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GASA4, One of the 14-Member *Arabidopsis* GASA Family of Small Polypeptides, Regulates Flowering and Seed Development

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Members of the plant-specific gibberellic acid-stimulated *Arabidopsis* (*GASA*) gene family play roles in hormone response, defense and development. We have identified six new *Arabidopsis GASA* genes, bringing the total number of family members to 14. Here we show that these genes all encode small polypeptides that share the common structural features of an N-terminal putative signal sequence, a highly divergent intermediate region and a conserved 60 amino acid C-terminal domain containing 12 conserved cysteine residues. Analysis of *promoter::GUS* (β-*glucuronidase*) transgenic plants representing six different *GASA* loci reveals that the promoters are activated in a variety of stage- and tissue-specific patterns during development, indicating that the *GASA* genes are involved in diverse processes. Characterization of *GASA4* shows that the promoter is active in the shoot apex region, developing flowers and developing embryos. Phenotypic analyses of *GASA4* loss-of-function and gain-of-function lines indicate that *GASA4* regulates floral meristem identity and also positively affects both seed size and total seed yield.

Keywords: Floral meristem identity — Gene regulation — Gibberellic acid-stimulated *Arabidopsis* (*GASA*) gene family — Seed development — Seed size.

Abbreviations: EST, expressed sequence tag; GASA, gibberellic acid-stimulated *Arabidopsis* (*GASA*); GUS, β-glucuronidase.

Introduction

Hormone-regulated gene families play key roles in diverse biological processes such as flower induction, seed development and germination in a wide range of monocotyledonous and dicotyledonous plant species. Members of the gibberellic acid-stimulated *Arabidopsis* (*GASA*) gene family form a plant-specific group of genes present in a wide range of species including tomato, rice, petunia, gerbera and *Arabidopsis*. Several *GASA*-like genes are hormone responsive, and are proposed to participate in pathogen responses as well as various aspects of plant development. The tomato gene *GAST1* was the first member of the family to be characterized, and its expression was induced upon application of exogenous gibberellin in a gibberellin-deficient background (Shi et al. 1992). A related tomato gene, *RSI-1*, shares high sequence identity with *GAST1* and is activated during lateral root formation (Taylor and Scheuring 1994). The *GASA* homologs *GIP1*, *GIP2*, *GIP4* and *GIP5* from petunia are activated by gibberellin in vitro (Ben-Nissan and Weiss 1996, Ben-Nissan et al. 2004), and *GIP2* RNAi lines displayed late flowering and reduced stem elongation, suggesting a developmental role in promoting internode elongation and the transition to flowering (Ben-Nissan et al. 2004). The *GASA* homolog *GEG* from *Gerbera hybrida* is expressed in corollas and carpels, and its expression coincides with the cessation of longitudinal cell expansion (Kotilainen et al. 1999). Plants ectopically expressing *GEG* developed shorter corollas with decreased cell length compared with the wild type, indicating a role for *GEG* as an inhibitor of cell elongation.

Eight *GASA* genes have previously been identified in *Arabidopsis thaliana*. *GASA1–GASA4* were first identified based on their similarity to tomato *GAST1* (Herzog et al. 1995). Expression data indicated that *GASA1* accumulates in flower buds and immature siliques, *GASA2* and *GASA3* in siliques and dry seeds, and *GASA4* in growing roots and flower buds. Further studies showed *GASA4* to be expressed in all meristematic regions—in shoots, in flower buds, and in primary and lateral roots (Aubert et al. 1998). Similarly to tomato *GAST1*, *GASA1* and *GASA4* are activated by the bioactive gibberellin GA_3 added exogenously in a gibberellin-deficient background (Aubert et al. 1998). In an attempt to unravel cross-talk between brassinosteroids and gibberellins, Bouquin et al. (2001) also found that gibberellin activates and brassinosteroids inhibit *GASA1* expression. Four additional *GASA* genes were uncovered based on sequence analysis: *GASA5* and

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GASA6 (Aubert et al. 1998), as well as *GASA7* and *GASA8* (Berrocal-Lobo et al. 2002). However, no function has been assigned to any of the *GASA* genes in *Arabidopsis*.

The observation that several *Arabidopsis GASA* genes are expressed in flower buds suggests a possible role for members of this gene family in flower development. In *A. thaliana*, flower development is controlled in four steps (Jack 2004), the first of which is a switch from vegetative to reproductive growth by the action of flowering time genes in response to various environmental and endogenous signals. The next step integrates the activities of flowering time pathways to activate a small group of genes that specify floral meristem identity. *LEAFY* (*LFY*) and *APETALA1* (*AP1*) are major floral meristem identity genes that specify lateral meristems to develop into flowers rather than axillary inflorescence shoots (Weigel et al. 1992, Mandel and Yanosky 1995, Weigel and Nilsson 1995). LFY is a key integrator of signals from pathways that promote flowering, including the gibberellin pathway (Blazquez and Weigel 2000). The meristem identity genes activate the class A [*AP1* and *APETALA2* (*AP2*)], class B [*APETALA3* (*AP3*) and *PISTILLATA* (*PI*)] and class C [*AGAMOUS* (*AG*)] floral organ identity genes that pattern discrete regions of the flower. Complexes of the floral organ identity transcription factors subsequently activate downstream 'organ-building' genes, which specify various cell types and tissues that will constitute the four organs of the mature flower. *AP2* is unique among these genes in that it also plays a key role in seed development controlling seed mass (Jofuku et al. 2005, Ohto et al. 2005). The expression of *GASA4* and *GASA6* is affected in plants with altered *LFY* or *AP3*/*PI* activity, respectively (Zik and Irish 2003, Wagner et al. 2004), suggesting a role for these *GASA* genes in flower meristem and/or flower organ development.

