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ORIGINAL ARTICLE

Arabidopsis mutants lacking asparaginases develop normally but exhibit enhanced root inhibition by exogenous asparagine

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Abstract Asparaginase catalyzes the degradation of L-asparagine to L-aspartic acid and ammonia, and is implicated in the catabolism of transported asparagine in sink tissues of higher plants. The *Arabidopsis* genome includes two genes, *ASPGA1* and *ASPGB1*, belonging to distinct asparaginase subfamilies. Conditions of severe nitrogen limitation resulted in a slight decrease in seed size in wild-type *Arabidopsis*. However, this response was not observed in a homozygous T-DNA insertion mutant where *ASPG* genes had been inactivated. Under nitrogen-sufficient conditions, the *ASPG* mutant had elevated levels of free asparagine in mature seed. This phenotype was observed exclusively under conditions of low illumination, when a low ratio of carbon to nitrogen was translocated to the seed. Mutants deficient in one or both asparaginases were more sensitive than wild-type to inhibition of primary root elongation and root hair emergence by L-asparagine as a single nitrogen source. This enhanced inhibition was associated with increased accumulation of asparagine in the root of the double *aspga1-1/-b1-1* mutant. This indicates that inhibition of root growth is likely elicited by asparagine itself or an asparagine-derived metabolite, other than the products of asparaginase, aspartic acid or ammonia. During germination, a fusion between the *ASPGA1* promoter and beta-glucuronidase was expressed in endosperm cells starting at the micropylar end. Expression was initially high throughout the root and hypocotyl, but became restricted to the root tip after three days, which may indicate a transition to nitrogen-heterotrophic growth.

Keywords Asparaginase · Asparagine · Mutants · Root elongation · Root hair formation · Nutrient sensing

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Introduction

Asparaginases (ASPG) (EC 3.5.1.1) catalyze the hydrolysis of Asn, releasing Asp and ammonia. The plant ASPGs belong to the superfamily of *N*-terminal nucleophile (Ntn) hydrolases (Michalska and Jaskolski 2006). They are heterotetramers of α- and β-subunits, formed by autoproteolytic cleavage of a polypeptide precursor, which exposes an *N*-terminal catalytic Thr residue. There are two ASPG subfamilies in higher plants, corresponding to biochemical subtypes previously defined on the basis of their dependence for K^+ (Sodek et al. 1980; Bruneau et al. 2006). Each subfamily is represented by a single gene in *Arabidopsis*. ASPGB1 (K^+-dependent ASPG, TAIR accession no. At3g16150) has an approximately 50-fold higher catalytic efficiency with Asn as substrate than ASPGA1 (K^+-independent ASPG, At5g08100) and is activated by K^+ (Bruneau et al. 2006). Similar findings have been reported for K^+-dependent (LjNSE1) and K^+-independent (LjNSE2) ASPGs from *Lotus japonicus* (Credali et al. 2011). Plant

ASPGs also act as specialized dipeptidases that cleave isoaspartyl linkages (Michalska and Jaskolski 2006) formed in protein especially during seed desiccation (Ogé et al. 2008).

There is a large body of evidence implicating ASPG in the metabolism of Asn transported to sink tissues. ASPG activity is high in pea expanding leaves (Sieciechowicz et al. 1985) and seed coat, and present, though at lower levels, in developing cotyledons (Sodek et al. 1980; Murray and Kennedy 1980). Expression analysis of a fusion between the promoter of a K^+-independent ASPG from *Lupinus angustifolius* and β-glucuronidase (GUS) revealed a close association with sink tissues having localized high levels of Asn, including the apical meristem, expanding leaves and developing seed (Grant and Bevan 1994). In *L. angustifolius* root, ASPG generates ammonia to support nodule development, and later on ASPG transcript and activity levels are suppressed upon nodule maturation (Vincze et al. 1994). Recent evidence implicates ASPG in the in situ mobilization of nitrogen (N) stored as free Asn. Analyses of metabolite and transcript profiles in *Arabidopsis* germinating seeds have identified that ASPGA1 is involved in the catabolism of free Asn stored during seed desiccation (Fait et al. 2006). In pine seedlings, ASPG associated with cambial cells of the secondary vascular system is involved in the metabolism of Asn stored in hypocotyl after germination (Cañas et al. 2006, 2007). In addition, several lines of evidence indicate that excess N can be stored as Asn in seedlings. In pine, excess ammonium elevates transcript levels of both asparagine synthetase and ASPG, which are expressed in different cell types (Canales et al. 2010). In *Arabidopsis*, Asn metabolism is part of a transcriptional regulatory network whereby excess organic N as Glu stimulates biosynthesis by ASN1 and represses catabolism by ASPGB1 (Gutiérrez et al. 2008).

Quantitative RT-PCR analysis of *ASPG* expression in Arabidopsis confirmed its association with sink tissues (Bruneau et al. 2006). *ASPGA1* and *–B1* have an overlapping pattern of expression in different tissues, suggesting a redundant function. In the tissues examined, transcript levels of *ASPGA1* were higher than those of *ASPGB1*, by up to 10-fold, and negatively correlated with the catalytic efficiency of the enzymes with Asn as substrate.

Beside ASPG, Asn can be metabolized by transamination via serine:glyoxylate aminotransferase (EC 2.6.1.45) (Ireland and Joy 1983). Barley and tobacco mutants deficient in serine:glyoxylate aminotransferase lack Asn aminotransferase activity in leaf (Havir and McHale 1988; Murray et al. 1987). In pea, this activity predominates over ASPG in older leaves, and is present in pods, but very low in developing seeds (Ireland and Joy 1981). As part of photorespiratory N recycling, Asn aminotransferase was hypothesized to contribute little to the net catabolism of Asn (Lea et al. 2007).

The objective of this study was to investigate ASPG function by characterizing insertional mutants in which *ASPG* genes are inactivated. Results revealed that ASPG activity is dispensable in *Arabidopsis*. Phenotypes uncovered in seed and roots are consistent with ASPG function in Asn catabolism. Expression analysis of promoter-GUS fusions showed a coincidence between these phenotypes and the sites of *ASPGA1* expression.

