Sixteen cytosolic glutamine synthetase genes identified in the *Brassica napus* L. genome are differentially regulated depending on nitrogen regimes and leaf senescence

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Abstract

A total of 16 *BnaGLN1* genes coding for cytosolic glutamine synthetase isoforms (EC 6.3.1.2.) were found in the *Brassica napus* genome. The total number of *BnaGLN1* genes, their phylogenetic relationships, and genetic locations are in agreement with the evolutionary history of *Brassica* species. Two *BnaGLN1.1*, two *BnaGLN1.2*, six *BnaGLN1.3*, four *BnaGLN1.4*, and two *BnaGLN1.5* genes were found and named according to the standardized nomenclature for the *Brassica* genus. Gene expression showed conserved responses to nitrogen availability and leaf senescence among the Brassiceae tribe. The *BnaGLN1.1* and *BnaGLN1.4* families are overexpressed during leaf senescence and in response to nitrogen limitation. The *BnaGLN1.2* family is up-regulated under high nitrogen regimes. The members of the *BnaGLN1.3* family are not affected by nitrogen availability and are more expressed in stems than in leaves. Expression of the two *BnaGLN1.5* genes is almost undetectable in vegetative tissues. Regulations arising from plant interactions with their environment (such as nitrogen resources), final architecture, and therefore sink–source relations *in planta*, seem to be globally conserved between *Arabidopsis* and *B. napus*. Similarities of the coding sequence (CDS) and protein sequences, expression profiles, response to nitrogen availability, and ageing suggest that the roles of the different *GLN1* families have been conserved among the Brassiceae tribe. These findings are encouraging the transfer of knowledge from the *Arabidopsis* model plant to the *B. napus* crop plant. They are of special interest when considering the role of glutamine synthetase in crop yield and grain quality in maize and wheat.

**Key words:** Alloploidization, *Brassica napus*, *Brassica oleracea*, *Brassica rapa*, nitrogen metabolism, nitrogen remobilization, senescence.

Abbreviations: GS, glutamine synthetase; GLN1, cytosolic glutamine synthetase gene; GSL, chloroplastic glutamine synthetase gene.

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Winter oilseed rape (*Brassica napus* L.) is the dominant oilseed crop in northern Europe, and nitrogen (N) fertilization is the main operational cost for farmers (50% of the total cost of production). When compared with other crops, oilseed rape is characterized by low nitrogen use efficiency (NUE) of production. When compared with other crops, oilseed is the main operational cost for farmers (50% of the total cost).

**Introduction**

Oilseed rape is characterized by early leaf shedding and unusual high N loss in senescing falling leaves. The plant can lose up to 15% of its entire N content in this way (Rossato et al., 2001). Leaf senescence generally corresponds to the mobilization of N reserves from source leaves to sink organs such as seeds (Masclaux-Daubresse et al., 2008). In oilseed rape, it has been shown that N can be remobilized from senescing leaves to expanding leaves at the vegetative stage (sequential senescence) as well as from senescing leaves to seeds at the reproductive stage (monocarpic senescence) (Malagoli et al., 2005).

The rate of senescence and remobilization of leaf N are related to the N nutrition status of the plant and to source–sink relations (Masclaux et al., 2000). N remobilization towards new developing organs is largely dependent on senescence-related catabolism events and translocation of leaf N compounds. Amino acids derived from protein catabolism are exported via the phloem to growing parts of the plant; the concentration of amino acids in the phloem sap increases during leaf senescence (Herrera-Rodriguez et al., 2006, 2007; Masclaux-Daubresse et al., 2006). In many species including *B. napus*, aspartate, glutamate, and their corresponding amides are the principal forms of amino N compounds transported in the phloem and play a key role in rendering N available for remobilization from senescing leaves (Tilsner et al., 2005). Enzymes involved in the biosynthesis and metabolism of amino acids destined for phloem loading are of special interest.

In plants, glutamine synthetase (GS; EC 6.3.1.2) is a key enzyme which catalyses an ATP-dependent conversion of glutamate to glutamine using ammonium derived from primary N uptake and various internal N recycling pathways including catabolic release of ammonium during senescence (Bernard and Habash, 2009). In a large variety of plants, induction of cytosolic glutamine synthetase (GS1) genes has been detected during leaf senescence, while chloroplastic synthetase isoenzyme (GS2) expression decreases (Masclaux et al., 2000; Guo et al., 2004; Martin et al., 2006). It has been proposed that in young photosynthetic leaves, the chloroplastic isoenzyme GS2 is mainly involved in the assimilation of ammonium provided by nitrate reduction and photorespiration through the GS/GOGAT cycle (Masclaux et al., 2001). In old senescing leaves, as chloroplasts are breaking down, glutamine to be exported would be synthesized by the newly expressed cytosolic GS1 isoforms (Masclaux-Daubresse et al., 2006).

The importance of GS1 in N management, growth rate, leaf senescence onset and severity, yield, and grain filling has been confirmed by co-location of quantitative trait loci (QTLs) and functional genomics approaches mainly performed on maize (Hirel et al., 2001; Martin et al., 2006) and rice (Tabuchi et al., 2005). In maize, *Gln1.4* is up-regulated during senescence (Martin et al., 2005). The *Gln1.4* knockout mutation led to a dysfunction in N export and a sharp reduction of kernel yield (Martin et al., 2006). *GLN1.4* was proposed to be involved in re-assimilation of ammonium released during leaf protein degradation. In rice, mutants lacking *OsGS1;1* are severely impaired in growth rate and grain filling, and glutamine levels in mutant leaf blades are reduced (Tabuchi et al., 2005). As the gene product is located in companion cells and parenchyma cells of leaf tissues, it has been proposed that *OsGS1;1* is responsible for generation of glutamine for remobilization via the phloem.

To date, all studies on plant genomes have revealed multi-genic families coding for several GS1 isoforms. In rice, three *GLN1* genes have been identified, with seven in wheat, five in maize, and five in *Arabidopsis thaliana*. Transcriptomic data showed that three *A. thaliana* genes, *AtGLN1.1*, *AtGLN1.2*, and *AtGLN1.4*, are induced during leaf ageing (Guo et al., 2004). Promoter::GFP (green fluorescent protein) fusions were used to investigate *AtGLN1* gene expression in roots. *AtGLN1.1* was localized at the root surface layer, whereas *AtGLN1.2*, *AtGLN1.3*, and *AtGLN1.4* were expressed in root vascular tissues (Ishiyama et al., 2004). Detailed expression of *AtGLN1* in leaves was only reported for *AtGLN1.2* that is induced in root and leaf tissues under a high N regime and is mainly expressed in veins and mesophyll cells in older leaf tissues (Lothier et al., 2011). In veins, *AtGLN1.2* protein was localized in the companion cells. The knock-out mutant phenotype led to the conclusion that *AtGLN1.2* is essential for N assimilation under ample nitrate supply and for ammonium detoxification (Lothier et al., 2011). For all plant species, it is clear that not all GS1 isoforms participate equally in N management and remobilization. Regulation of expression is then a key clue towards the identification of *GLN1* genes potentially involved in N remobilization.

Accumulation of GS1 and a decrease in GS2 polypeptides were observed in *B. napus* leaves after onset of leaf senescence (Ochs et al., 1999). Up to now, four closely related genes coding for GS1 isoenzymes, *BnGSR1-1*, *BnGSR1-2*, *BnGSR2-1*, and *BnGSR2-2*, have been identified using *B. napus* root-derived expressed sequence tag (EST) libraries (Ochs et al., 1999). Analysis of different tissue types has also revealed that these genes are expressed in senescing leaves (Buchanan-Wollaston and Ainsworth, 1997). Recent studies of Brassicaceae genomes show that the genome of *B. napus*, which is a recent allotetraploid (2n=4x=38, AACC) arising from the natural hybridization of monogenomic diploids *Brassica rapa* (AA) and *Brassica oleracea* (CC) (Nagaharu, 1935), contains additional genes coding for GS1 isoenzymes. Analysis of *Brassica* lineage genomes revealed that a whole-genome triplication occurred shortly after their divergence from *Arabidopsis* (Parkin et al., 2005). Therefore, gene families are more frequent, larger, and more complex in *B. napus*.
than in *A. thaliana*. Brassicaceae genome sequences are also highly conserved and many synthetic regions have been identified (Paterson et al., 2001; Parkin et al., 2005; Schranz et al., 2006), allowing the identification of ‘true’ orthologous genes between *A. thaliana* and *B. napus*.