In this work, we identify six new members of the *GASA* gene family in *A. thaliana*, *GASA9–GASA14*. Analyses of the *GASA1*, *GASA3*, *GASA4*, *GASA8*, *GASA10* and *GASA14* promoters show that all are activated in root tissue and the vasculature, although in distinct spatial and temporal patterns, as well as in other tissues including meristems, flowers and seeds. Through phenotypic analysis of mutant and transgenic plants, we show that *GASA4* plays a role in regulating floral meristem and floral organ identity, and promotes seed size and weight. This is the first assignment of a function to the *Arabidopsis GASA* genes, and suggests that members of the *GASA* gene family have regulatory roles in addition to the structural roles indicated by previous studies.

Results

The GASA gene family consists of 14 genes

In an effort to analyze the *GASA* gene family thoroughly, in *A. thaliana*, we identified six new members of the family through whole-genome data mining. We named them *GASA9–GASA14* according to the nomenclature established in the literature, and propose that the *GASA* gene family in *Arabidopsis* consists of 14 members (Table 1). The genes were identified using the criteria of overall nucleotide sequence similarity, and a common predicted protein structure consisting of an N-terminal signal sequence and a C-terminal conserved cysteine-rich domain (Herzog et al. 1995). We discovered that *GASA11* (At2g18420/*GASA11*) was incorrectly

Table 1 Overview of the *GASA* family, presenting *GASA* number, *Arabidopsis* gene number, current annotation in databases, expression information based on isolated clones and predicted signal sequence cleavage sites

GASA gene number	*Arabidopsis* gene number	Annotated as	Expression	Signal sequence cleavage after amino acid
GASA1	At1g75750	GASA1	cDNA	23
GASA2	At4g09610	GASA2	cDNA	25
GASA3	At4g09600	GASA3	cDNA	26
GASA4	At5g15230	GASA4	cDNA	25
GASA5	At3g02885	GASA5	cDNA	27
GASA6	At1g74670	Gibberellin-responsive protein, putative	cDNA	23
GASA7	At2g14900	Gibberellin-regulated family protein	cDNA	23
GASA8	At2g39540	Gibberellin-regulated family protein	cDNA	25
GASA9	At1g22690	Gibberellin-responsive protein, putative	EST	24
GASA10	At5g59845	Gibberellin-regulated family protein	cDNA	25
GASA11	At2g18420	Gibberellin-responsive protein, putative	Our cDNA	23
GASA12	At2g30810	Gibberellin-regulated family protein	EST	22
GASA13	–	Non-annotated	–	23
GASA14	At5g14920	Gibberellin-regulated family protein	cDNA	21

GASA9–GASA14 have been defined as part of the *GASA* family in this work.

annotated. While gene predictions based on the *Arabidopsis* genomic sequence annotated the putative gene product as containing 25 additional amino acid residues downstream of the conserved cysteine-rich domain, we found by cDNA cloning and sequencing that *GASA11* encodes a stop codon after the last residues of the conserved domain, as is the case for the other 13 *Arabidopsis GASA* genes. However, the gene we have named *GASA13* lies in a region currently annotated as not encoding genes. Although the putative *GASA13* gene sequence corresponds perfectly to the structure of the genes in the *GASA* family, there is no confirmation of expression data in the databases and nor were we successful in cloning a corresponding cDNA product. Thus it remains unclear whether this genomic sequence encodes a functional gene product.

All 14 *Arabidopsis* GASA family members have structural characteristics of secreted polypeptide molecules. Each putative protein contains an N-terminal 21–27 amino acid hydrophobic region that is predicted by SignalP v.2.0 to act as a cleavable signal sequence (Table 1), designating them to be secreted into the extracellular space. This information suggests that the functional roles of the *GASA* gene family are related to processes in the extracellular environment. Downstream of the signal sequence, the putative *GASA* gene products contain a region displaying high divergence between family members. This region is both divergent in amino acid composition, from hydrophilic to hydrophobic, and ranges in sequence length from 190 amino acid residues in GASA14 to only three residues in GASA8. The primary structural feature of the *GASA* gene family constitutes a 59–60 amino acid conserved domain at the C-terminal end of the predicted proteins. A multiple sequence alignment of this C-terminal cysteine-rich region illustrates the conservation of this domain (Fig. 1a). Approximately 30% of the domain, 19 amino acid residues, is perfectly conserved between the predicted 14 proteins of the *GASA* family. Among these 19 residues are 12 conserved cysteines, with the possibility of six disulfide bridges being formed between them. In addition, several proline and glycine residues are fully or partially conserved.

The evolutionary relationship within the GASA family was examined by constructing a phylogenetic tree based on multiple sequence alignments of the C-terminal conserved cysteine-rich region (Fig. 1b). The GASA family members appear to form four primary phylogenetic branches supported by high bootstrap values. One clade contains five members formed by two pairs of closely related proteins, plus the more divergent GASA9. These two pairs, GASA2/GASA3 and GASA1/GASA11, display >60% amino acid identity between them. The second group is formed by the closely related GASA8 and GASA10, together with the somewhat evolutionarily more distant GASA7. The third cluster contains GASA5, GASA12, GASA13, GASA4 and GASA6. The highly divergent GASA14 does not group with any other GASA protein and forms a phylogenetic branch of its own.