Materials and methods

Plant growth and growth assays

Arabidopsis thaliana plants were grown as previously described, in Promix PGX (Premier Tech Horticulture, Rivière-du-Loup, QC) and fertilized with 20:20:20 (3 g l^{-1}) unless otherwise noted (Bruneau et al. 2006). Substrate without pre-added nutrient was Sunshine Mix 2 (Sun Gro Horticulture, Vancouver, BC). Plants were fertilized weekly unless otherwise noted. A similar volume of nutrient solution was applied to each genotype. Nutrition with a single N source made use of a defined nutrient solution or defined medium as described in Wang et al. (2003). Asn (Sigma Life Sciences 11149) and Gln (Sigma G3126) were filter sterilized and added to the medium after autoclaving. Seeds used for seedling growth assays had been produced and harvested at the same time. Seed size was measured as per Herridge et al. (2011). Seeds were scanned using a Powerlook 1120 scanner (UMAX, Dallas, TX) at a resolution of 1200 dpi and images analyzed with the ImageJ software (http://rsbweb.nih.gov/ij/). A *Brassica napus* seed whose diameter was measured with a caliper was scanned and used to set a millimetric scale as per software instructions. Germination was scored as radicle protrusion. Root elongation was measured with a caliper.

Plant materials and genotype analysis

Seeds of *Arabidopsis* insertion mutants for *ASPGA1* and *−B1* were obtained from the Arabidopsis Biological Resource Centre at Ohio State University (http://www.arabidopsis.org/abrc). Homozygous individuals were identified by PCR genotyping of progeny from heterozygous plants, using gene-specific and T-DNA specific primers. Genomic DNA was extracted from leaf tissue using the DNeasy Plant Mini Kit (Qiagen, Mississauga, ON). Position of insertions was determined by sequencing of PCR products amplified with Platinum PCR Super Mix High Fidelity (Invitrogen, Burlington, ON), subjected to a reaction with the Klenow fragment of DNA polymerase I and cloned into the pCR-BluntII-TOPO vector (Invitrogen). Sequencing was performed with a 3130XL Genetic

Analyzer (Applied Biosystems, Streetsville, ON). The following primers were used for genotyping, for *aspga1-1*: SALK087F, 5′-ATGGCAGCTGGTCGGATCCATGCGT TTGA-3′, SALK087R, 5′-AAGCACAAGGCAAGGGG AGGATTATATCAT-3′ and JMLB2, 5′-TTGGGTGAT GGTTCACGTAGTGGG-3′; for *aspga1-2*: SALK087F, -R and LB2, 5′-GCTTCCTATTATATCTTCCCAAATTACC AATACA-3′; for *aspgb1-1*: SALK082F, 5′-GAAAGAGA GAGAATATGGGTGGGTGGGCAA-3′, SALK082R, 5′-AATCCGGTAATCAAACTGGTGAGAAAGCAA-3′ and JMLB1, 5′-GGCAATCAGCTGTTGCCCGTCTCACTGG TG-3′; and for *aspgb1-2*, SALK082F, -R and p745, 5′-AA CGTCCGCAATGTGTTATTAAGTTGTC-3′. The absence of transcript in homozygous mutants was confirmed by RT-PCR of total RNA isolated from flowers in reference with the parental ecotype using the following gene-specific primers: for *ASPGA1*, At5g08100F, 5′-AATTGGATCCATGGTC GGGTGGGCGAT-3′ and At5g08100R, 5′-AATTCTGCA GTCAATTGTTTGGCCAGATTGCGA-3′; for *ASPGB1*, At3g16150F, 5′-AATTGGTACCATGGGTGGGTGGGCA AT-3′ and At3g16150R, 5′-AATTCTGCAGTCACTCCCA AATAGCAA-3′; and *Act7* (*At5g09810*) as control for RNA input, with primers Atactin01, 5′-CTCACAGAGGCAC CTCTTAAC-3′, and Atactin02, 5′-GTTGTTTCATGGAT TCCAGGAG-3′. RNA extraction and RT-PCR were performed as previously described (Bruneau et al. 2006). RNA was quantified with a Nanodrop 1000 spectrophotometer (Fisher Scientific, Ottawa, ON).

Elemental analysis

Elemental analysis was performed by dry combustion with a LECO CNS-2000 Elemental Analyzer on approximately 40 mg dry seed samples as described by Taylor et al. (2008).

Amino acid analysis

Free and total amino acids were quantified from 50 mg seed samples as previously described (Taylor et al. 2008). For quantification of Asn in roots, tissue was harvested, rinsed with water, blotted and weighed. Samples were flash frozen in liquid nitrogen and transferred to 2 ml tubes on dry ice containing five zirconium grinding beads of 2.5 mm in diameter. Tubes were placed in a frozen grinding vessel and samples disrupted in a TissueLyser II (Retsch, Newton, PA) by rapid agitation twice for 2.5 min at 30 Hertz. Free amino acids were extracted as referenced above and Asn quantified by HPLC after derivatization with *o*-phthalaldehyde and 3-mercaptopropionic acid according to Molnár-Perl and Vasanits (1999). Lyophilized amino acids were dissolved in HPLC grade water at 1 ml per 100 mg tissue. The derivatization reagent was prepared by combining 200 µl *o*-phthalaldehyde-methanol, 800 µl of 0.2 M potassium borate, pH 9.3 and 2 µl of 3-mercaptopropionic acid at least 90 min before use. Equal amount of sample and derivatization reagent were mixed and incubated for 5 min before injection. The chromatography system consisted of a Waters 600E system controller and 717 Plus Autosampler (Mississauga, ON). Samples were separated on a 3.9 × 150 mm Nova-Pak C18 4 µm id column coupled with a 3.9 × 20 mm Nova-Pak guard column. Asn was eluted with a gradient of sodium acetate, pH 7.0 and methanol flowing at 1 ml per min. Solvent A was 50 mM sodium acetate, pH 7.0 and solvent B was 50 mM sodium acetate, pH 7.0 and methanol (50:50). The gradient conditions were a linear increase from 100% A to 100% B during 10 min, a 6 min hold at 100% B followed by a return to 100% A at 19 min and a 7 min equilibration time. Fluorescence was monitored at 455 nm after excitation at 340 nm with a Shimadzu RF-551 fluorescence detector (Columbia, MD). Data was acquired and processed with Empower 2 software. Asn was detected on the basis of retention time established for standard amino acids. Linearity of the peak areas at different concentrations was determined. Calculations were based on the area under the peak established for a known concentration.

