four BnaP5CS1 genes were significantly decreased in leaves having a very strong source leaf status compared with the L15 leaf, in accordance with the previously observed decrease in proline content (Figs 1F, 2A–E). The expression levels of BnaA.P5CS1b are not shown since they were too weak to be repeatedly detected in our samples. BnaC.P5CS1c showed the strongest expression levels compared with other BnaP5CS1 genes, suggesting a preponderant role in controlling endogenous proline levels (Fig. 2B). All BnaP5CS2 transcript levels were not significantly changed between the different leaf ranks (Fig. 2F; expression levels for each BnaP5CS2 gene are available in Supplementary Fig. S3). However, all BnaP5CS genes (except BnaA.P5CS1b) were significantly overexpressed in light conditions compared with dark conditions for all the leaf ranks.

In order to connect the variations of BnaP5CS1 transcript levels with the variations of proline content observed in the different leaf ranks, we performed 15N labelling experiments on leaf discs isolated from the four leaf ranks. This experiment aimed at comparing in vivo the capacity for net proline biosynthesis between the different leaf ranks using the repartition of the 15N as a proxy. We took advantage of the leaf capacity to assimilate de novo N using 15NH4 to label glutamate, the major precursor for proline biosynthesis (Fig. 3A). We analysed net 15N enrichment and contents of amino acids using an UPLC-TQD system. After 4 h of 15N labelling in isolated leaf discs, we found that net 15N was in great part allocated to glutamine (Fig. 3A). This allocation was significantly higher in L15 compared with other leaves, showing that L15 has a higher capacity for free ammonia assimilation compared with leaves having a very strong source status. Analysis of 15N labelling in glutamine showed similar values for each leaf rank (no statistical differences), with ~30–35% for the M+1 isotopologue and 50–55% for the M+2 isotopologue (Fig. 3B). The 15N labelling in the glutamate molecule was also statistically identical between each leaf rank, with values ranging between 48% and 60%. Interestingly, the net 15N labelling in the proline molecule was significantly lower between the different leaf ranks (~10% in L15, 7% in L11, and 2–0.5 in L7 and L3). Since the 15N labelling in glutamate was similar between the different leaf ranks, our results suggested that the commitment of de novo assimilated 15N toward proline biosynthesis was lowered in leaves having a very strong source status. To quantitatively evaluate this commitment, we calculated the net 15N allocation to proline by
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assuming that proline commitment toward the protein fraction between the different leaf ranks was negligible (15N labelling was undetectable in the protein fraction) (Fig. 3A). Although the net 15N allocation to proline underestimates the true net proline biosynthesis flux, it is still a reliable tool to compare net flux variations. We found that net 15N allocation to proline was significantly lowered from L15 to L11, L7, and L3, showing values of ~200 nmol 15N g–1 DW 4 h–1 in L15 to drop to 75 in L11 and then nearly 0 in L7/L3 (Fig. 3C). The results were consistent with a decrease of the net proline biosynthesis flux in source leaves. Such a decrease was strongly correlated with the decrease of *BnaP5CS1* transcript levels.

Increase of *BnaA.ProDH2a* transcript levels in source leaves of *B. napus* does not correlate with variations of maximal proline degradation capacity