GASA14, the protein that is most divergent from the other members of the GASA family, contains an intermediate domain displaying significant similarities with known protein domains, having segmental sequence identity with a range of proline-rich putative proteins in *Arabidopsis* and other plant species. The sequences similar to GASA14 are mostly annotated as cell wall-associated proteins such as extensins and arabinogalactans (Fig. 1c). This compositional similarity suggests that GASA14 may be located in the cell wall after secretion, by means of association of its long proline-rich region with the cell wall.

In silico searches within the *GASA* upstream sequences for *cis*-regulatory elements known to be of importance throughout the plant kingdom, using PLACE (Higo et al. 1999), identified several gibberellin-responsive elements such as GARE, the pyrimidine box and the TATCCAY elements (e.g. Gubler and Jacobsen 1992, Gubler et al. 1999, Yanagisawa and Schmidt 1999) in all 14 *GASA* promoters. Moreover, all the *GASA* upstream sequences contain multiple elements known to direct seed- and root-specific expression (e.g. Dickinson et al. 1988, Opsahl-Sorteberg et al. 2004, Nakabayashi et al. 2005). Eight *GASA* upstream sequences also contain one or both of the LFY and WUSCHEL (WUS) sequence elements found in the *AG* intron (Lenhard et al. 2001, Lohmann et al. 2001). The *GASA5* upstream region contains one putative LFY and two WUS sequence-binding elements, and the *GASA4* upstream region contains two putative LFY-binding elements.

The GASA promoters are activated in a diverse range of tissues and developmental stages

Among the 14 identified *GASA* family members, promoter–reporter gene analyses of only *GASA1* and *GASA4* have been reported to date (Table 2). To investigate the range of *GASA* promoter activation more thoroughly, we examined the spatial localization of the β-*glucuronidase* (*GUS*) gene product driven by the *GASA1*, *GASA3*, *GASA4*, *GASA8*, *GASA10* and *GASA14* promoters. The results shown here represent consistent patterns validated by examining multiple independent transgenic lines. Our analysis of p*GASA4::GUS* plants (Fig. 2) confirms the pattern established by Aubert et al. (1998) and provides additional data. Consistent with the previous study, which used a different construct and region of the promoter, we detected promoter activity (GUS) predominantly in rapidly dividing cells and meristematic tissues. p*GASA4*-directed GUS was detected in vegetative shoot apical meristems and initiating leaves (Fig. 2a, b), as well as in the vasculature of cotyledons (Fig. 2c), hypocotyls and rosette leaves