Generation and analysis of transgenic lines expressing ASPG promoter-GUS fusions

Two constructs were designed for each *ASPG* gene generating a translational fusion with the *uidA* reporter gene encoding GUS. One construct included the first exon and intron. The promoter sequence was considered as the interval between the last nucleotide of the previous reading frame (ORF) and the start codon of the *ASPG* gene. The interval between the start codon of *ASPGA1* and the stop codon of the preceding ORF (*At5g08000*), both on the reverse strand of chromosome 5, is equal to 267 base pairs. The length of the cloned *ASPGA1* promoter fragment was slightly longer at 426 base pairs. The distance between *ASPGB1* and the preceding ORF (*At3g16140*), on opposite strands of chromosome 3, is equal to 2,379 base pairs. The length of the cloned *ASPGB1* promoter was equal to 2,486 base pairs. Promoter fragments were PCR amplified from Columbia genomic DNA and cloned into the pCR-BluntII-TOPO vector as described above. Primers introduced *Hin*dIII and *Nco*I restriction sites at the 5′ and 3′ end, respectively: for *ASPGA1*, At5g08100piFw, 5′-AATTA AGCTTCAGCCATCATGATCATGCAGGTAACC-3′ and At5g08100proRv, 5′-AATTCCATGGGCACGCCGTGTA GCGCAAT-3′ or At5g08100piRv, 5′-AATTCCATGGC ACGACCTTACAAATGGTGATCG-3′; and for *ASPGB1*, At3g16150piFw, 5′-AATTAAGCTTTCAGGCGGGAAGG CTTGAT-3′, and At3g16150proRv, 5′-AATTCCATGGC CCACCACCCATATTCT-3′ or At3g16150piRv, 5′-AAT

TCCATGGGTTCCAATTCTCTAATCTGTGTTAATC-3′. Fragments were digested with *Hin*dIII and *Nco*I, and ligated into the same sites of the pCAMBIA1302tZ-PrxN-GUS vector using T4 DNA ligase. This vector was designed to avoid interference between the constitutive promoter driving the hygromycin resistance marker and the tissue-specific ASPG promoter driving reporter gene expression. The pCAMBIA1302tZ-PrxN-GUS vector was modified from vector L12 in Gudynaite-Savitch et al. (2009) where a 2.7 kb *lacZ* fragment found in vector L10 was inserted immediately 5′ to the promoter. The constructs were electroporated into *Agrobacterium tumefaciens* C58 cells, which were used to transform ecotype Columbia plants by the floral dip method (Clough and Bent 1998). Transformants were selected on MS media with hygromycin (50 μg ml^{-1}). For each construct, twenty resistant seedlings were transplanted to soil. These lines were screened for GUS activity in half expanded leaves, flower and silique by histochemical staining (Jefferson et al. 1987). Histochemical staining was performed in incubation buffer containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc), 50 mM sodium phosphate pH 7.2 and 0.5% Triton X-100. Plant material was visualized under an SMZ1500 stereomicroscope equipped with a DXM-1200C digital camera (Nikon Instruments, Melville, NY). Progeny of positive lines was screened for the presence of a single insertion by segregation analysis of hygromycin resistance using approximately 100 individuals, and the results analyzed by Chi-Square goodness of fit. A homozygous individual was identified by screening progeny at the following generation. Further experiments were carried out with a single, representative line, designated as ASPGA1PI::GUS-20 and ASPGB1P::GUS-19.

Statistical analysis

Unpaired, two-tailed *t* test assuming homogeneity of the variances was performed with the Excel software (Dytham 1999). ANOVA was performed using the SuperANOVA statistical program (Abacus Concepts, Berkeley, CA) and SAS version 9.2 (Toronto, ON). Homogeneity of the variances was inspected by residual graphic analysis. When variances were not homogeneous, depending on their distribution, variables were transformed as $\log(x + 1)$.

Results

Insertion mutants of ASPGs display increased sensitivity to inhibition of root elongation and root hair formation by Asn as a single N source

Homozygous T-DNA insertion lines were isolated for *ASPGA1*, SALK_087377 (*aspga1-1*) (Alonso et al. 2003) and SAIL_690_E07 (*aspga1-2*) (Sessions et al. 2002), and for *ASPGB1*, SALK_082112 (*aspgb1-1*) and WISCDsLox 382A10 (*aspgb1-2*) (Woody et al. 2007). These insertions were all localized in the second exon (Fig. 1a). To inactivate both *ASPG* genes simultaneously, a homozygous double mutant *aspga1-1/-b1-1* was isolated through a cross. To confirm that the *ASPG* genes were inactivated by the T-DNA insertions, total RNA from floral tissue of homozygous plants was analyzed by reverse transcription (RT)-PCR, which revealed the absence of detectable transcripts in mutant lines when compared with the wild-type, parental Columbia ecotype (Fig. 1b, c). To test whether single and double mutant lines were impaired in Asn catabolism, plants were grown on vertical plates containing defined media without N or with 5 mM Asn as a single N source and root length was measured after 10 days. In controls without N, root elongation was equal to approximately 80% of that with 2.5 mM ammonium nitrate or 5 mM Gln. Root elongation was inhibited by Asn, and *aspg* mutants were more sensitive to Asn than wild-type (Table 1; Fig. 1d). The percentage of root elongation of *aspga1-1/-b1-1* was significantly lower than *aspga1-1* or *aspgb1-1* suggesting an additive effect of the single mutations. Independent T-DNA insertion lines were assayed in a separate experiment. Their percentage of root elongation was also significantly lower than wild-type, confirming that this phenotype was due to the inactivation of *ASPG* genes.