In the present study, advantage is taken of the Brassicaceae genome structure and of its recent sequencing (published in order to identify all *BnaGLN1* genes coding for GS1 iso-enzymes. It is demonstrated that they are differentially regulated depending on tissue type, senescence, and N availability. The potential role of the *BnaGLN1* genes in N remobilization during leaf senescence, the impact of whole-genome duplications and merging on the evolution of the *GLN1* multigenic family in the Brassicaceae tribe, and strategies based on knowledge transfer from *A. thaliana* to crop plants are discussed.

### Materials and methods

#### Brassica gene identification

Sequence searches by similarity to *A. thaliana* AtGLN1 coding sequences were performed in the GenBank and Genoplante databases using the BLAST algorithm (Altschul and Lipman, 1990) and the *A. thaliana* AtGLN1 coding sequences AtGLN1.1 (NM_123119, At5g37600), AtGLN1.2 (NM_105291, At1g66200), AtGLN1.3 (NM_112663, At3g17820), AtGLN1.4 (NM_121663, At5g16570), and AtGLN1.5 (NM_103743, At1g48470). The BlastN option was used to recover *B. napus*, *B. rapa*, and *B. oleracea* ESTs and complete mRNAs as well as genomic sequences of *B. napus* and *B. rapa* (Cheng et al., 2011; http://brassicadb.org/bradi/). Contigs of ESTs were built using the CAP3 algorithm (Huang and Madan, 1999) and validated through multiple sequence alignments with ESTs and AtGLN1 coding sequences, allowing a manual proofreading. Alignments were generated with the ClustalW algorithm (Thompson et al., 1994) using Clustalw-sequence and Clustalw-profile options available at the MOBYLE platform (http://mobyle.pasteur.fr). ESTs included in each contig are listed in Supplementary Data File 1 available at *JXB* online.

*BnaGLN1* contigs were then enriched and/or their coding sequence completed with new cDNA sequences: clones from Genoplante oilseed rape cDNA libraries and the ADIZ-MPIZ 021 library corresponding to ESTs were sequenced when available (Supplementary Table S1 at *JXB* online). When the coding sequence from cDNAs was incomplete or no clone was available, specific primers were designed to clone the total or missing coding region (Supplementary Table S2). The amplified fragments were cloned into pGEM-T Easy plasmids (Promega) according to the recommendation of the supplier, and sequenced. Universal T6 and SP7 primers, as well as specific primers were used to sequence the clones on the positive and negative strands (Supplementary Table S3). All DNA sequencing was performed by Cogenics (Grenoble, France) and sequences were submitted to GenBank (accession numbers are given in Supplementary Table S1).

#### Sequence analysis

A global alignment of coding sequences from mRNA, inferred coding sequences, or newly created contigs from ESTs was generated with ClustalW (Thompson et al., 1994). Distance matrices were computed using the Dnadist algorithm with a Kimura 2 nucleotide substitution model, and bootstrap analysis was performed with 1000 iterations. A consensus unrooted tree was then generated using the Neighbor-Joining method. All algorithms are contained in the Phylip 3.67 package available at the MOBYLE platform (http://mobyle.pasteur.fr). The NCBI Conserved Domain Database (Marchler-Bauer et al., 2011) was searched with translated *B. napus* and *A. thaliana* GLN1-coding sequences. A multiple protein sequence alignment was generated with the ClustalW algorithm.

#### Genetic mapping and genome or chromosome assignment

Genetic mapping of *BnaGLN1* genes was realized using three different *B. napus* double haploid (DH) populations. The Stellar×Drakkar (SD), Darmor×Samourai (DS), and Darmor-bzh×Yudal (DY) populations consist, respectively, of 94, 134, and 445 genotype DH lines described by Lombard and Delourme (2001) and Delourme et al. (2006). Gene-specific primers were designed and selected for a presence/absence polymorphism in one of the three populations (Supplementary Table S2 at *JXB* online). Linkage analyses were performed as previously described by Auger et al. (2009) using MAPMAKER/EXP version 3.0b (Lander et al., 1987) and framework maps constructed in Lombard and Delourme (2001) and updated in Delourme et al. (2006). *BnaGLN1* genes were assigned to a linkage group using the ASSIGN command (LOD threshold=8.0) and then placed in the most confident interval with the PLACE command (LOD threshold=2.0). Recombination frequencies were converted into centiMorgans (cM) with the Kosambi function (Kosambi, 1944).

*BnaGLN1* gene assignment to A or C Brassica genomes was performed using a panel of diverse *B. napus*, *B. oleracea*, and *B. rapa* genotypes available in the authors’ group. The panel contains genomic DNA from *B. napus* genotypes Darmor-bzh, Yudal, Stellar, Drakkar, Samourai, aburamassari, Aviso, Tenor, Express, Montego; *B. rapa* genotypes Z1, C1.3, Chifu; and *B. oleracea* genotypes HDEM and C102. This panel was PCR screened with specific but not polymorphic gene markers (Supplementary Table S2 at *JXB* online).

#### Plant material and growth conditions

*Brassica napus* L. plants from the Darmor-bzh genotype were grown in a greenhouse at INRA Versailles, France. Seeds were sown on sand and watered with nutritive solution during 2 weeks in order to allow germination and subsequent growth of plantlets. When the first two true leaves appeared, plantlets were transferred into pots containing sand and were separated into two groups with contrasting N fertilization, setting the potential yield for LN and HN regimes at 20 and 50 m–2, respectively according to Albert et al. (2012). At 56 d after sowing, four plants of each nutrition regime were harvested and sampled. For each plant, all leaf ranks were collected: primary and secondary veins were separated from the rest of the leaf, described as the limb. All fresh samples were frozen immediately in liquid nitrogen and stored at −80 °C.

*Brassica napus* L. plants from the Express genotype were grown in field trials in 2009–2010, in Le Rheu (Brittany), France. Seeds were sown on 7 September 2009 with plant density set at 40 plants m–2. The field trial was conducted with contrasting N fertilization regimes. Plant N status was monitored over the vegetative stage by calculating the nitrogen nutrition index (NNI) (Colnenne et al., 1998). The balance-sheet method was used as a decision tool for N fertilization, setting the potential yield for LN and HN regimes at 20_q ha–1 and 35_q ha–1, respectively (Makowski et al., 2005). LN plants did not receive N fertilizer, while the HN plants received a total input of 110kg N ha–1 spread at two different times (12 February and 19 March 2010). LN and HN plants were harvested, respectively, on 9 and 12 April, at the beginning of the flowering period when half the plants of the plot had their first flowers open on the main stem (F1,
or 60 on the BBCH scale), and 400 degree-days later (base 0) on 17 and 20 May at the beginning of the seed filling period (G2, or 71–73 on the BBCH scale).

Plants from 0.5 m² per plot (~20 plants) were harvested in the early morning and sampled during the subsequent hour. For each batch, plants were ranked according to their length and developmental stage: the six median plants were selected for sampling. On the main stem, the lowest leaf starting to yellow (Old) and the highest leaf at least 5 cm long (Young) were selected, the petiole and main vein were removed, and limbs were sampled. The stems above young and old leaf insertions were selected and sampled over 2 cm and 4 cm, respectively. All fresh samples were frozen immediately in liquid nitrogen and stored at −80 °C.

Nucleic acid manipulation

PCR reactions were conducted in a 20 μl mixture containing 2–10 ng of DNA, 0.25 mM dNTPs (Promega), 0.5 μM of each primer (Eurogentec, Angers, France), and 0.5 U of Taq DNA polymerase (Promega) in the appropriate buffer supplemented with 2.5 mM MgCl₂. The amplification program was run on a PTC-225 thermocycler (MJ Research, Waltham, MA, USA) with the following conditions: 35 cycles of denaturation at 94 °C for 30 s (3 min for the first cycle), annealing at 55–60 °C for 30 s, and elongation at 72 °C for 1–2 min (10 min for the last cycle).

Total RNAs were extracted with the SV Total RNA Isolation System (Promega) from 70 mg fresh weight (FW) of ground frozen tissue. First, samples were homogenized in the RNA lysis buffer (400 μl) using TissueLyserII from Qiagen. Then, all cell debris was eliminated by filtering the lysate through a ‘Nucleospin 96 RNA filter Plate’ (Macherey-Nagel). The manufacturer’s protocol was then followed. In order to remove any remaining DNA traces, 1.5 μg of RNA was treated with DNase using the Turbo DNA-free kit (Ambion) according to the manufacturer’s protocol. The quality of RNA was assessed by an electrophoresis on agarose gel (1.3%, w/v), and the absence of DNA contamination in samples was confirmed by PCR amplification. First-strand cDNA was synthesized using 2 μg of total RNA with oligo(dT)₁₂–₁₈ primers and SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Two independent reverse transcription reactions were performed as technical replicates. cDNA samples were diluted 26-fold with sterile water before use.