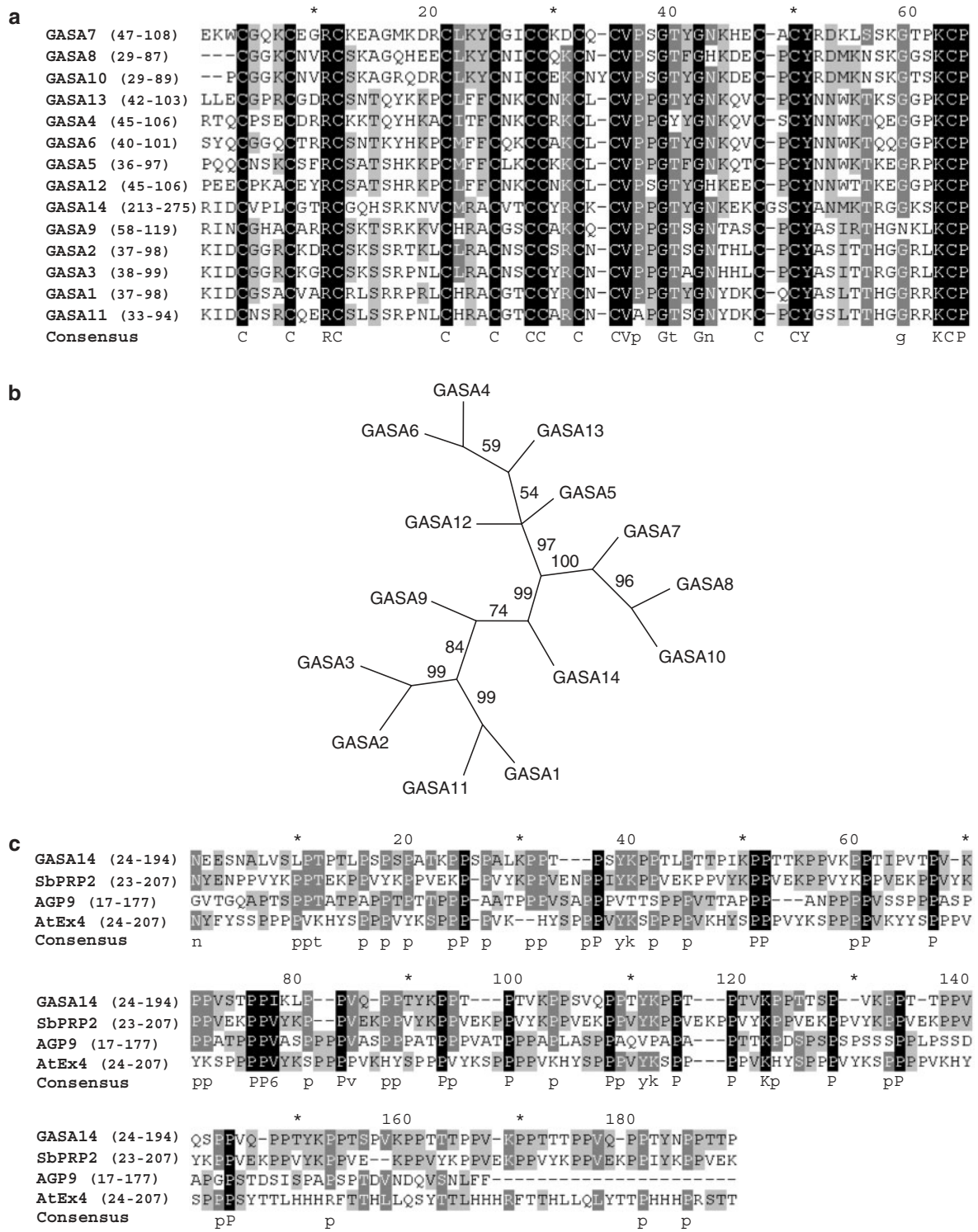

Fig. 1 The *Arabidopsis GASA* gene family. (a) Multiple sequence alignment of the conserved C-terminal domain of the 14 putative GASA proteins. Identical residues are shown in black and conserved residues are shaded, with dark gray indicating 75–99% conservation and light gray 55–74% conservation. The specific amino acids included in the alignment for each GASA protein are indicated in parentheses. (b) An unrooted phylogenetic tree constructed using neighbor-joining. The branches were reconfirmed by 1,000 bootstrap resamplings, and the bootstrap value of each branch is indicated. (c) Multiple sequence alignment of the proline-rich regions from GASA14, soybean proline-rich protein 2, SbPRP2 (AAA34011); *Arabidopsis* arabinogalactan protein 9, AGP9 (NP973463); and *Arabidopsis* extensin 4, AtEx4 (NP849895). The amino acids included are indicated in parentheses.

Table 2 Patterns of GUS localization from Arabidopsis GASA promoters as determined by Raventos et al. (2000) (Rv), Aubert et al. (1998) (A) and this work (R), and Northern expression analysis of GASA genes as determined by Hertzog et al. (1995).

GASA gene	Roots	Vasculature	Hypocotyl/cotyledon	Leaves	Seeds	Shoot apex	Abscission zone	Flowers
GASA1	+(Rv,R)	+(Rv,R)	+(Rv,R)	+(Rv,R)	−(R)	+(R)	+(R)	+(H,Rv,R)
GASA2	−(H)	−(H)	−(H)	−(H)	+(H)	−(H)	−(H)	−(H)
GASA3	+(R)	+(R)	+(R)	+(R)	+(H)	−(R)	−(R)	−(R)
GASA4	+(H,A,R)	+(R)	+(A,R)	+(R)	+(R)	+(A,R)	−(R)	+(H,A,R)
GASA8	+(R)	−(R)	−(R)	−(R)	+(R)	−(R)	−(R)	−(R)
GASA10	+(R)	+(R)	+(R)	+(R)	+(R)	−(R)	−(R)	−(R)
GASA14	+(R)	+(R)	+(R)	−(R)	−(R)	−(R)	+(R)	+(R)

The absence or presence of expression/promoter activity is indicated by − for not detected and + for detected, respectively.

(data not shown). In the root, GUS was observed in the distal region of the root apical meristem and the columella root cap cells (Fig. 2d), emerging lateral roots (Fig. 2e) and in the phloem of the vasculature (Fig. 2f, g). The flowers of p*GASA4::GUS* plants displayed GUS in the stylar region of the female reproductive organs, as well as strong staining in the stamen filaments and the petal vasculature (Fig. 2h, i). In seeds, p*GASA4*::*GUS* was detected in developing embryos (Fig. 2j, k). The GUS signal appeared to be uniformly distributed in the embryo and low or absent in the endosperm and maternal tissues of the seed.

p*GASA1*-driven GUS was observed in the vasculature of rosette leaves (Fig. 3a), cotyledons and hypocotyls (data not shown) as well as in the vegetative shoot apex (Fig. 3b). The flowers displayed strong GUS staining in the style, the stamen filaments and the vascular tissue of the sepals (Fig. 3c). A strong p*GASA1*::*GUS* signal was also observed in the flower abscission zone (Fig. 3d). In the underground tissue, staining was observed in the root apical meristem initials and the distal region of the root meristem (Fig. 3e), as well as in the root vasculature and emerging lateral roots (Fig. 3f). Cross-sections of the roots pinpointed GUS to the phloem cells of the vasculature (Fig. 3g).

The novel GUS localizations obtained with four additional *GASA* promoters are distinct, with only a few features in common (Fig. 4). GUS localization driven by the *GASA8* promoter was restricted to the underground tissue in the vegetative stage. Strong GUS signals were detected in the zone of elongating cells above the root tip, gradually becoming weaker in the more mature parts of the root tissue (Fig. 4a, b). In addition, p*GASA8*-driven GUS was detected in large parts of the developing seeds (Fig. 4c). p*GASA14::GUS* seedlings displayed high GUS levels in the elongating hypocotyl and at the base of the cotyledons and leaves (Fig. 4d). Flowers displayed p*GASA14*-driven GUS in the abscission zone, the style and the filaments of the stamens, resulting in a floral pattern very similar to that of *GASA1* and *GASA4* (Fig. 4e). However, p*GASA14::GUS* was not detected in sepals or petals. The roots of p*GASA14::GUS* plants displayed GUS in the young dividing root vascular cells above the root tip, and faded in the more mature vascular cells of the root (Fig. 4f). Emerging lateral roots showed strong p*GASA14::GUS* staining (Fig. 4g). p*GASA3*-driven GUS was detected in the vascular tissues of leaves from young rosette plants as well as in the root (Fig. 4h, i). At later developmental stages, p*GASA3*-driven GUS became considerably weaker and was not detected in inflorescences. p*GASA10*-directed GUS was detected in the vasculature of both rosette leaves and roots (Fig. 4j, k), and in the tips of the cotyledons and roots (Fig. 4k, l). p*GASA10*::*GUS* was also detected in a wide area in the basal parts of developing seeds (Fig. 4m).