This root phenotype was further characterized by comparing Columbia and the *aspga1-1/-b1-1* double mutant. To evaluate whether differences in root elongation were due to variable rates of germination, seeds were germinated on defined media without N as control or with 2.5, 5 or 10 mM Asn and germination scored at 30, 48, 52 and 72 h after sowing. Under control conditions without N,

Table 1 Percentage of root elongation after 10 days of growth on defined media containing 5 mM Asn as a single N source compared with control conditions without N

Experiment 1		Experiment 2	
Genotype	Percent elongation (%)	Genotype	Percent elongation (%)
Columbia	49.1	Columbia	60.4
aspga1-1	35.4	*aspga1-2*	46.9
aspgb1-1	21.0	*aspgb1-2*	39.0
aspga1-1/-b1-1	9.0		
ANOVA *p* value	0.0001		0.001
LSD	7.7		6.4

$n = 4$; *ANOVA* analysis of variance, *LSD* Fisher's protected least significant difference at $p \leq 0.05$; each experimental unit consisted of a plate with approximately 25 seedlings

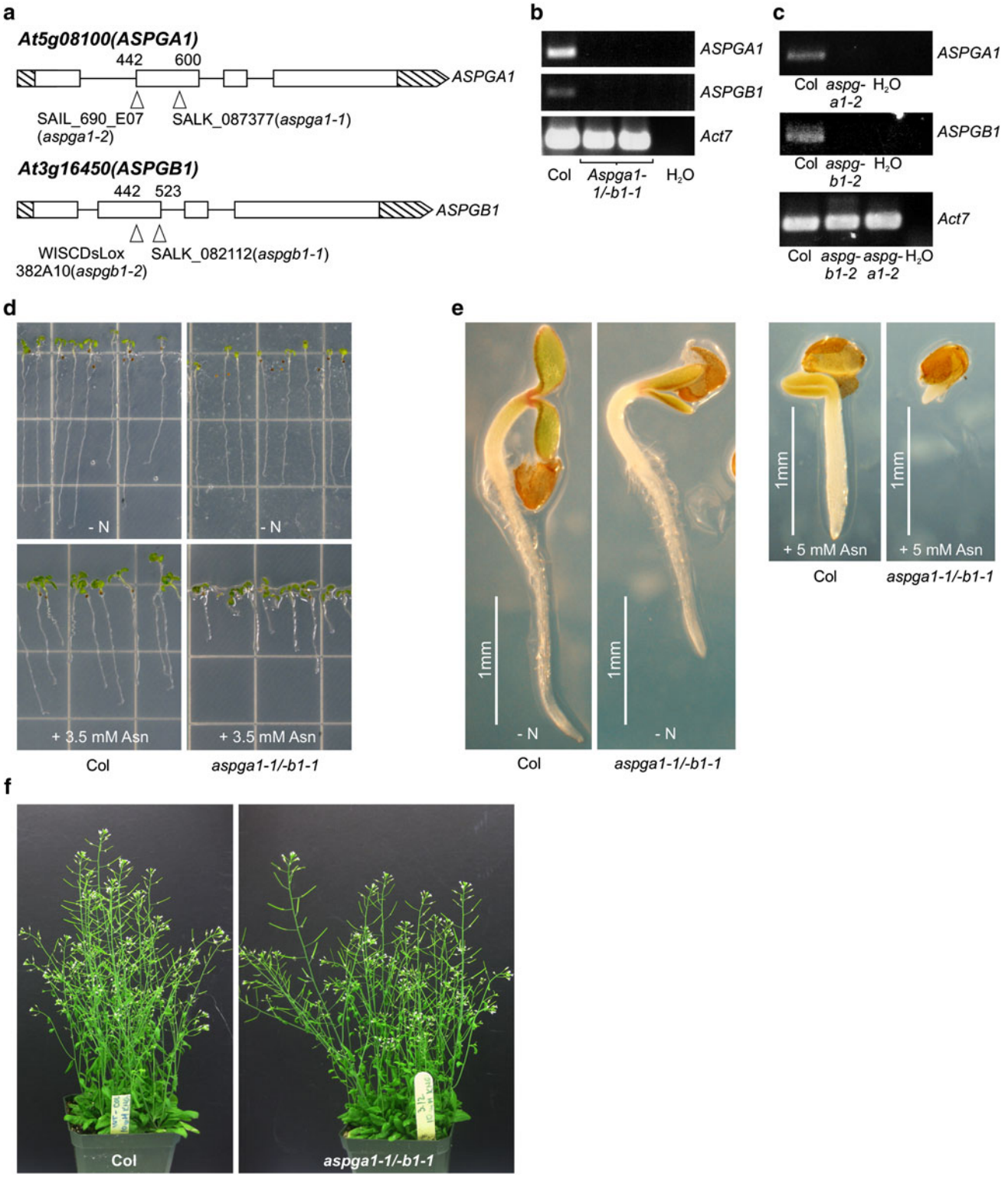


Fig. 1 Characterization of T-DNA insertion mutants of asparaginases. **a** Schematic representation of *ASPGA1* and *-B1* loci, showing the position of T-DNA insertions. *Bars* represent exons, *hatched regions* represent untranslated regions, *triangles* indicate the site of T-DNA insertion. Position of insertions with respect to beginning of first exon is indicated in bp. RT-PCR analysis shows absence of *ASPG* transcripts in floral tissue of *aspga1-1/-b1-1* (**b**), *aspga1-2* and *aspgb1-2* (**c**). **d** Root elongation phenotype of wild-type, Columbia and *aspga1-1/-b1-1* seedlings grown for 10 days on defined media without N or with 3.5 mM Asn. **e** Root hair phenotype of wild-type and *aspga1-1/-b1-1* seedlings after 72 h of growth on defined media without N or with 5 mM Asn. **f** Columbia and *aspga1-1/-b1-1* plants grown under long day conditions (16 h light, 8 h dark) and fertilized with 10 mM potassium nitrate