Gene expression analysis with qPCR

Quantitative PCR (qPCRs) were set up with the LightCycler 480 SYBR Green I Master mix (Roche Diagnostics) and 4 μl of diluted cDNA in a final volume of 12 μl. The concentration of specific forward and reverse primers was set at 0.42 μM. qPCRs were run on a Light Cycler LC480 (Roche Diagnostics) under the following conditions: an initial step at 95 °C for 10 min, then 50 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 20 s.

Primer pairs were designed for short and specific amplification of individual members of the multigene BnaGLN1 family and five reference genes (Supplementary Table S4 at JXB online). Identification of B. napus reference genes for reverse transcription–qPCR analysis was based on EST sequence similarity to A. thaliana genes with verified expression stability over a wide range of tissues and growing conditions. Arabidopsis thaliana genes were selected from among the list established by Czechowski et al. (2005). Their respective coding sequences were used to retrieve highly similar B. napus ESTs from GenBank using the BlastN algorithm. One B. napus EST per reference gene was selected to design qPCR primers (Supplementary Table S4).

For each run, single product amplification was confirmed by melt curve analysis. PCR products from each primer pair and genotype were sequenced in a preliminary analysis. The amplification efficiency was assessed for each genotype, with each primer pair using a dilution curve method over six orders of magnitude, on a pool of cDNAs from different tissues and modalities. Selected primer pairs have efficiencies >1.8.

The results reported were obtained from four biological replicates and two reverse transcriptions as technical replicates. All samples, including reverse transcription and biological replicates, were run at the same time for each primer pair. Raw fluorescence data were collected and analysed with the R package ‘qpcR’ (Ritz and Spiess, 2008). The ‘perbatch’ function was used to select sigmoid models for the fluorescence curves and then allowing the determination of the intrinsic amplification efficiency (sig.eff) and threshold cycle (sig.CpD2) at the second derivative maximum (Rutledge, 2004). For each run, cDNA relative quantity (RQ) was calculated using the efficiency mean value from the two technical and the four biological repetitions (mean sig.eff), and the run-specific threshold cycle as: RQ=1/(mean sig.eff) (sig.CpD2).

The most stable reference genes were selected using the GeNorm method from Vandesompele et al. (2002) available through the ‘SLqPCR’ R package (Dr Matthias Kohl SIRS-Lab GmbH). The four reference genes BnaX.PFB, BnaX.SAND, BnaX.PP2A, and BnaX.UFC21 from the five tested were retained with an average M value equal to 0.33. For each cDNA sample, a normalization factor (NF) was calculated as the geometrical mean of RQ from the four selected genes, and normalized RQ (NRQ) was then calculated as NRQ=RQ/NF. The mean of both technical replicates was then calculated for each sample.

Results

Identification of GLN1 coding sequences in EST databases of Brassica napus and its progenitors

Brassica oleracea and Brassica rapa

A total of 588 B. napus ESTs, 126 B. rapa ESTs, and 36 B. oleracea ESTs highly similar to one or several of the five AtGLN1.1–AtGLN1.5 mRNA-coding sequences from A. thaliana were isolated from public and private Genoplante databases (Supplementary Data File S1 at JXB online). Sequence assembly and alignments with AtGLN1 mRNA sequences revealed different groups of transcripts that allowed ESTs to be grouped and 16 individual contigs that might correspond to different BnaGLN1 genes of B. napus to be extracted (Supplementary Data File S2). Eight contigs for B. rapa and seven contigs for B. oleracea were also isolated (Supplementary Data Files S1, S2). The BnaGLN1, BnaGLN1, and BolGLN1 contigs from B. napus, B. rapa, and B. oleracea, respectively, show high levels of sequence similarity with all the five AtGLN1 genes (Table 1). The analysis of similarity levels and a phylogenetic tree (Fig. 1) allowed homologous sequences for each AtGLN1 mRNA to be clearly identified in B. napus, B. rapa, and B. oleracea. The phylogenetic tree reveals that the GLN1 mRNA sequences are divided into two distinct groups for monocotyledonous (wheat, maize, and rice) and dicotyledonous species. The Brassicaceae sequences are divided into five clusters, each one including one A. thaliana and one or several B. napus, B. oleracea, and B. rapa sequences (Fig. 1). Each B. napus sequence is closely related to one sequence from either B. oleracea or B. rapa progenitors, illustrating the ancestral relationship with the A and C Brassica genomes. Each B. napus sequence is also closely related to one of the five A. thaliana AtGLN1 mRNA sequences, allowing the identification of homologous related sequences between the
Table 1. *BnaGLN1* proteins and nucleotide sequence identities

The percentage identity within the *BnaGLN1* and *AtGLN1* family between contig nucleotide coding sequences (top right), and between translated protein sequences (bottom left). Contig names are used for the column index and protein names for the line index.

| AtGLN1.1 | BnaGLN1.1_C1 | BnaGLN1.1_C2 | BnaGLN1.1_C3 | BnaGLN1.1_C4 | BnaGLN1.1_C5 | BnaGLN1.1_C6 |
|----------|--------------|--------------|--------------|--------------|--------------|--------------|
| AtGLN1.2 | 92.2         | 92.2         | 91.1         | 91.4         | 91.8         | 78.1         |
| BnaGLN1.1 | 92.7         | 93.9         | 92.7         | 95.5         | 99.1         | 77.7         |
| BnaGLN1.1 | 93.3         | 93.3         | 93.3         | 95.5         | 99.4         | 78.1         |
| AtGLN1.3 | 85.8         | 86.3         | 85.8         | 83.4         | 84.9         | 84.9         |
| BnaGLN1.3 | 84.6         | 85.5         | 85.2         | 83.8         | 84.3         | 84.4         |
| BnaGLN1.3 | 84.6         | 85.5         | 85.2         | 83.8         | 84.3         | 84.4         |
| BnaGLN1.3 | 83.2         | 84.4         | 83.5         | 83.0         | 84.1         | 83.5         |
| BnaGLN1.3 | 83.2         | 84.4         | 83.5         | 83.0         | 84.1         | 83.5         |
| BnaGLN1.3 | 83.8         | 85.2         | 84.9         | 82.7         | 83.2         | 83.2         |
| BnaGLN1.3 | 83.2         | 86.4         | 84.4         | 82.1         | 82.7         | 82.7         |
| AtGLN1.4 | 88.5         | 87.4         | 87.7         | 86.6         | 87.4         | 88.0         |
| BnaGLN1.4 | 88.5         | 88.3         | 88.5         | 88.0         | 88.3         | 88.8         |
| BnaGLN1.4 | 86.0         | 87.7         | 88.0         | 87.9         | 88.0         | 88.5         |
| BnaGLN1.4 | 89.1         | 88.9         | 88.3         | 88.0         | 88.5         | 89.1         |
| BnaGLN1.4 | 88.5         | 89.0         | 88.3         | 87.7         | 88.5         | 89.1         |
| AtGLN1.5 | 78.8         | 80.4         | 80.4         | 78.8         | 79.1         | 79.6         |
| BnaGLN1.5 | 79.6         | 80.4         | 80.4         | 78.8         | 79.1         | 79.6         |

Light grey indicates percentage identity between *A. thaliana* and *B. napus* orthologous genes.
Dark grey indicates identity between *B. napus* homeologous genes.
Identities >95% are in bold.
four species. The contigs were then named according to the AtGLN1.x (x from 1–5) gene with the highest sequence similarity. Since for each AtGLN1.x sequence several BnaGLN1 sequences were found, contig names were also extended by a copy number (Cn): BnaGLN1.x_Cn (Tables 1, 12; Fig. 1). Similarly, the names of the B. rapa and B. oleracea contigs follow the same rules (BraGLN1.x_Cn and BolGLN1.x_Cn, respectively; Table 2).