GASA4 affects shoot branching, floral determinacy and floral organ identity

To determine the functional roles of *GASA4*, we analyzed the phenotypes of plants with altered *GASA4* activity. We identified a *GASA4* T-DNA insertion line, *gasa4-1*, in the Col-0 ecotype. *gasa4-1* plants produced no detectable *GASA4* mRNA (Fig. 5a) and thus we conclude that *gasa4-1* represents an RNA null allele. To assess the phenotypic effects of *GASA4* gain of function, we generated *35S::GASA4* overexpression lines in the Wassilewskija (Ws) ecotype, which produced highly elevated levels of *GASA4* mRNA compared with wild-type plants (Fig. 5b).

The main phenotypic aberration observed in the *gasa4-1* plants was a significant increase in the number of axillary inflorescence shoots produced prior to flower formation (Fig. 6a). Both the total number of higher order axillary inflorescence shoots originating from the primary shoot and the number of secondary inflorescences emerging directly from the primary shoot were approximately 50% higher in *gasa4-1* plants than in wild-type plants (Fig. 6a). However, the number of leaves produced during vegetative development was similar in the *gasa4-1* plants compared with wild-type plants, suggesting that the

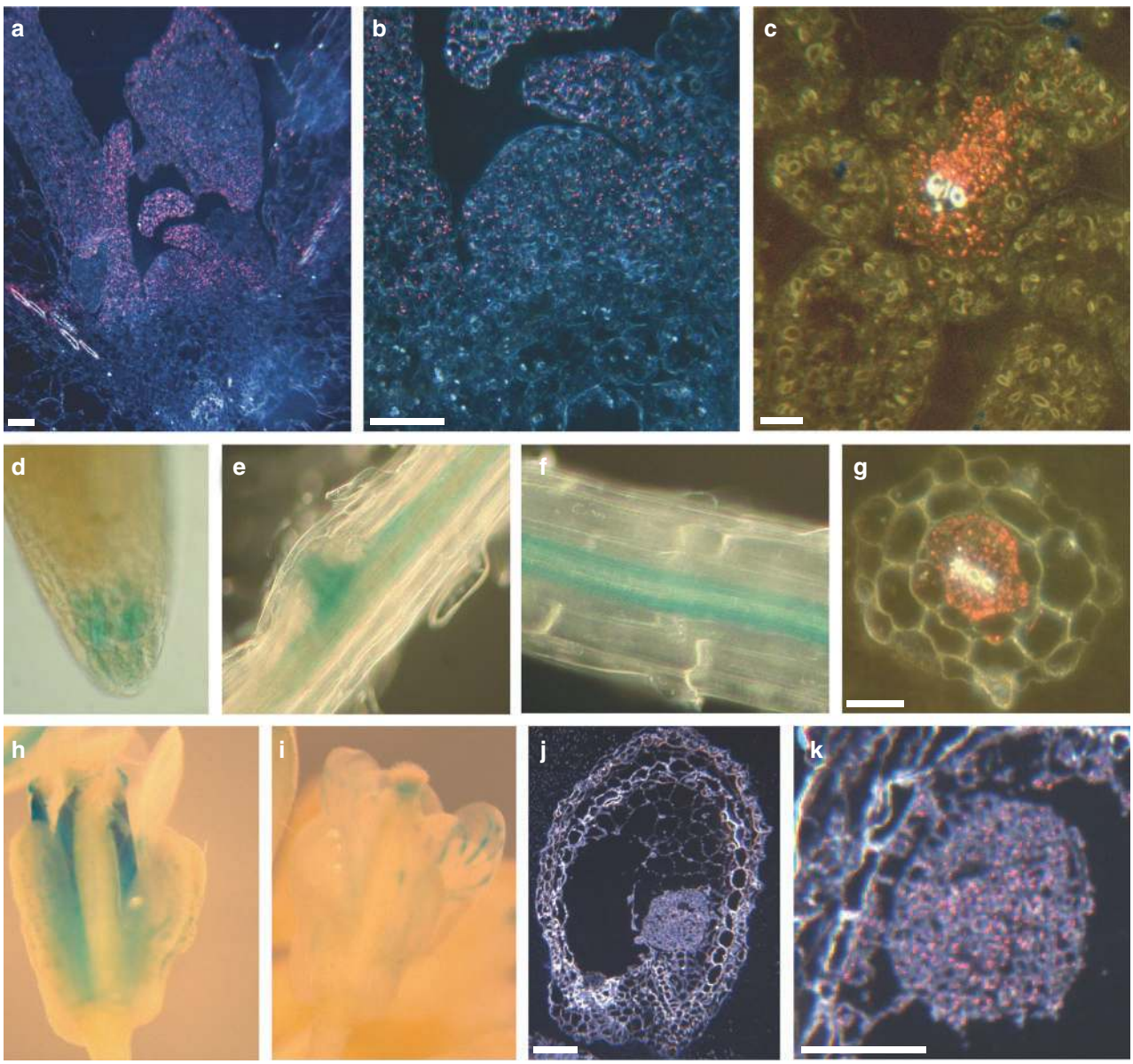

Fig. 2 Histochemical localization of GUS activity in p*GASA4::GUS* plants. (a) Dark-field image showing GUS in the vegetative shoot apical meristem and in initiating and emerging leaves. (b) Higher magnification image of GUS uniformly distributed throughout the SAM. (c) Cross-section of a cotyledon at 18 d after germination showing GUS specifically in the phloem cells of the vasculature. (d) Root tip showing GUS in the root cap cells and the distal parts of the root meristem. (e) GUS in emerging lateral roots. (f) Whole roots exhibit GUS in the vascular tissue. (g) Cross-section of the root revealing GUS in the phloem. (h) Flower showing very strong GUS in the stamen filaments. (i) Flower showing GUS in the vascular tissue of the petals and in the style. (j) Dark field image of a developing seed, displaying GUS in the embryo. (k) Higher magnification showing GUS uniformly distributed throughout the globular stage embryo. Scale bars = 20 µm.

observed phenotypic effect is specific to reproductive development.