percentages of germination were similar between genotypes ($n = 3$; evaluated by one-way ANOVA at $p \leq 0.05$). For each Asn concentration, the percentage of germination at a given time point was compared to the control without N by Dunnett's one-tailed test. Values significantly different from the control were observed at 30 h with 5 mM Asn, and at all four time points at 10 mM ($p \leq 0.05$). For each dose, these values were summed, treated as data from a factorial experiment with genotype and dose as variables, and analyzed by two-way ANOVA (at 5 mM Asn, the sum was equal to 12 and 10% for Columbia and *aspga1-1/-b1-1*, respectively, and at 10 mM Asn, 132 and 117%). Asn caused a significant, dose-dependent delay in germination (D; $p \leq 0.0001$). However, there was no significant difference in germination rate between genotypes (G; not significant). A similar experiment was performed where root length was measured after 10 days (Table 2). Under control conditions without N, root elongation of the wild-type and *aspga1-1/-b1-1* was similar. Results indicated a dose-dependent inhibition of root elongation by Asn (D; $p \leq 0.0001$). They confirmed that *aspga1-1/-b1-1* was more inhibited than wild-type (G; $p \leq 0.0001$). To test the hypothesis that increased inhibition of root elongation by Asn in the mutant may be related to a higher accumulation of the amide due to the lack of ASPGs, seedlings were grown for 12 days on control, defined media without N or with 2 mM Asn, free amino acids were extracted from root tissue and Asn quantified. No Asn was detected from seedlings grown on media without N. However for the Asn treatment, roots from *aspga1-1/-b1-1* seedlings contained approximately twice as much free Asn as those from wild-type seedlings (90.3 ± 2.6 vs. 48.6 ± 2.0 nmol mg^{-1} fresh weight; average ± standard deviation; $n = 4$; t test p value ≤ 0.00002). A second effect of Asn on root growth was an inhibition of root hair formation. To analyze this response, wild-type and *aspga1-1/-b1-1* were grown on control, defined media without N or with different concentrations of Asn. The percentage of seedlings having root hairs in Asn treated versus control conditions was measured at different time points (Table 3). For each Asn concentration, values at different time points were summed and treated as data from a factorial experiment as described above. Asn caused a dose-dependent inhibition of root hair formation (D; $p \leq 0.01$). The *aspga1-1/-b1-1* mutant was more sensitive than wild-type to this inhibition (G; $p \leq 0.0005$). This inhibition likely reflects, at least in part, a general delay in root growth (Fig. 1e).

Table 2 Root length (mm) of *Arabidopsis* seedlings grown on different concentrations of Asn as a single N source for 10 days

[Asn] (mM)	Root length (mm)	
	Columbia	*aspga1-1/aspgb1-1*
0	37.55[a]	35.17
2.5	22.72	13.63
5	18.66	7.35
10	11.24	4.62
Sources of variation	*df*	ANOVA p value
Dose (D)	2	0.0001
Genotype (G)	1	0.0001
D × G	2	0.04[b]
Error	12	

$n = 3$; values for treatments (2.5, 5 and 10 mM Asn) were significantly different from controls without N according to Dunnett's one-tailed test at $p \leq 0.05$ and were analyzed by two-way ANOVA

[a] Controls without N were not significantly different between genotypes according to one-way ANOVA at $p \leq 0.05$

[b] After $\log(x + 1)$ transformation of the data; graph of D × G interaction indicated an overlap of standard deviations of the means for Columbia at 2.5 and 5 mM Asn (Supplementary Figure 1a)

Lack of ASPG impacts free amino acid profiles in mature seed exclusively under conditions of low C and high N supply

Due to the likely functional redundancy of ASPGA1 and -B1, phenotypic characterization focused on the double *aspga1-1/-b1-1* mutant, which should be devoid of ASPG activity. The effect of *ASPG* inactivation on growth and seed characteristics was examined under different conditions of N regime and photoperiod. Growth was initially compared under long day conditions (16 h light, 8 h dark) and fertilization with 0, 0.25 or 2.5 mM ammonium nitrate. Under the control, 0 mM ammonium nitrate condition, plants relied exclusively on fertilizer pre-added to the substrate for their N nutrition. Seed yield was similar between genotypes, of 63 ± 22 and 74 ± 30 mg seed per plant at 2.5 mM ammonium nitrate, 52 ± 11 and 50.5 ± 12 at 0.25 mM, and 3 ± 2 and 5 ± 3 at 0 mM for Columbia and *aspga1-1/-b1-1*, respectively ($n = 10$; not significant by t test at $p \leq 0.05$). Seed size was similar between genotypes at 0.25 and 2.5 mM ammonium nitrate (Table 4). However, at 0 mM, seed size was significantly reduced in Columbia, by approximately 12%, but not in *aspga1-1/-b1-1* (significant D × G interaction, $p \leq 0.03$). Under this condition, *aspga1-1/-b1-1* seeds were significantly larger than wild-type, by approximately 16%.