After analysing the relationship between the net proline biosynthesis capacity and the transcriptional regulation of *P5CS* genes, we focused our work on the proline degradation pathway. Consequently, we quantified ProDH transcript levels with respect to dark–light transition and the sink/source balance of the leaves. Since the *BnaC.ProDH2a* gene has already been reported to be expressed in the midveins of senescent leaves (Faës *et al*., 2015) and as ProDH genes were overexpressed during dark periods in Arabidopsis (Hayashi *et al*., 2000), we also analysed *BnaProDH* gene expression in the midveins of leaves sampled in the dark. Out of the six *BnaProDH1* genes, *BnaA&C.ProDH1a* and *BnaA&C.ProDH1b* genes were the most expressed and were significantly overexpressed in the lamina of leaves L7 and L3 compared with L15 (Fig. 4A–C). This overexpression was dependent on the light/dark condition (*BnaA&C.ProDH1a* genes for light, *BnaA&C.ProDH1b* genes for dark). *BnaA&C.ProDH1c* gene expression was significantly increased in leaves having a very low source status. However, those last genes were only detected in the lamina sampled in the light and were poorly expressed. Considering the two *BnaProDH2* genes, their expression levels were only detected in the lamina sampled in the light. The *BnaA.ProDH2a* gene was the most expressed and was significantly overexpressed in the lamina of the L3 senescing leaves (Fig. 4D, E). No *BnaProDH* gene was overexpressed in the midveins of leaves sampled in the dark compared with other conditions/tissues (Fig. 4). To summarize, *BnaA&C.ProDH1b* and *BnaA.ProDH2a* genes were overexpressed in leaves having a very strong source status, and showed a maximal expression value in the L3 senescent leaves. Since the net 15N allocation to proline was close to 0 in the L3 leaf rank (Fig. 3C), our results suggested that proline degradation could be activated in these tissues. Therefore, we also performed 15N labelling experiments.
on leaf discs isolated from the four leaf ranks using $^{15}\text{N}$proline. This experiment aimed at comparing in vivo the maximal proline degradation capacity between the different leaf ranks, by measuring the incorporation of the $^{15}\text{N}$ (resulting from proline) into other amino acids (Fig. 5A). After 4 h of $^{15}\text{N}$proline labelling in isolated leaf discs, net $^{15}\text{N}$ labelling in the proline molecule (M+1 isotope analogue) was ~87–93% in the different leaf ranks (Fig. 5B). Although this enrichment was significantly lower in the L15 leaf rank compared with the other leaf ranks, the values were relatively similar. This suggested that $^{15}\text{N}$proline might have been similarly incorporated in the leaf discs during the labelling experiment. From the net $^{15}\text{N}$ labelling and quantification of amino acids, we calculated the net $^{15}\text{N}$ allocation from proline to free amino acids (Fig. 5C). We found that net $^{15}\text{N}$ allocation to glutamate and other amino acids (except proline) was significantly reduced from the leaf ranks L15 to L7. However, net $^{15}\text{N}$ allocation to glutamine was significantly higher in L3 compared with other leaf ranks. Considering the total net $^{15}\text{N}$ allocation from proline to free amino acids, our results showed that the maximal proline degradation capacity was decreased from L15 to L7, but was restored in L3 leaves (similarly to L15 leaves). Overall, our results suggest that proline catabolism is weakly activated in leaves having a very strong source status and may not be specifically controlled by variations of BnaProDH transcript levels.

Some BnaP5CS and BnaProDH genes are specifically involved in development and stress response processes

We have shown that multiple copies of P5CS and ProDH genes were differentially expressed according to the sink/source status of the leaves in B. napus. Since the variation of P5CS and ProDH gene expression can control proline contents during some stress conditions, we decided to test whether specific BnaP5CS and BnaProDH genes were differentially expressed during multiple processes (sink/source status, dark to light transition, and stress response). Consequently, we analysed the gene-specific expression of BnaP5CS and BnaProDH genes during two stressing conditions known to regulate proline metabolism: an osmotic shock followed by a recovery period and a dark-induced senescence kinetic experiment (Trotel et al., 1996; Dietrich et al., 2011; Launay et al., 2019). We decided to perform this experiment on the L7 leaf, since its source but not senescing leaf status allows a rapid entry into senescence during the dark-induced senescence experiment.
First, leaf discs from L7 were subjected to a PEG-induced hyperosmotic stress (−1.88 MPa) for 18 h under light conditions, followed by a 6 h recovery phase in a hypo-osmotic medium. The evolution of the leaf disc water content in the PEG treatment condition compared with the control condition confirmed that tissue dehydration occurred during hyperosmotic treatment whereas full tissue rehydration was restored after the recovery phase (Fig. 6A). A strong accumulation...
of proline was observed in the stressed tissues compared with the control after 18 h of PEG treatment. However, after 6 h of recovery, there was no significant decrease in proline content. Out of the four BnaP5CS1 genes detected, the expression of the three genes BnaA.P5CS1c, BnaC.P5CS1b, and BnaC.P5CS1c was significantly highly induced (log2 FC variation >7) during the osmotic shock and was significantly repressed (log2 FC variation less than –4) during the post-stress recovery phase (Fig. 6B). BnaC.P5CS1a gene expression was moderately induced during the stress phase and repressed during the post-stress recovery phase. Surprisingly, we found that BnaC.P5CS2b gene expression was also moderately induced by the osmotic shock, whereas BnaA.P5CS2a gene expression was moderately repressed (Fig. 6C). During the recovery from hyperosmotic stress, BnaA.P5CS2b and BnaC.P5CS2b gene expression was significantly repressed. Regarding BnaProDH genes, only BnaA&.C.ProDH1a and BnaA&.C.ProDH1b gene expression was repressed during osmotic shock then induced during the recovery phase (Fig. 6D). Overall, our results demonstrated that three BnaPCS1 genes were specifically involved in the control of proline content according to the sink/source balance of the leaves and during a dark–light transition and an osmotic stress and recovery. Next, we performed a time-course dark-induced senescence experiment on leaf discs from L7 to explore the differential expression of BnaProDH genes during stress-induced senescence processes. After 2 d of incubation, chlorophyll content was not significantly different in the control compared with the prolonged darkness condition (Fig. 7A). After 4 d, a significant decrease in chlorophyll content was observed in the prolonged darkness condition compared with the control. Concomitantly, transcription of the senescence marker genes BnaA&.C.SAG12-1&2 was strongly
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induced by prolonged darkness from day 2 to day 4 compared with the control (Fig. 7B). Those results confirmed that dark-induced senescence only operated from day 2 to day 4 in our experimental set-up. As a result, except for *BnaA&C.ProDH1c*, all *BnaProDH* genes were significantly induced (log2 FC variation >3) by dark-induced senescence compared with the control (Fig. 7C). Therefore, our results demonstrated that *BnaA&C.ProDH1a* gene expression was specifically controlled during a dark–light transition, an osmotic shock, recovery, and a dark-induced senescence, whereas *BnaA&C.ProDH1b* and *BnaA.ProDH2a* gene expression was specifically controlled by senescence (natural or dark induced).