In contrast to *gasa4* loss of function, ectopic expression of *GASA4* under the control of the cauliflower mosaic virus (CaMV) *35S* promoter did not affect secondary inflorescence branching. Unlike *gasa4-1* plants, *35S::GASA4* lines occasionally exhibited meristem identity changes in which the plants underwent the transition to flowering and produced floral meristems, followed by a reversion to normal indeterminate inflorescence development. *35S::GASA4* flowers exhibited mosaic floral organs, most frequently ectopic carpel or stamen structures. Abnormalities such as the development of structures with part carpel and part inflorescence identity were also

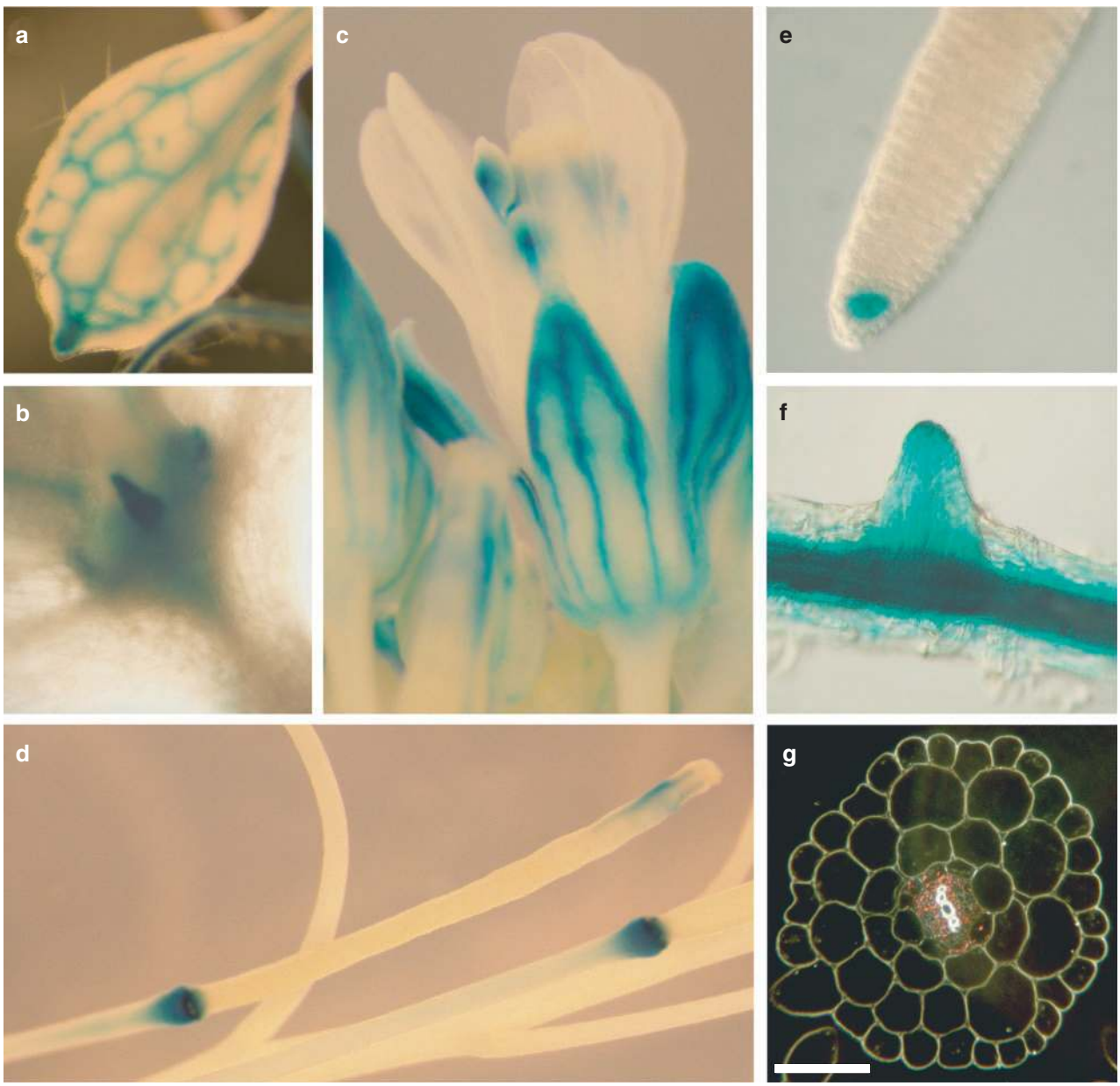

Fig. 3 Histochemical localization of GUS activity in p*GASA1::GUS* plants. (a) GUS activity in the vascular tissue of leaves. (b) Whole seedling showing GUS in the region around the shoot apex. (c) Flowers displaying GUS in the vascular tissue of the sepals, in the stamen filaments and in the style. (d) Developing siliques exhibit strong GUS activity in the abscission zone. (e) GUS localization in the root meristem. (f) GUS localized to emerging lateral roots. (g) Cross-sections of the root localize GUS to the phloem cells of the vasculature. Scale bar = 20 μm.

observed, but at a very low frequency. The latter phenotypes are likely to be due to partial co-suppression of endogenous *GASA4* activity in the *35S::GASA4* lines. The average occurrence of flowers with developmental abnormalities in two independent *35S::GASA4* lines was 13 and 20 flowers per 10 plants, while a third line exhibited seven abnormal flowers per 10 plants.