Plants were subsequently grown in a substrate without pre-added nutrients and fertilized with 3 or 10 mM potassium nitrate. Bi et al. (2005) and Peng et al. (2007) showed that under these conditions, fertilization with 10 mM potassium nitrate constitutes an N-sufficient condition whereas 3 mM potassium nitrate restricts growth at the reproductive stage. During the different growth experiments, no visual differences were observed between genotypes (see Fig. 1f, for an example at 10 mM potassium

Table 3 Ratio of seedlings having root hairs over total number of seedlings in Asn treated versus control without N conditions (%)

[Asn] (mM)	Genotype	48 h	52 h	72 h	7 day	8 day	9 day	10 day
3.5	Columbia	49	66	97	97	98	98	100
	aspga1-1/-b1-1	10	16	25	65	80	91	93
5	Columbia	9	12	46	93	91	91	93
	aspga1-1/-b1-1	3	4	8	22	27	30	40
Sources of variation		*df*	ANOVA *p* value					
Dose (D)		1	0.01					
Genotype (G)		1	0.0005					
D × G		1	0.0001[a]					
Error		12						

$n = 4$; two-way ANOVA was performed on the sum of values at different time points

[a] Graph of D × G interaction indicated an overlap of standard deviations of the means for Columbia at 5 mM Asn and *aspga1-1/-b1-1* at 3.5 mM (Supplementary Figure 1b)

Table 4 Seed size of wild-type Columbia and *aspga1-1/-b1-1* fertilized with different concentrations of ammonium nitrate

[Ammonium nitrate] (mM)	Genotype	Seed size (mm^2)	
0	Columbia	0.379 ± 0.011	
	aspga1-1/-b1-1	0.439 ± 0.017	
0.25	Columbia	0.422 ± 0.004	
	aspga1-1/-b1-1	0.460 ± 0.014	
2.5	Columbia	0.432 ± 0.015	
	aspga1-1/-b1-1	0.443 ± 0.017	
Sources of variation		*df*	ANOVA *p* value
Dose (D)		1	0.0001
Genotype (G)		2	0.003
D × G		2	0.03
Error		12	

$n = 3$; average ± standard deviation; each replicate consisted of 50 seeds from an individual plant

nitrate). Free amino acids were extracted and quantified from mature seed. The levels of free Asn and total free amino acids were higher at 10 mM than 3 mM potassium nitrate. However, there were no significant differences between genotypes (data now shown). Plants were then grown under low illumination of 70 µmol photons m^{-2} s^{-1} instead of the standard illumination of 115 µmol photons m^{-2} s^{-1}. For this experiment, plants were grown in a substrate with pre-added nutrient and fertilized bi-weekly with 20:20:20. Growth under low illumination, resulted in lower carbon (C) and a decreased C:N ratio in mature seed (Supplementary Table 1). These results are consistent with the observation that low illumination reduces oil content in *Arabidopsis* seed (Li et al. 2006). Under each condition, wild-type and mutant seed had a similar C, sulfur (S) and N content. Analysis of free amino acid profiles in mature seed revealed significant differences between genotypes under low illumination. Free Asn levels were increased by 2.6-fold in *aspga1-1/-b1-1* when compared with wild-type, and total free amino acids were increased by 1.9-fold (Table 5). This experiment was repeated with weekly instead of biweekly fertilization and similar results were obtained. Free Asn content was raised from 9.8 ± 0.5 nmol per mg seed weight in Columbia to 18.6 ± 0.5 nmol per mg seed weight in *aspga1-1/-b1-1* ($n = 4$; p value ≤ 0.00001), while total free amino acids were raised from 30.3 ± 2.0 to 42.9 ± 3.0 nmol per mg seed weight ($p \leq 0.0004$). Analysis of total amino acid profiles revealed no significant differences between genotypes (Supplementary Table 2) consistent with results obtained for N content.

Developmental expression of ASPG

To investigate the relationship between these phenotypes and *ASPG* gene expression, transgenic lines expressing PASPG-GUS fusions were generated and characterized. For *ASPGA1*, inclusion of the first exon and intron was required to visualize reporter gene expression by histochemical staining, a common occurrence in plant genes (Rose et al. 2008), but this did not influence *ASPGB1* promoter-driven expression. Expression of PASPGB1-GUS was detected exclusively in developing pollen grains

Table 5 Free amino acid profile of mature seed from wild-type and *aspga1-1/-b1-1* mutant grown under low illumination

	Columbia	*aspga1-1/ aspgb1-1*	*p* value
Asp	0.32 ± 0.08	0.47 ± 0.10	0.01
Glu	0.98 ± 0.22	1.4 ± 0.3	0.01
Asn	1.7 ± 0.8	4.4 ± 2.8	0.02
Ser	0.20 ± 0.06	0.33 ± 0.10	0.005
Gln	0.39 ± 0.13	0.76 ± 0.22	0.001
Gly	0.12 ± 0.04	0.23 ± 0.05	0.0001
His	0.029 ± 0.009	0.051 ± 0.017	0.006
Arg	0.21 ± 0.11	0.46 ± 0.26	0.02
γ-Amino butyric acid	0.067 ± 0.025	0.14 ± 0.04	0.001
Thr	0.12 ± 0.05	0.22 ± 0.07	0.006
Ala	0.46 ± 0.15	0.62 ± 0.14	0.03
Pro	0.66 ± 0.46	1.2 ± 0.4	0.03
Amino-*N*-butyric acid	0.035 ± 0.017	0.066 ± 0.020	0.005
Tyr	0.065 ± 0.018	0.096 ± 0.019	0.004
Val	0.20 ± 0.07	0.34 ± 0.08	0.004
Met	0.12 ± 0.05	0.20 ± 0.05	0.006
Ile	0.10 ± 0.04	0.16 ± 0.04	0.004
Leu	0.060 ± 0.020	0.11 ± 0.02	0.001
Phe	0.066 ± 0.027	0.14 ± 0.06	0.005
Ornithine	0.002 ± 0.001	0.001 ± 0.001	n.s.
Lys	0.021 ± 0.013	0.036 ± 0.021	n.s.
Total free amino acids	5.93 ± 1.90	11.3 ± 4.1	0.005

Values are expressed in nmol per seed weight ± standard deviation; $n = 4$; p value determined by t test

(Fig. 2m), where PASPGA1-GUS was also expressed (Fig. 2k, l), consistent with the results of Grant and Bevan (1994) and microarray data of Honys and Twell (2004) indicating peak expression at the tricellular stage [visualized with the Arabidopsis eFP Browser; http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi (Winter et al. 2007)]. The lack of PASPGB1-GUS detection in tissues other than developing pollen grains is consistent with the lower absolute levels of *ASPGB1* transcripts when compared with *ASPGA1* in *Arabidopsis* tissues (Bruneau et al. 2006). Expression driven by PASGB1 is probably below the threshold necessary to detect GUS reporter activity in other tissues.