Most of the EST assemblies have been confirmed through sequencing partial or full-length cDNA clones when available (Supplementary Table S1 at JXB online). For the few EST assemblies that cannot be confirmed in this way, the cloning of missing coding sequences (CDS) was performed by designing primers from the B. rapa and B. oleracea homologous contig sequences (see the Materials and methods). This allowed the completion of the BnaGLN1.4_C3 and BnaGLN1.4_C4 sequences.
Table 2. Names of contigs of ESTs, mRNA, and AtGLN1 homologous genes in Brassica napus, B. oleracea, and B. rapa

The names of genes encoding each contig were assigned according to the Ostergaard and King (2008) nomenclature, taking into account A or C genome location, and the closest AtGLN1 sequence homology.

| A. thaliana | Brassica napus | Brassica oleracea | Brassica rapa |
|------------|----------------|------------------|--------------|
| Gene name  | Name of contig of ESTs; mRNA name | Gene name | Name of contig of ESTs; mRNA name | Gene name |
| AtGLN1.1 | BnaGLN1.1_C1; X82997 (BnGSR2.1) | BnaA.GLN1.1.a | BraGLN1.1_C1 | BnaA.GLN1.1.a |
| (At5g37600) | BnaGLN1.1_C2; Y12460 (BnGSR2.2) | BnaC.GLN1.1.a | BoGLN1.1_C1 | BnaC.GLN1.1.a |
| AtGLN1.2 | BnaGLN1.2_C1; X76736 (BnGSR1.1) | BnaA.GLN1.2.a | BraGLN1.2_C1; EU499383; AT3G73089 | BnaA.GLN1.2.a |
| (At1g66200) | BnaGLN1.2_C2; Y12459 (BnGSR1.2) | BnaC.GLN1.2.a | BoGLN1.2_C1; EU822334; EU822335 | BnaC.GLN1.2.a |
| AtGLN1.3 | BnaGLN1.3_C2; JK036693 | BnaA.GLN1.3.a | BraGLN1.3_C1 | BnaA.GLN1.3.a |
| (At3g17820) | BnaGLN1.3_C1; JK036690 | BnaA.GLN1.3.a | BraGLN1.3_C1 | BnaA.GLN1.3.a |
|  | BnaGLN1.3_C4 | BnaA.GLN1.3.b | BraGLN1.3_C1 | BnaA.GLN1.3.b |
|  | BnaGLN1.3_C3 | BnaA.GLN1.3.b | BraGLN1.3_C1 | BnaA.GLN1.3.b |
|  | BnaGLN1.3_C5; JK036694 | BnaA.GLN1.3.c | BraGLN1.3_C2* | BnaA.GLN1.3.c |
|  | BnaGLN1.3_C6 | BnaC.GLN1.3.c | BoGLN1.3_C2* | BnaC.GLN1.3.c |
| AtGLN1.4 | BnaGLN1.4_C1; JK036697; JK036692* | BnaA.GLN1.4.a | BraGLN1.4_C1 | BnaA.GLN1.4.a |
| (At5g16570) | BnaGLN1.4_C2; JK036695 | BnaA.GLN1.4.a | BraGLN1.4_C1 | BnaA.GLN1.4.a |
|  | BnaGLN1.4_C4; JK036700* | BnaA.GLN1.4.b | BraGLN1.4_C2 | BnaA.GLN1.4.b |
|  | BnaGLN1.4_C3; JK036698* | BnaA.GLN1.4.b | BraGLN1.4_C2 | BnaA.GLN1.4.b |
| AtGLN1.5 | BnaGLN1.5_C2 | BnaA.GLN1.5.a | BraGLN1.5_C1* | BnaA.GLN1.5.a |
| (At1g48470) | BnaGLN1.5_C1; JK036691* | BnaA.GLN1.5.a | BraGLN1.5_C1* | BnaA.GLN1.5.a |

* Annotation and localization on the linkage group from BRAD, the Brassica rapa genome sequencing project consortium (Wang et al., 2011).
* Incomplete CDS sequence when compared with the A. thaliana CDS.
* SNP insertion disrupting the ORF when compared with the A. thaliana CDS reference sequence and the BnaGLN1 contig.

Except for the four BnaGLN1.1_C1, BnaGLN1.1_C2, BnaGLN1.2_C1, and BnaGLN1.2_C2 contigs, no other BnaGLN1 sequences have ever been described previously in the literature or reported in databases as glutamine synthetase gene products. While the BnaGLN1.1_C1, BnaGLN1.1_C2, BnaGLN1.2_C1, and BnaGLN1.2_C2 sequences have been identified as BnGSR2-1, BnGSR2-2, BnGSR1-1, and BnGSR1-2 mRNA, respectively (Table 2; Ochs et al., 1999), contig analysis allowed the completion of the 3′ end (untranslated region and coding sequence) of the BnGSR2-2 sequence that was previously missing (Supplementary Data File S3 at JXB online).

Genetic localization of the BnaGLN1 loci on the A or C Brassica genome using PCR and gene name annotation

Phylogenetic analyses showed a strong relationship between each BnaGLN1 gene and a gene from one or other of the progenitors B. rapa and B. oleracea, suggesting a common ancestral origin on the A or C Brassica genome, respectively. The phylogenetic tree also shows that each B. napus sequence, related to one sequence from either progenitor, is also related to another B. napus sequence, itself related to the other progenitor. The two B. napus homologous genes, the B. rapa and the B. oleracea genes, are thus defining in this way a homeology group (a, b, or c). It was found therefore that each of the AtGLN1.1, AtGLN1.2, and AtGLN1.5 genes is related to one homeology group, while the AtGLN1.3 and AtGLN1.4 genes are related to three and two groups, respectively. It has to be noted that the b group related to AtGLN1.3 is incomplete as no BoGLN1.3 expressed sequence has been identified. Both the homeology groups and the Brassica genome were used to ascribe names to the BnaGLN1 genes; thus, the genes are named Bna[A or C genome]GLN1.x[a, b, or c homeology group] according to Ostergaard and King (2008). A similar notation was used for the BnaGLN1 and BoGLN1 genes (Table 2).

In order to identify the A or C genome origin, the potential localization of the BnaGLN1 genes on linkage groups
and/or chromosomes known to arise from the A or C *Brassica* genomes was then investigated. Specific primer pairs were designed to localize each *BnaGLN1* gene (Supplementary Table S1 at JXB online). Five genes were mapped in this way on at least one of the three mapping populations available (Stellar×Drakar, Darmor×Samouraï, and Darmor-bzh×Yudal), recording the presence/absence of polymorphism. In good agreement with the phylogenetic tree analysis, the three genes *BnaA.GLN1.3.c*, *BnaA.GLN1.5.a*, and *BnaA.GLN1.5.5.a* were localized on linkage groups associated with the A genome on chromosomes A03, A05, and A06, respectively. The two genes *BnaC.GLN1.2.a* and *BnaC.GLN1.2.c* were localized on linkage groups associated with the C genome on chromosomes C02 and C03 (Table 3; Supplementary Fig. S1 at JXB online).

For the other members of the *BnaGLN1* gene family, an attempt to assign the *BnaGLN1* genes to the A or C genomes using mapping populations was unsuccessful. Therefore, a panel of various Brassicaceae genotypes (Supplementary Fig. S2 at JXB online) was used in order to detect the *BoGLN1* and *BraGLN1* orthologous genes, using specific *BnaGLN1* primers (Supplementary Table S1). The number of genotypes used for each *Brassica* species was adjusted in order to take into account the possible allelic variations and to detect the presence/absence of polymorphism. Furthermore, additional lines carrying the full A genome and one or several *B. napus* C chromosomes were used in order to determine precisely the localization of the *BnaC.GLN1* genes (Auger *et al.*, 2009) (Supplementary Fig. S3).