### Discussion

Proline metabolism plays an essential role in plant adaptation to multiple environmental stress conditions, specifically when they are facing biotic and abiotic stresses (Szabados and Savouré, 2010). The tight control of proline content, generally achieved by regulating the *P5CS/ProDH* balance at the transcriptional level, allows the plants to tolerate the stress condition (Slama et al., 2015). However, proline metabolism can also participate in plant developmental events, by contributing to proline accumulation and/or degradation during specific growth phases. In particular in reproductive organs and tissues with actively dividing cells, proline accumulation was shown to be stimulated (Mattioli et al., 2009; Biancucci et al., 2015). Conversely, at the vegetative stage, several pieces of evidence suggested that proline catabolism could be activated during leaf senescence and source leaf status acquisition in *B. napus* and could fuel mitochondrial respiration (Faës et al., 2015; Clément et al., 2018; Launay et al., 2019). Such a phenomenon of metabolic reconversion of proline can be of functional interest for N recycling and nutrient remobilization processes through the phloem only if it is adequately fed with proline arising from neosynthesis or proteolysis.

In this study, we have shown that four *BnaP5CS1* genes were underexpressed in leaves having a very strong source status during the vegetative growth phase, whereas transcript levels of two *BnaProDH* genes were moderately overexpressed in the L3 senescent leaves (Figs 2B–E, 4B, D). These changes in transcript levels were strongly correlated with the depletion in proline content and with the reduction in commitment of *de novo* assimilated N towards proline biosynthesis, whereas maximal proline degradation capacity was not different between L15 (very low source status) and L3 (very strong source status) leaves (Figs 1H, 3C, 5C). We also found that transcription of three *BnaP5CS1* genes was strongly induced during an osmotic stress, and was repressed during the post-recovery phase (Fig. 6B). In particular, these changes were associated with strong variations of proline contents (Fig. 6A).

In Arabidopsis, during an osmotic stress and the post-recovery phase, the modulation of proline content was associated with the overexpression of the *P5CS1* gene, whereas expression of the *P5CR* gene was not changed at all (Sharma and Verslues, 2010). Indeed, *P5CR* from Arabidopsis has a higher protein stability compared with *P5CR* from *Escherichia coli*, which may explain why this gene is less transcribed (Funck et al., 2012). Therefore, our results suggest that the variations of *P5CS* transcript levels in *B. napus* may actively affect the biosynthesis of proline.