During germination on media without N, PASPGA1-GUS expression appeared in endosperm cells prior to its rupture, starting at the micropylar end (Fig. 2a). Immediately after rupture of the endosperm, GUS was expressed throughout the root and hypocotyl and in immature root hairs (Fig. 2b, c). Expression was strong in the hypocotyl and in the elongation zone of the root 2 days post sowing (Fig. 2d). From day 3, expression became restricted to the root tip (Fig. 2e). When grown on MS media containing 40 mM ammonium nitrate, GUS expression was detected

Fig. 2 Expression of PASPGA1- (**a–l**, **n**) and PASPGB1-GUS (**m**) fusions visualized by histochemical staining. Plants were grown on defined media without N and assayed 24–30 h (**a–c**), 2 day (**d**) and 4 day post sowing (**e**). **a** Expression in endosperm. Expression was observed in the root, hypocotyl and immature root hairs (**b**, **c**). Plants were grown on MS media and assayed after 8 (**f**) or 11 days (**g**, **h**). Expression was observed in lateral root primordia (**f**), tip of lateral root (**g**), immature trichomes and shoot apex (**h**). Plants were grown in soil (**i–n**) and expression was detected at the shoot apex and in leaf vasculature after 17 days (**i**), inflorescence stem after 28 days (**j**), pollen grains (**k–m**), and developing seeds 3 days after fertilization (**n**)

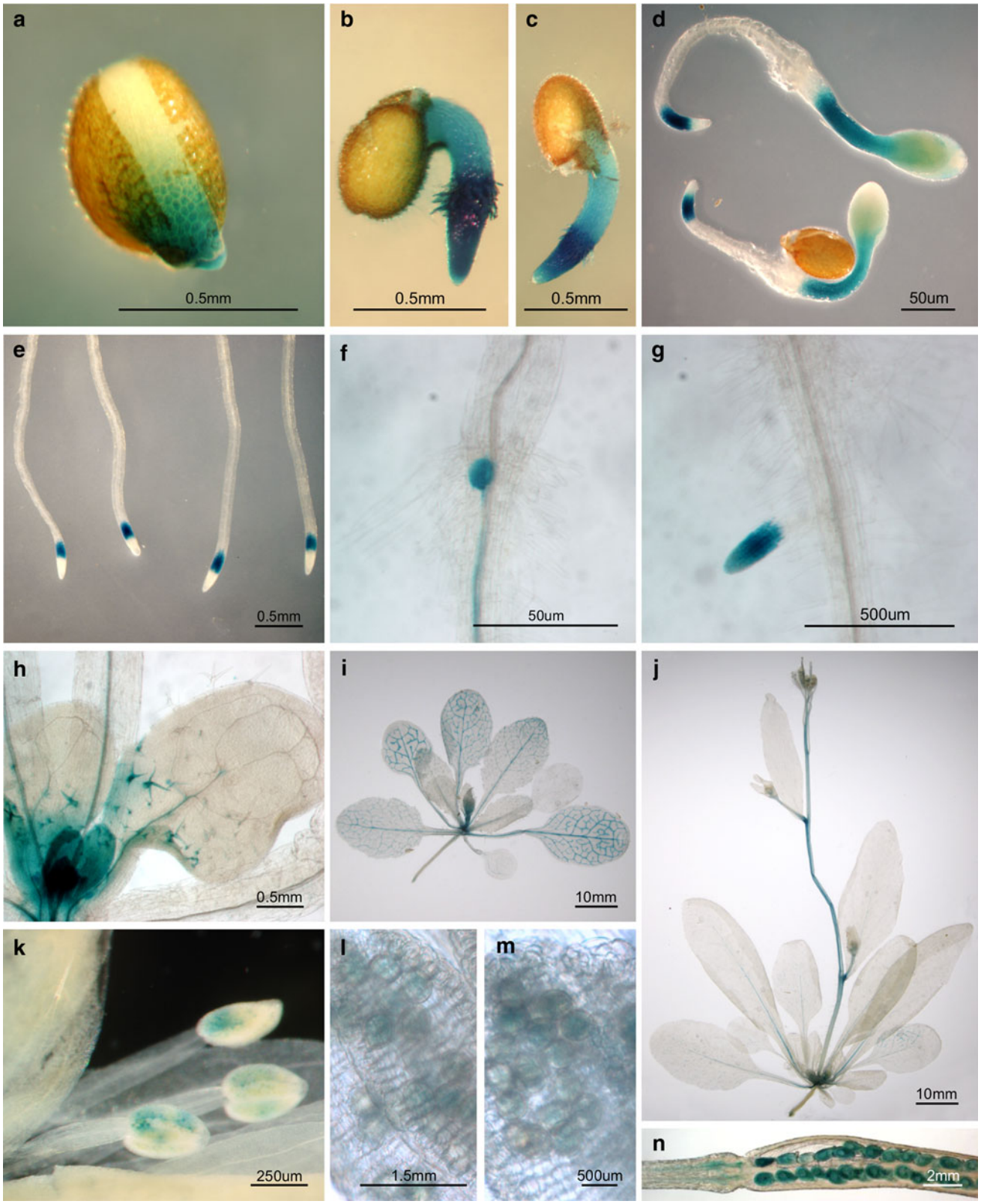
in quiescent lateral root primordia and at the tip of young lateral roots (Fig. 2f, g). Expression was particularly strong near the shoot apical meristem, and also present in immature trichomes at the base of the leaf (Fig. 2h). In soil grown plants, expression was observed in leaf vascular tissue (Fig. 2i). Shortly after bolting, GUS staining was present throughout the upper part of the inflorescence stem (Fig. 2j). During reproductive development, expression was observed in developing seeds 3 days after fertilization (Fig. 2n).