### Identification of GLN1 genes of *Brassica rapa* and *Brassica napus* genomes

The recently sequenced and annotated *B. rapa* genome (BRAD; Cheng *et al.*, 2011) was used to perform BLAST searches and sequence alignments using the *BraGLN1* contig sequences identified here. Analysis revealed eight annotated *BraGLN1* genes (Table 2). Alignments between *BraGLN1* gene sequences and contigs revealed potential splicing variants. Indeed, the CDS deduced from the *BraGLN1.3.C2* contig appeared incomplete at the 5′ end. The most highly similar *Bra001686* annotated gene on the A03 chromosome also appeared incomplete when compared with the *AtGLN1.3* CDS, as it is missing the first exon. The BLAST search on A03 chromosome v1.1 revealed the presence of a sequence highly similar to the *AtGLN1.3* first exon, 4kb upstream of the identified *Bra001686* sequence (bp 17 854 801 to 17 854 849). According to the BRAD annotation, this inserted region has been described as an long terminal repeat (LTR) transposon of 3746 bp on the minus strand. The identified

### Table 3. Genetic mapping of BnaGLN1 genes

| BnaGLN1 | Contig name | Mapping population | Linkage group (previous name) | LOD | Upstream marker | Downstream marker |
|---------|-------------|--------------------|-------------------------------|-----|-----------------|-------------------|
| BnaA.GLN1.1.a | BnaGLN1.1.C1 | Panela | A | 13.5 | PFM504 21.8 | J15.1200 9.1 |
| BnaA.GLN1.2.a | BnaGLN1.1.C2 | Addb | C06 | 17 | E1M4.21 2.7 | BN614 13.5 |
| BnaA.GLN1.3.a | BnaGLN1.2.C1 | Panela | A | 16.1 | PFM193 3.8 | BN466 7.9 |
| BnaA.GLN1.3.b | BnaGLN1.2.C2 | Addc | C01 | 25.9 | BN04C 1.3 | IGF0193c 2.2 |
| BnaA.GLN1.3.c | BnaGLN1.3.C5 | Addc | C03 (SD717) | 8.9 | nr | nr |
| BnaA.GLN1.4.a | BnaGLN1.3.C6 | ex | C03 (SD717) | 21.8 | Bn57463 | Bn57463 |
| BnaA.GLN1.4.b | BnaGLN1.3.C7 | Panela | B | 8.9 | nr | nr |
| BnaA.GLN1.5.a | BnaGLN1.5.C1 | Panela | C | 21.8 | Bn57463 | Bn57463 |

**Gene name** and **Contig name**

- A Panel of *B. napus*, *B. oleracea*, and *B. rapa* genotypes.
- B Monosomic and polysomic addition lines obtained from the Darmor-bzh×C1.3 cross.
- C Stellar×Drakar mapping population.
- D Darmor×Samouraï mapping population.
- E Darmor-bzh×Yudal mapping population.
B. rapa EST (EX089134) that allowed identification of the 5’ region of the BraGLN1.3_C2 contig starts in the transposon region and continues into the first identified exon of Bra001686 which corresponds to the second AtGLN1.3 exon.

The sequencing programme performed at URGV allowed identification of BnaGLN1 sequences in the B. napus Darmor-Bzh genome (SEQ-POLYNAP, ANR-09-GENM-021). The BnaGLN1 protein sequences were deduced from the identified BraGLN1 genes using the BRAD tool, and used to search the database of protein sequences built from B. napus genomic sequence analysis (unpublished data). From the BnaGLN1 protein sequences identified, genomic sequences were recovered (Supplementary Data File S4). Interestingly, 16 putative BnaGLN1 genes and two putative BnaGSL genes (coding for the GS2 isoform) were found. The 16 BnaGLN1 genomic sequences (Supplementary Data File S4) were used to analyse similarities with the genomic sequences (Supplementary Data File S4). Similarities were estimated using BLAST (NCBI) and exons using FGENESH software available at the SoftBerry website (http://linux1.softberry.com/berry.phtml?topic=fgenesh&group=programs&subgroup=gfind), the SoftBerry website (http://linux1.softberry.com/berry.jsp?topic=fgenesh&group=programs&subgroup=gfind), and allowed the gene structures to be deduced (Fig. 2). Except for BnaGLN1.3_C5 and BnaGLN1.3_C6, similarities between mRNA and associated contigs were near 100% (Table 4). Knowing that contig sequences and mRNA sequences are obtained from different B. napus genotypes (Supplementary Data File S1), this indicates that there is almost no polymorphism between the different BnaGLN1 coding sequences regarding the various genotypes of B. napus used for genome and EST sequencing. The BnaGLN1 genes contained between seven and 12 exons. GLN1.4 and GLN1.5 genes have the same number of exons in both B. napus and Arabidopsis (Table 4). For the other GLN1 genes, exon numbers are different between Arabidopsis and B. napus, but quite close; for example, AtGLN1.3 and BnaGLN1.3 contain fewer exons than other AtGLN1 and BnaGLN1 genes.

BnaGLN1 protein sequence conservation

Protein sequences of the BnaGLN1 family deduced from the coding sequences of contigs or from the deduced mRNA are similar. The BnaGLN1 proteins share between 93% and 96.6% identity with the AtGLN1 proteins encoded by their respective orthologous genes (Table 1). Within each homeology group, the A and C BnaGLN1 proteins share 98.3–100% identity.

In all BnaGLN1 protein sequences, two conserved pfam domains specific to glutamine synthetase enzymes (pfam 03951 and pfam 00120) were identified (Fig. 3). The residues involved in the ammonium/glutamate-binding pocket (Eisenberg et al., 2000) are also strictly conserved. In contrast,

| Gene name | Contig name | Contig length (bp) | Deduced mRNA name | Putative mRNA length (bp) | % similarity between contig and mRNA | No.of exons |
|-----------|-------------|--------------------|------------------|--------------------------|-----------------------------------|-------------|
| AtGLN1.1  | BnaA.GLN1.1.a | BnaGLN1.1.C1 | 1374 mRNA.BnaA.GLN1.1.a | 1494 | 100 | 9 |
| BnaA.GLN1.1.a | BnaGLN1.1.C2 | 1367 mRNA.BnaA.GLN1.1.a | 1474 | 100 | 11 |
| AtGLN1.2  | BnaA.GLN1.2.a | BnaGLN1.2.C1 | 1430 mRNA.BnaA.GLN1.2.a | 1499 | 100 | 10 |
| BnaA.GLN1.2.a | BnaGLN1.2.C2 | 1431 mRNA.BnaA.GLN1.2.a | 2587 | 100 | 9 |
| AtGLN1.3  | BnaA.GLN1.3.a | BnaGLN1.3.C1 | 1336 mRNA.BnaA.GLN1.3.a | 1341 | 99 | 11 |
| BnaA.GLN1.3.a | BnaGLN1.3.C2 | 1487 mRNA.BnaA.GLN1.3.a | 2012 | 100 | 8 |
| BnaA.GLN1.3.a | BnaGLN1.3.C3 | 1273 mRNA.BnaA.GLN1.3.a | 1555 | 99 | 8 |
| BnaA.GLN1.3.a | BnaGLN1.3.C4 | 1275 mRNA.BnaA.GLN1.3.a | 2051 | 99 | 7 |
| BnaA.GLN1.3.a | BnaGLN1.3.C5 | 1253 mRNA.BnaA.GLN1.3.a | 1803 | 100 | 7 |
| BnaA.GLN1.3.a | BnaGLN1.3.C6 | 1245 mRNA.BnaA.GLN1.3.a | 1840 | 96 | 9 |
| AtGLN1.4  | BnaA.GLN1.4.a | BnaGLN1.4.C1 | 1273 mRNA.BnaA.GLN1.4.a | 1269 | 96 | 9 |
| BnaA.GLN1.4.a | BnaGLN1.4.C2 | 1259 mRNA.BnaA.GLN1.4.a | 1461 | 100 | 12 |
| BnaA.GLN1.4.a | BnaGLN1.4.C4 | 1102 mRNA.BnaA.GLN1.4.a | 1415 | 99 | 12 |
| BnaA.GLN1.4.a | BnaGLN1.4.C3 | 1123 mRNA.BnaA.GLN1.4.a | 2582 | 99 | 12 |
| AtGLN1.5  | BnaA.GLN1.5.a | BnaGLN1.5.C1 | 1392 mRNA.BnaA.GLN1.5.a | 1307 | 100 | 10 |
| BnaA.GLN1.5.a | BnaGLN1.5.C2 | 1380 mRNA.BnaA.GLN1.5.a | 1329 | 99 | 10 |

Table 4. Comparison between contigs of ESTs and the mRNA sequences deduced from the BnaGLN1 genomic sequences
Fig. 2. Structure of BnaGLN1 genes. For each BnaGLN1 gene, the length of the 5' and 3' untranslated regions (UTRs) (white boxes), exons (black boxes), and introns (black lines) is represented by a number corresponding to base pairs.
the polar amino acids Q49 and S174, shown to be involved in the ammonium high affinity properties of AtGLN1 and AtGLN1.4 (Ishiyama et al., 2006), are not strictly conserved in all the BnaGLN1.1 and BnaGLN1.4 proteins. The polar Q49 was converted into an acidic glutamate E49 in all the BnaGLN1.1 and BnaGLN1.4 sequences, and the S174 is conserved only in the two BnaGLN1.4.b sequences but was converted into an A174 in the BnaGLN1.4.a and BnaGLN1.1.a sequences. Depending on the effect of such amino acid modifications, it might be possible that ammonium affinity properties have not been conserved within the BnaGLN1.1 and BnaGLN1.4 protein families. In contrast, the residues K49 and A174 present in the low affinity enzymes AtGLN1.2 and AtGLN1.3 are conserved in all the BnaGLN1.2 and BnaGLN1.3 protein sequences, suggesting the conservation of the low ammonium affinity properties in those two protein families (Fig. 3).