GASA4 activity increases seed size and total seed yield

Analysis of plants with altered *GASA4* expression levels showed that *GASA4* affects seed size, seed weight and total seed yield (Fig. 6b, c). Seeds from *gasa4-1* null mutant plants had significantly reduced seed weight, while, conversely, seeds from *35S::GASA4*-overexpressing lines had significantly increased seed weight (Fig. 6b). In addition

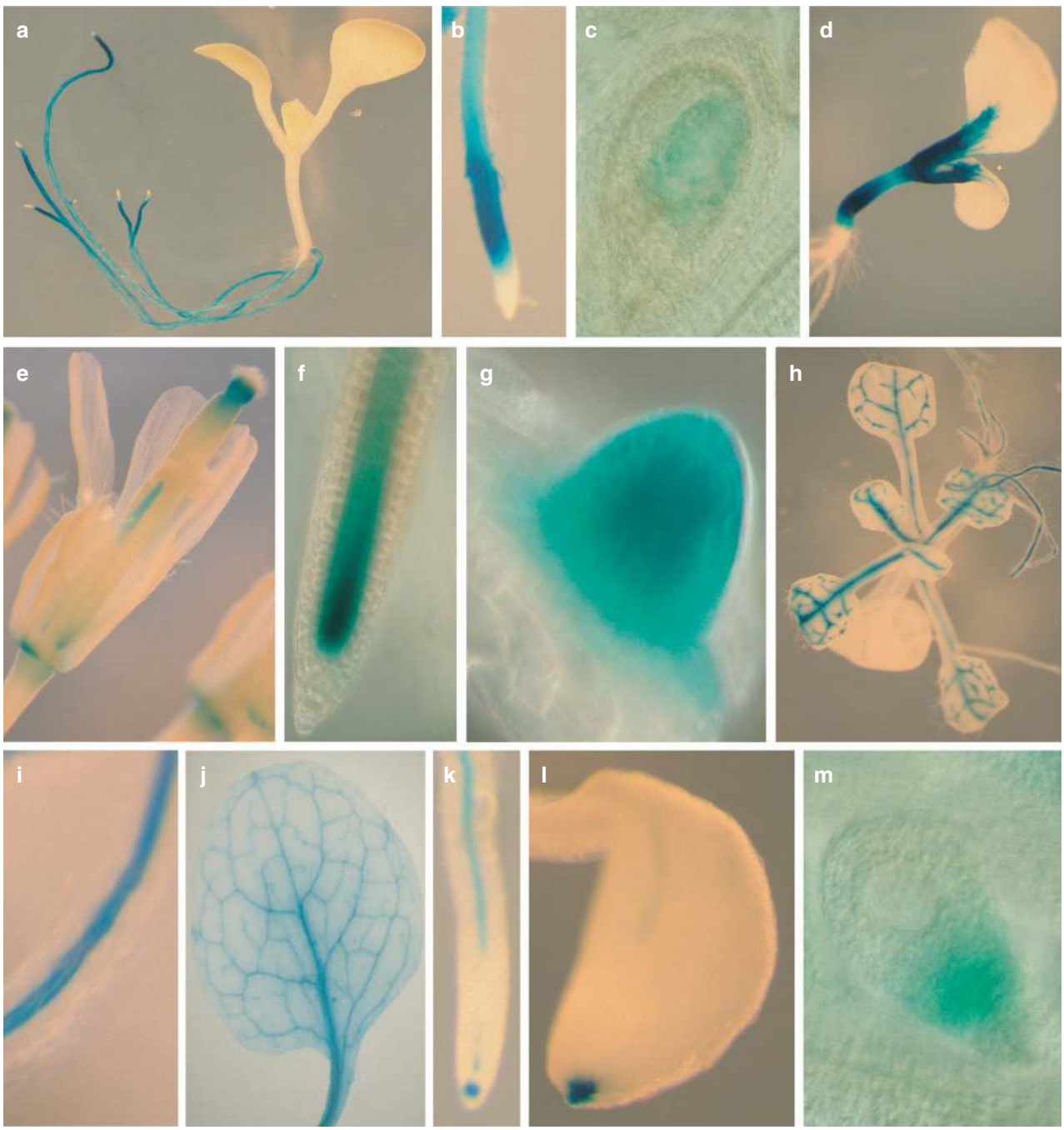

Fig. 4 GUS patterns from the *GASA8*, *GASA14*, *GASA3* and *GASA10* promoters. (a) At the seedling stage, p*GASA8::GUS* activity is restricted to roots. (b) p*GASA8::GUS* activity is strong in the cell elongation zone of the root, but it declines in the more mature root cells closer to the hypocotyl and is absent from the root tip. (c) Developing seeds show p*GASA8::GUS* activity in a domain covering large parts of the seed. (d) p*GASA14::GUS* activity is strong in the hypocotyl and the base of the cotyledons in 9-day-old seedlings. (e) Flowers displaying strong p*GASA14::GUS* activity in the abscission zone, the stamen filaments and the style. (f) p*GASA14::GUS* activity is observed specifically in the root vasculature, with higher accumulation towards the root tip. (g) p*GASA14::GUS* activity is localized to initiating lateral roots. (h) p*GASA3::GUS* activity is exclusively in the vascular tissue throughout the developing seedling. (i) p*GASA3::GUS* activity in the root vasculature. (j) p*GASA10::GUS* activity is localized in patches in the rosette leaf vascular tissue. (k) The root displays p*GASA10::GUS* activity in the vascular tissue and at the root tip. (l) p*GASA10::GUS* activity is detected at the tip of the cotyledons. (m) Developing seeds have *GASA10*-driven GUS in a wide domain in the basal parts of the seed.