Discussion

ASPG function in vivo

Analyses with promoter-GUS fusions revealed sites of *ASPG* gene expression that were previously not described, such as in germinating seeds, root tip, quiescent lateral root primordia, immature root hair and trichome. The observation that PASPGA1-GUS expression in endosperm cells is initiated at the micropylar end, prior to endosperm rupture suggests that N stored in protein bodies of the endosperm (Sanders et al. 2009) is rapidly mobilized to sustain early stages of germination, similar to the mobilization of storage lipids via gluconeogenesis (Penfield et al. 2004). In castor, Gln is the main amino acid transported from the endosperm to cotyledons in germinating seed (Robinson and Beevers 1981). Ammonia liberated by ASPG may be re-assimilated via the glutamine synthetase-glutamate synthase cycle in endosperm cells prior to release to the apoplast. The high level of expression in hypocotyl and root of germinating seedlings indicates that ASPGA1 may be involved in the catabolism of Asn transported from cotyledons following the mobilization of storage protein. The early decline in PASPGA1-GUS expression in seedlings at 3 days and its spatial restriction to the root tip suggests a transition to N-heterotrophic growth taking place at this developmental stage.

The results of bioassays performed with single and double *aspg* insertion mutants grown on Asn as a sole



source of N suggest that ASPGs are the main enzymes involved in Asn catabolism in root. The increased accumulation of Asn in *aspga1-1/-b1-1* compared to wild-type reveals a partial blockage in Asn catabolism.

The significance of the effect of *aspg* mutations on seed size at severely limiting N levels is unclear. Further analyses are required to understand how Asn catabolism by ASPGs is linked to the slight decrease in size in wild-type

seeds under these conditions. Nonetheless, under N-sufficient conditions, the lack of ASPGs in the double mutant led to a surprisingly mild biochemical phenotype in mature seed, considering the function hypothesized for these enzymes in the supply of N to developing seeds. The increased free Asn content was conditional on growth conditions resulting in a low ratio of C:N translocated to the seed. The fact that Asn is not dominant in the phloem sap of Brassicaceae, accounting for about 5% of total amino acids, when sampled in the second half of the light period (Lohaus and Moellers 2000), may partially account for this weak phenotype. The results point to the conclusion that an alternative enzyme can metabolize Asn in the absence of ASPGs. A primary candidate is serine:glyoxylate aminotransferase (At2g13360) (Liepman and Olsen 2001; Kendziorek and Paszkowski 2008). Transcript levels of serine:glyoxylate aminotransferase are low in the root of seedlings, but parallel those of ASPGs in seed and silique, being highest at early developmental stages (Schmid et al. 2005). They are also high in dry seed but decline rapidly during seed imbibition (Nakabayashi et al. 2005). The results highlight the metabolic plasticity of central amino acid pathways in higher plants.

Root responses to Asn

The results presented here identified several growth responses to Asn. The delay in seed germination occurred at high concentrations and was not influenced by ASPG deficiency, indicating that the onset of inhibition occurred prior to *ASPG* expression. The degree of inhibition of root elongation and root hair formation was correlated with the level of internal Asn accumulation as observed in the *aspga1-1/-b1-1* mutant. This suggests that inhibition of root growth is elicited by Asn itself, or an Asn-derived metabolite, distinct from the products of the ASPG reaction, Asp and ammonia. These responses are distinct from inhibition of primary root elongation and stimulation of root branching by Glu, which involves local perception at the root tip, without a global increase in Glu concentration (Walch-Liu et al. 2006).

The present results extend several recent observations on the uptake of amino acids and the effects of Asn on growth of *Arabidopsis*. Plants are capable of organic N uptake from the soil in the form of amino acids (Näsholm et al. 2009). *Arabidopsis* LHT1 and AAP1 have been characterized as high and low affinity transporters for uptake of neutral amino acids in root, respectively (Hirner et al. 2006; Svennerstam et al. 2007; Lee et al. 2007). Asn, Gln, Glu, Asp and γ-aminobutyric as single N sources at a concentration of 5 mM were previously shown to support the growth of the Columbia ecotype for up to 21 days, although Asn had an inhibitory effect compared with the four other amino acids (Hirner et al. 2006). This inhibition was less pronounced in the *lht1-1* mutant, and enhanced in P35S-LHT1 transgenic plants suggesting a direct link with internal Asn accumulation. Forsum et al. (2008) reported that Asn at a concentration equivalent to 3 mM N stimulated growth measured at 20 days in comparison with a control without N, but not as much as Gln, meaning that plants grown on media without N will eventually deplete their internal reserves, whereas Asn can be utilized as a suitable N source. The present study focused on shorter growth periods of 10–12 days. The findings reported here are also consistent with the prior observation that Asn at 5 mM with 0.5 mM potassium nitrate inhibits primary root growth at 14 days, when compared with 0.5 mM potassium nitrate alone, in combination with Gln, or 10 mM potassium nitrate (Tranbarger et al. 2003). In the present study, root elongation and root hair formation appeared sensitive to an excess of internal Asn. Analysis of gene expression using a promoter-GUS fusion suggested that ASPGA1 catabolizes Asn in the root tip, near the elongation zone of the root, thereby controlling its local concentration. Whether this sensitivity is linked to a possible role for Asn as an N-satiety signal in root is unknown. Asn has recently been hypothesized as a feedback signal regulating N fixation in *Medicago truncatula*, by comparing phloem and root nodule amino acid profiles under growth conditions resulting in reduced N fixation activity (Sulieman et al. 2010). A similar signal has been postulated to regulate N uptake, whose rate is usually far below capacity (Miller et al. 2008; Imsande and Touraine 1994). Cycling of amino acids in phloem has been shown to be involved in this regulatory process.

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Conflict of interest The authors declare that they have no conflict of interest.

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