Expression of BnaGLN1 genes is modified depending on the nitrogen regime and leaf senescence

A first analysis of EST distribution between libraries and BnaGLN1 contigs led to the conclusion that BnaGLN1 genes are probably differentially expressed according to tissue and developmental stage (Supplementary Data File S1 at JXB online).

The BnaGLN1 gene expression was monitored at the vegetative stage measuring transcript levels by quantitative real-time RT–PCR in samples of taproot, crown, limbs, and veins of plants grown under low or high nitrate conditions. Plants grown under low or high nitrate conditions grew 13 and 17 leaves, respectively. $F_v/F_m$ and SPAD measurements on all the leaf ranks (numbered from the bottom leaf to the top leaf) were done to estimate the relative leaf senescence status of each leaf. From both SPAD and $F_v/F_m$ as senescence markers, six leaves were selected from each nitrate condition presenting differential senescence levels to perform further experiments (Fig. 4). Leaves of rank 3, 5, 6, 7, 9, and 11 were harvested on plants grown under low nitrate conditions. Leaves 3, 5, 6, 9, 12, and 15 were harvested on plants grown under high nitrate conditions. To simplify the presentation of further results, the collected leaf ranks were renamed 1, 2, 3, 4, 5, and 6, respectively, with 1 designating the bottom-most and oldest collected leaf and 6 the youngest collected leaf. Leaves dissected as limbs, and primary and secondary veins were used to measure BnaGLN1.1 gene expression levels in the different
In addition to *BnaGLN1.1* expression, the expression of *BnaGSL1* and *BnaGSL2* encoding the chloroplastic GS2 isoenzymes was also monitored and used as a control for leaf senescence as it is known that genes encoding GS2 isoenzymes are down-regulated with leaf ageing in all the plant species studied so far (Masclaux-Daubresse et al., 2008). *BnaGSL1* and *BnaGSL2* expression levels confirmed the differential senescence phenotype of the chosen leaf ranks. Leaves 1, 2, and 3 can be considered as senescing leaves, 4 and 5 as mature leaves, and 6 as a young leaf according to Masclaux et al. (2000) (Fig. 4E, F).

Genes that are preferentially expressed under high or low nitrate conditions were identified. The results showed that regarding the N regime, all the members of the same gene family...
BnaGLN1 genes are differentially expressed at the reproductive stage depending on plant organs or leaf age

In order to monitor BnaGLN1 gene expression at the reproductive stage, plants were grown in field conditions under low or high N regimes. Two leaf ranks (young top leaf and old bottom leaf) and the two corresponding stem sections (also referred to hereafter as young and old) were collected at flowering and during grain filling.

Globally, effects of N limitation on BnaGLN1 expression were similar to those found at the vegetative stage, except that the magnitude of gene repression or induction was lower than that observed at the vegetative stage (Supplementary Table S5 at JXB online).

Figure 7 reports the effect of senescence on the expression of the BnaGLN1 genes in leaves and stems of plants grown under a sufficient N regime. As a control of leaf senescence stages, the BnaGSL1 and BnaGSL2 genes are significantly more highly expressed in the young tissues than in older tissues (Fig. 7N, O). There was a sharp decrease in BnaGSL gene expression at the flowering stage, while at the seed filling stage the magnitude of BnaGSL repression was much lower but still significant.

As observed at the vegetative stage, the BnaGLN1.1 genes were up regulated with leaf and stem senescence, but this was only observed at the flowering stage (Fig. 7A, B). During seed filling, expression in leaves and stems was higher than during flowering, showing an effect of plant ageing. However, no difference was observed between the young and old leaves, suggesting that both types of leaves had become senescent between flowering and seed filling. The two BnaGLN1.1 genes were expressed more highly in leaf blades than in stems at both the flowering and seed filling stages. Similarly the BnaGLN1.2 genes were more expressed in leaf blades than in stems (Fig. 7C, D). The effect of senescence on BnaGLN1.2 genes was opposite to the effect observed on BnaGLN1.1 genes. BnaGLN1.2 expression decreased 2- to 3-fold in old leaf blades and old stems compared with young leaf blades and young stems, respectively. The bifasic profile obtained for BnaA.GLN1.2 at the vegetative stage was also observed at the flowering stage (data not shown). As observed with the BnaGLN1.1 genes, the effect of senescence was no more significant at seed filling.

Among the four BnaGLN1.4 genes, only BnaA.GLN1.4.a and BnaC.GLN1.4.a shared similar expression profiles (Fig. 7E–H). They are preferentially expressed in leaf blades rather than in stems. In contrast to the vegetative stage, BnaA.GLN1.4.a and BnaC.GLN1.4.a tend to be repressed by senescence in leaf blades but induced by senescence in stems. This trend was especially significant at the flowering stage. In contrast, BnaA.GLN1.4.b was induced by senescence in leaf blades and stems at the flowering stage but repressed during seed filling (Fig. 7G). Finally, BnaC.GLN1.4.b expression was higher in leaf blades than in stems and was repressed by senescence at the flowering stage, similarly to the two BnaA.GLN1.4.a and BnaC.GLN1.4.a genes (Fig. 7H). In contrast to the vegetative stage, the members of the BnaGLN1.4 family have developed specificities and are differentially expressed at the flowering and seed filling stages. It is likely that they have different roles and influences on N metabolism after flowering.

Among the BnaGLN1.3 members, similar profiles were observed for BnaA.GLN1.3.a, BnaA.GLN1.3.b, and BnaC.GLN1.3.b (Fig. 7I, K, L). These three genes are
Fig. 5. Expression of BnaGLN1 genes is modified depending on nitrate availability and leaf ageing. The relative expression level of BnaGLN1 genes was monitored in limbs and secondary veins of six leaf ranks harvested on vegetative plants grown under low (white bars) or high (black bars) nitrate conditions. Leaf ranks represented as number 1 (bottom and older leaf) to 6 (top and younger leaf) showed differential senescence symptoms. Mean and standard deviation of four plant repeats are shown.
down-regulated in old leaves and stems compared with young leaves at the flowering stage. However, their expression increased sharply in old limbs at the seed filling stage. The other BnaC.GLN1.3.a and BnaA.GLN1.3.c/ BnaC.GLN1.3.c expression profiles did not show any modification associated with leaf or stem senescence (Fig. 7J, M). All the BnaGLN1.3 genes appeared to be more highly expressed in stems than in leaves, especially at the flowering stage.

It was not possible to measure BnaA.GLN1.5.c/BnaC. GLN1.5.c gene expression, possibly due to the very low expression level in vegetative tissues that cannot be accurately measured in field-grown plants.

Results obtained at the flowering and seed filling stages confirm results from the vegetative stage. BnaGLN1 genes are generally similarly regulated according to their orthology group, although exceptions were observed particularly at the seed filling stage, such as with BnaA.GLN1.4.b and BnaC. GLN1.3.a (Fig. 7G, J).