The specific control of proline content by the transcript levels of *BnaP5CS1* genes according to the sink/source status of the leaves may be explained by different physiological processes. First, growth and development of young leaves requires protein biosynthesis whereas old leaves having a very strong...
source status degrade their proteins and recycle their macromolecules. Increasing proline biosynthesis in young leaves compared with old source leaves could support the demand for proline to synthesize proteins in young leaves and to fuel high-energy-consuming cell division processes (Kavi Kishor and Sreenivasulu, 2014). Secondly, old leaves having a very strong source status are more hydrated than young leaves (Musse et al., 2013; Sorin et al., 2015). We have shown that the transcription of BnaP5CS1 genes was decreased during tissue hydration after a hyperosmotic shock. Therefore, the decrease of net proline biosynthesis flux in source leaves may be a side effect of the tissue hydration. In addition to these factors, BnaP5CS1 may also be down-regulated by other factors, including a cross-talk between senescence and the redox balance, regarding its role performed in Arabidopsis (Hayashi et al., 2019).

Concerning the sub-/neofunctionalization of proline metabolism-associated gene copies, we have shown that some BnaP5CS1 and BnaProDH genes shared conserved multiple functions in B. napus. The expression of three out of six BnaP5CS1 genes was controlled by both the sink/source status of the leaf and osmotic stress (Figs 2B, D, E, 6B), and the expression of four out of six ProDH1 genes was controlled by both osmotic stress and dark-induced senescence (Figs 6D, 7C). Since parental ancestors of oilseed rape have undergone a whole-genome triplication events (Girousse et al., 1996; Albert et al., 2012). To date, no cell to cell proline transporter has been identified to play a role in proline remobilization at the vegetative stage in Brassica species. However, one proline-specific transporter (AtProT2) and one amino acid permease (AAP1) can both contribute to proline uptake under normal or stress conditions (Lehmann et al., 2011; Wang et al., 2017).

Concerning the sub-/neofunctionalization of proline metabolism-associated gene copies, we have shown that some BnaP5CS1 and BnaProDH genes shared conserved multiple functions in B. napus. The expression of three out of six BnaP5CS1 genes was controlled by both the sink/source status of the leaf and osmotic stress (Figs 2B, D, E, 6B), and the expression of four out of six ProDH1 genes was controlled by both osmotic stress and dark-induced senescence (Figs 6D, 7C). Since parental ancestors of oilseed rape have undergone a whole-genome triplication
event, the presence of multiple copies was expected to relax natural and artificial selective pressures on any individual gene copy, thereby facilitating the emergence of novel gene function (Conant and Wolfe, 2008). In the oilseed rape genome, duplicated flowering time genes were shown to be preferentially retained compared with other genes, and ~70% of them had a divergent expression patterns relative to each other across development, suggesting neo- or subfunctionalization (Jones et al., 2018). Such conserved multifunctionalization of P5CS and ProDH genes in *B. napus* clearly reflects the importance of finely controlling both phases of proline metabolism (biosynthesis and degradation) to face issues related to both development and stress conditions.

In conclusion, we have shown that the depletion of proline content in source leaves in *B. napus* was associated with a decrease in the net proline biosynthesis flux rather than an activation of proline catabolism. Such flux variations were correlated with the strong underexpression of four *BnaP5CS1* genes, whereas the expression of three *BnaDH* genes was moderately increased in source leaves to senescent leaves. Since *BnaProDH* genes were strongly overexpressed during dark-induced senescence (C starvation condition), our results suggested that proline catabolism may play a role in *B. napus* leaves but during late stages of senescence, perhaps to contribute to proline-dependent mitochondrial respiration. Consequently, the proline arising from protein degradation in source leaves might be remobilized toward young leaves and sink tissues through the phloem. Recent works showed that N use efficiency and seed yields could be dramatically increased in crops by enhancing the expression of the amino acid transporter AAP1, which can contribute to proline uptake (Perchlík and Tegeder, 2017; Wang et al., 2017). Future research should focus on the possible source–sink proline transport at the vegetative stage to identify potential new targets for improvement of N use efficiency.

**Supplementary data**

Supplementary data are available at JXB online.

Fig. S1. FW and DW per leaf area and FW/DW ratio of the four leaf ranks having a different sink/source balance (L15, L11, L7, L3).

Fig. S2. Molecular phylogenetic analysis of the *Brassica napus* P5CS coding sequences.

Fig. S3. Relative expression for each *BnaP5CS2* gene in four leaf ranks having a different sink/source balance (L15, L11, L7, L3) harvested under either dark or light conditions.

Table S1. Amino acid contents in the laminae of four leaf ranks having a different sink/source balance (L15, L11, L7, L3) sampled 3 h after the beginning of the illumination period.

Table S2. List of primers used in this study for qPCR analysis and primer efficiency.

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