Discussion

Glutamine synthetase is a key enzyme of N metabolism involved in ammonium assimilation and remobilization. Recent studies highlight the important role of GS1 cytosolic isoenzymes for N management linked to yield establishment and seed filling in monocotyledonous crops (Tabuchi et al., 2005; Martin et al., 2006; Bernard and Habash, 2009; Swarbreck et al., 2011). The GS1-coding genes are therefore
Table 5. Nitrogen and senescence effects observed on the BnaGLN1 expression levels at vegetative, flowering and seed filling stages

Significant positive and negative effects of nitrogen limitation (A) and ageing (B) on the expression of the BnaGLN1 genes recorded at vegetative, flowering, and seed filling stages are reported. The average level of expression of each BnaGLN1 gene as the mean of the data recorded in all the samples analysed is reported in column 2 (relative to the BnaX.SAND gene).

| Gene(s) name | Mean relative expression level | (A) Nitrogen limitation effect | (B) Age effect |
|--------------|-------------------------------|-------------------------------|----------------|
|              | [Vegetative stage]             | Flowering                     | Seed filling    |
|              | [Limb | Primary vein | Secondary vein | Tap root | Crown | Limb | Stem | Limb | Stem | Limb | Primary vein | Secondary vein | Limb | Stem | Limb | Stem |
| BnaA.GLN1.1.a | 4.59                          | +                              | +              | +        | +      | +    | +    | +    | +    | +    | +              | +              | +    | +    | +    | +    |
| BnaC.GLN1.1.a | 9.64                          | +                              | +              | +        | +      | +    | +    | +    | +    | +    | +              | +              | +    | +    | +    | +    |
| BnaA.GLN1.2.a | 2.69                          | –                              | –              | –        | –      | –    | –    | –    | –    | –    | –              | –              | –    | –    | –    | –    |
| BnaC.GLN1.2.a | 2.59                          | –                              | –              | –        | –      | –    | –    | –    | –    | –    | –              | –              | –    | –    | –    | –    |
| BnaA.GLN1.3.a | 0.26                          | +                              | +              | +        | +      | +    | +    | +    | +    | +    | +              | +              | +    | +    | +    | +    |
| BnaC.GLN1.3.a | 2.07                          | +                              | +              | +        | +      | +    | +    | +    | +    | +    | +              | +              | +    | +    | +    | +    |
| BnaA.GLN1.3.b | 0.06                          | –                              | –              | –        | –      | –    | –    | –    | –    | –    | –              | –              | –    | –    | –    | –    |
| BnaC.GLN1.3.b | 0.06                          | –                              | –              | –        | –      | –    | –    | –    | –    | –    | –              | –              | –    | –    | –    | –    |
| BnaA.GLN1.3.c | 4.82                          | +                              | +              | +        | +      | +    | +    | +    | +    | +    | +              | +              | +    | +    | +    | +    |
| BnaA.GLN1.4.a | 9.54                          | +                              | +              | +        | +      | +    | +    | +    | +    | +    | +              | +              | +    | +    | +    | +    |
| BnaC.GLN1.4.a | 38.29                         | +                              | +              | +        | +      | +    | +    | +    | +    | +    | +              | +              | +    | +    | +    | +    |
| BnaA.GLN1.4.b | 14.18                         | +                              | +              | +        | +      | +    | +    | +    | +    | +    | +              | +              | +    | +    | +    | +    |
| BnaC.GLN1.4.b | 10.65                         | +                              | +              | +        | +      | +    | +    | +    | +    | +    | +              | +              | +    | +    | +    | +    |
| BnaA.GLN1.5.a | <0.01                         | +                              | +              | +        | +      | +    | +    | +    | +    | +    | +              | +              | +    | +    | +    | +    |

aRelative expression level associated to both A and C genes.
good candidates for improving yield traits and grain quality (Masclaux-Daubresse et al., 2008). The complexity of studying glutamine synthetases arises from the fact that two isoenzymes exist, one in the chloroplast and the other in the cytosol, and that several isoforms exist for the cytosolic enzyme. The numerous isoforms are encoded by a multigenic family, and the five GLN1 genes in A. thaliana are likely to present different roles depending on plant organs and nitrate availability in the soil (Lothier et al., 2011). Similarly the five maize GLN1 genes do not participate equally in N management at the whole-plant level (Martin et al., 2006; Hirel et al., 2007).

The aim of this study was to identify the whole BnaGLN1 gene family and to characterize the expression of the different genes depending on nitrate availability as well as depending on ageing and leaf and stem tissue senescence.

Using the sequences obtained from EST libraries and genome sequencing, a total of 16 genes belonging to the BnaGLN1 family, eight genes from each of the A and C genomes, were found. In accordance with the history of B. napus genome formation (Nagaharu, 1935), it was found that each BnaGLN1 gene is closely related to a BraA.GLN1 or BolC.GLN1 gene depending on its A or C genome location. Therefore, it can be stated with confidence that all the GLN1.1 genes of B. napus have been described in this report. Sequence analyses also showed that B. napus coding sequences are highly conserved between the A and C genomes and also between B. napus genotypes. The level of sequence divergence observed in the BnaGLN1 family is between 0.9% and 2.9% SNPs (single nucleotide polymorphisms) in CDS, which is less than the preliminary observation showing sequence divergences of ~3–5% SNPs in CDS from SLR1 (Inaba and Nishio, 2002).

It is well known that B. napus shows a high degree of collinearity to its diploid progenitors B. rapa and B. oleracea (Rana et al., 2004). Many studies have investigated the segmental structure of the Brassica genomes and led to the identification and genetic mapping of synteneic blocks between A. thaliana and the Brassica genomes (Parkin et al., 2005; Schranz et al., 2006; J. Wang et al., 2011). The number of potential BnaGLN1 genes and their localization on linkage groups can then be predicted depending on the number of times the blocks are replicated and on their localization on each B. napus linkage group. According to the whole-genome triplication event that occurred in Brassicaceae genome species after divergence from Arabidopsis (Lysak et al., 2005; Parkin et al., 2005), and the recent hybridization between B. rapa and B. oleracea leading to the appearance of B. napus, each AtGLN1 gene could have been found in triplicate in each A and C genome from B. napus to form three pairs of homeologous genes. From EST and genome sequence analyses, it is revealed here that
only one homeology group exists for *AtGLN1.1, AtGLN1.2*, and *AtGLN1.5* and two groups for *AtGLN1.4, AtGLN1.3* is the only GLN1 gene for which the six *BnaGLN1* orthologous genes were retained in the *B. napus* genome. This illustrates the massive gene loss that occurred in the *Brassica* lineage after the whole-genome triplication event. Indeed, using *A. thaliana* as an outgroup, *Town et al.* (2006) found that 35% of genes inferred to be present when genome tripliation occurred in the *Brassica* lineage have been lost in *B. oleracea*. Similarly, whole-genome analysis of *B. rapa* revealed a high rate of gene loss, from 30% to 64% depending on the degree of fractionation of the region considered (X. Wang et al., 2011). *BnaGLN1* families are a good example of this.

With the exception of *BnaGLN1.4.b* genes, the present study points out that pairs of *BnaGLN1* homeologous genes share very similar transcription profiles. Furthermore, within one orthology group, when several groups of homeologues were retained, paralogous genes conserved similar expression profiles (*BnaGLN1.3* and *BnaGLN1.4*). This suggests that coding but also regulatory sequences were essentially conserved after genome merging of *B. napus* progenitors, but also after the whole-genome duplication (WGD) and diploidization events that occurred in the *Brassica* lineage after the divergence from the *Arabidopsis* genus. WGD is generally thought to provide raw material for gene neo- and subfunctionalization, extending resilience to deleterious mutations, increasing the net speciation rate and species richness (Soltis et al., 2009), as well as providing the adaptive advantage for colonizing harsh and unstable environments (Franzke et al., 2011). On the other hand, maintenance of redundancy can confer robustness against mutations (De Smet and Van de Peer, 2012) and/or a selective advantage in increasing the abundance of encoded proteins (Bekaert et al., 2011). As GS1 is an essential enzyme of primary N metabolism, linked to central carbon metabolism via the GS/GOGAT cycle that might also play a role in the adaptation of plant to nutrient deficiency and pathogen attack (Brauc et al., 2011; Seifi et al., 2013), maintenance of multicopy of GLN1 could confer robustness against mutations. Nevertheless, GLN1 expression profiles have not been exhaustively investigated and there might be particular environmental or developmental conditions allowing the differentiation of expression profiles between homeologous and/or paralogous genes. Partially overlapping profiles could provide robustness against mutations, but also adaptive advantages for colonizing harsh and unstable environments.

Allopolyploidy involves the merger of two different, and often divergent, genomes whose recombination in a common nucleus often leads to myriad changes, including unequal expression of the two merging genomes. Biased expression among homeologues has been found in cotton and wheat (Pumphrey et al., 2009; Rapp et al., 2009). Previous studies have suggested bias toward the *B. rapa* A genome in the transcriptional expression of rRNA genes (Chen and Pikaard, 1997). In the present study it is shown that most of the *BnaGLN1* homeologous pairs display similar expression levels in the various tissues studied. Differences in mRNA contents observed between homeologous pairs of *A* and *C* genome origins were generally very small. No systematic bias towards the *B. oleracea* C parental genome or *B. rapa* A genome can be identified in this study. Bias towards the *B. oleracea* C parental genome was identified for *BnaGSL* (at flowering and seed filling stages), *BnaGLN1.3.a* (especially at the vegetative stage), and *BnaGLN1.4.a* homeologues, and in favour of the *B. rapa* parental genome for *BnaGLN1.1.a* and *BnaGLN1.2.a*. These results are in agreement with the recent finding that nearly 7% of the potentially identified homeologous genes expressed in a leaf extract are displaying a differential expression level in favour of the *A* or *C* parental genome for 1/3 and 2/3 of the pairs, respectively, and that genes involved in metabolic processes tend to be over-represented (Higgins et al., 2012).

Beside their intrinsic expression levels, it was found that the *BnaGLN1* genes are similarly regulated depending on their orthology group and that they are differentially regulated between groups. Overall it was found that specificities of expression are conserved between *BnaGLN1* genes and their respective *AtGLN1* orthologues, raising the hypothesis of conserved physiological functions.

In *Arabidopsis*, several studies have shown that *AtGLN1.1* is highly induced in leaves during senescence (Guo et al., 2004) and is up-regulated when exogenous N sources are limiting (Lothier et al., 2011). Up-regulation of *AtGLN1.1* under nitrate starvation is in good agreement with the kinetic properties described by Ishiyama et al. (2004) that suggested that the high affinity of *AtGLN1.1* for ammonium is correlated with a role for the enzyme under low N conditions. The induction of the expression of the two *BnaGLN1* genes in older stems and leaves is conserved, as already shown by Buchanan-Wollaston and Ainsworth (1997) and Ochs et al. (1999). It was also found that the two *BnaGLN1.1* genes are overexpressed under low nitrate conditions especially at the vegetative stage. As the amino acid residues known to be involved in the high affinity of *AtGLN1* towards ammonium are not conserved in any of the *BnaGLN1* proteins, no information about the potential kinetic properties can be extrapolated from the protein sequence. Regarding expression profiles, it is at least suspected that *BnaGLN1.1* and *AtGLN1.1* proteins might have similar roles.

In a previous study, it was found that *AtGLN1.2* is slightly induced by leaf ageing and that the expression profile is biphasic, with an increase from young to mature leaves and then a decrease in strongly senescing leaves (Diaz et al., 2008; Lothier et al., 2011). In addition, it was found that *AtGLN1.2* is mainly expressed in roots and leaves under a high N regime (Guo et al., 2004; Ishiyama et al., 2004; Lothier et al., 2011). A detailed functional analysis led to the conclusion that *AtGLN1.2* was involved in primary ammonium assimilation under high N regimes (Ishiyama et al., 2004; Lothier et al., 2011). *BnaGLN1.2* genes (also named *BnGSR2.1* and *BnGSR2.2*) are also more highly expressed in roots than in shoots (Ochs et al., 1999). It is shown here that the two *BnaGLN1.2* genes are more expressed in young leaves than in old leaves and are overexpressed under high N regimes, suggesting a similar role to *AtGLN1.2* in primary ammonium assimilation. Interestingly, a *BraA.GLN1.2* gene, named *BcGSI* (Sun et al., 2010), was found also to be expressed in root and induced under high N regimes, suggesting the conservation of the regulation in the Brassiceae tribe.
Similarly to AtGLN1.3, the BnaGLN1.3 genes are not induced in older leaves at the vegetative stage and do not respond to differential N regimes (Guo et al., 2004; Lothier et al., 2011). The hypothesis about the physiological roles of AtGLN1.3 in N export via phloem tissues in roots is supported by the high capacity of the enzyme for glutamine synthetase and by the location of AtGLN1.3 expression in the root vasculature (Ishiyama et al., 2004). It has to be noted that BnaGLN1.3 genes are the only BnaGLN1 genes preferentially expressed in stem tissues compared with leaf blades. As stems are richer in vascular tissues than leaf blades, the higher expression of BnaGLN1.3 in stems might be related to a potential vascular localization that remains to be explored.

AtGLN1.4 is one of the markers used for leaf senescence (Guo et al., 2004; Wingler et al., 2009). AtGLN1.4 is induced by N limitation or starvation in both the root and shoot (Ishiyama et al., 2004; Lothier et al., 2011). AtGLN1.4 protein exhibits high affinity towards ammonium and is expressed in the pericycle cells of roots (Ishiyama et al., 2004). Evidence for BnaGLN1.4a root expression was found in EST libraries. However, induction of gene expression in leaves in response to ageing and low N regime is not well conserved among the four BnaGLN1.4 orthologues in regards to developmental stages. The BnaGLN1.4 genes are significantly overexpressed in senescing leaves and under low nitrate conditions at the vegetative stage. However, this trend is not conserved at the flowering and seed filling stages. BnaAGL1.4a, BnaCGLN1.4a, and BnaCGLN1.4b are clearly and significantly less highly expressed in old than in young leaves at these stages. Furthermore, as the residues conferring the high affinity property are partially conserved in BnaGLN1.4 proteins, it might be suspected that BnaGLN1.4 genes have a role when N resources are low. Surprisingly, in contrast to Arabidopsis in which AtGLN1.4 expression is one of the lowest, BnaGLN1.4 expression levels are the highest found among all the BnaGLN1 genes.

In Arabidopsis, AtGLN1.5 expression is known as the lowest of the AtGLN1 gene family. Expression was mainly found in seeds (Lothier et al., 2011), and very little is known about AtGLN1.5. BnaGLN1.5 gene expression is also very low, and it was not possible to measure it in leaf and stem tissues at flowering and vegetative stages. Similarly to AtGLN1.5, BnaGLN1.5 ESTs were found in reproductive tissues and seeds, thus suggesting specific roles during seed maturation.

**Conclusion**

A total of 16 B. napus GLN1 genes were identified, among which 12 have never been described. The total number of BnaGLN1 genes, their phylogenetic relationships, and genetic location are in agreement with the evolutionary history of Brassica species. Some specificities of expression seemed to be conserved among the Brassicaceae tribe and especially between A. thaliana and B. napus. Regulations arising from plants interactions with their environment (such as N resources), final architecture, and therefore sink–source relations in planta, seem to be globally conserved when compared with data available from the Arabidopsis model. Considering the architectural, size, and lifespan differences between A. thaliana and B. napus, it is not surprising to find some differences in gene expression profiles. Also, due to the higher number of GLN1 genes conserved in the B. napus genome, it seems correct to find some specificities in the expression of each BnaGLN1 in contrast to genes involved in flavonoid biosynthesis that display highly conserved expression profiles between A. thaliana and B. napus during seed development and are highly dependent on tissue differentiation (Auger et al., 2009). A more detailed localization of BnaGLN1 gene expression would refine the hypothesis concerning their physiological role. Indeed, the present expression study relied on leaf blade and stem samples consisting of different tissues with contrasting physiological roles, in particular parenchyma and vascular tissues. Such a localization study could be advantageously performed in Arabidopsis and B. napus to provide a new basis for comparison of the evolution of this gene family.

**Supplementary data**

Supplementary data are available at *JXB* online

**Figure S1.** BnaGLN1 gene localization on the A or C genome using a panel of various Brassica genotypes.

**Figure S2.** BnaGLN1 gene localization on LGs using mono- and polysomic additional lines.

**Figure S3.** Positions of BnaGLN1 genes on the B. napus genetic map.

**Figure S4.** Alignments of the genomic sequence, deduced mRNA sequence, and contig of EST sequences are reported for each BnaGLN1 gene.

**Figure S5.** Expression of BnaGLN1 genes is modified depending on nitrate availability and ageing in primary veins of vegetative B. napus plants.

**Table S1.** Clones from Genoplante and ADIS-MPIZ oilseed rape cDNA libraries used for BnaGLN1 mRNA sequencing.

**Table S2.** Primers used for cloning and genetic mapping of the BnaGLN1 gene.

**Table S3.** Specific primers used for sequencing BnaGLN1 cDNAs.

**Table S4.** qPCR primers used for BnaGLN1 gene expression analysis.

**Table S5.** Induction of BnaGLN1 gene expression under low N fertilization in field-grown plants.

**Data File S1.** List and description of EST sequences belonging to each B. napus, B. oleracea, and B. rapa GLN1 contig, and tables of the distribution of ESTs between libraries according to their BnaGLN1 contigs.

**Data File S2.** BnaGLN1 contig sequences in fasta format.

**Data File S3.** Global multiple alignment of nucleotide sequences in the study.

**Data File S4.** Genomic sequences of the BnaGLN1 genes.

**Data File S5.** Deduced BnaGLN1 mRNA sequences.

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