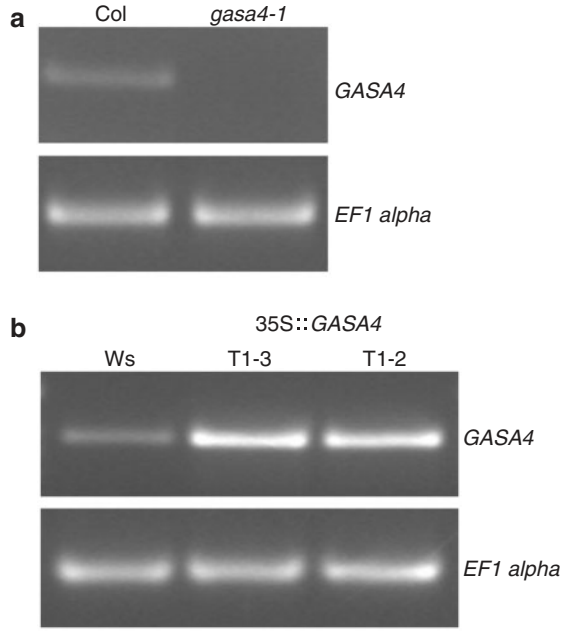

Fig. 5 RT–PCR analyses of plants with reduced or increased *GASA4* expression. (a) *GASA4* transcripts are detected in wild-type Col but not in *gasa4-1* T-DNA insertion mutant plants. (b) Relative *GASA4* steady-state transcript levels in two independent *35S::GASA4*-overexpressing plants compared with wild-type Ws plants. EF1α was amplified as a control. The amplification reactions were performed with RNA isolated from inflorescence tissues containing developing flower buds for 26 cycles.

to an increase in individual seed size, total seed yield was also increased in plants overexpressing *GASA4* (Fig. 6c). These data indicate that the increased seed weight is a direct effect of the ectopic *GASA4* activity and not a result caused by allocating more resources per seed due to lower seed set. Even *gasa4-1* plants, with smaller seeds, still exhibited higher total seed yields than wild-type plants, accounted for by increased branching leading to an increased total number of seeds (data not shown). These results indicate a function for *GASA4* in controlling seed development and seed yield through independent effects, where *GASA4* directly affects seed size in addition to affecting inflorescence branching and seed set, both of which contribute to increasing the total seed mass production.

Discussion

The Arabidopsis GASA genes encode putative secreted polypeptides with a conserved cysteine-rich domain

In this work we have identified six novel members of the *GASA* gene family in *Arabidopsis* by whole genome data mining, bringing the total number of family members to 14. The conservation lies primarily in 12 cysteine residues distributed throughout the C-terminus of all 14 predicted GASA proteins (Fig. 1a). This domain probably provides a conserved mode of action at the protein level. The distribution of the cysteines throughout the domain suggests that a specific globular protein structure is formed by the generation of up to six disulfide bridges between the cysteine residues (http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF02704). The variable positions between the conserved cysteines, in addition to the variation in the intermediate domain, may give rise to differences in functions for the GASA proteins.

The phylogenetic tree generated based on alignment of the C-terminal conserved domain of the 14 *Arabidopsis GASA* gene products depicts four phylogenetic clusters. Several *GASA* genes form closely related pairs: *GASA2* and *GASA3*, *GASA1* and *GASA11*, *GASA8* and *GASA10*, and also *GASA4* and *GASA6*. These pairs might have related functions, as many genes in *Arabidopsis* have close homologs with which they are functionally redundant (e.g. Ohgishi et al. 2004, Shpak et al. 2004). The use of out-group sequences would probably have provided a more informative phylogenetic tree. However, other sequences related to the GASA family, such as *GAST*, *GIP* and *GEG* sequences, are all found in other related angiosperms, and thus are not evolutionarily distant enough to root the tree.

The GASA promoters drive specific and distinct GUS localization patterns

The data obtained from our *GASA* promoter::GUS analyses reveal that the different *GASA* promoters are active in distinct tissues and developmental stages, suggesting that they fulfill distinct functions in the plant. Even though all *GASA* promoters investigated are active in roots and in the vasculature, the patterns are highly specific and different with respect to cell type and temporal regulation. The novel regulation patterns reported here for *GASA3*, *GASA8*, *GASA10* and *GASA14* further demonstrate the variability within the family. In addition, we have expanded the data available on *GASA1* and *GASA4* (Aubert et al. 1998, Raventos et al. 2000). Our results reveal that there is considerable overlap between the GUS patterns driven by the *GASA1* and *GASA4* promoters. Even though GASA1 and GASA4 belong to different phylogenetic clusters within the GASA family, the overlapping GUS localizations suggest that they may provide partially overlapping functions. In light of the proposed role of the *GASA* homolog *GEG* in *G. hybrida* cell elongation (Kotilainen et al. 1999), our GUS analyses identify *GASA8* and *GASA14* as candidates for being involved in similar processes to *GEG* because the two promoters are highly active in zones typically associated with cell expansion, such as growing cotyledons and cells above the root initials.

Extensive research has established an important function for gibberellin to induce stem elongation, leaf

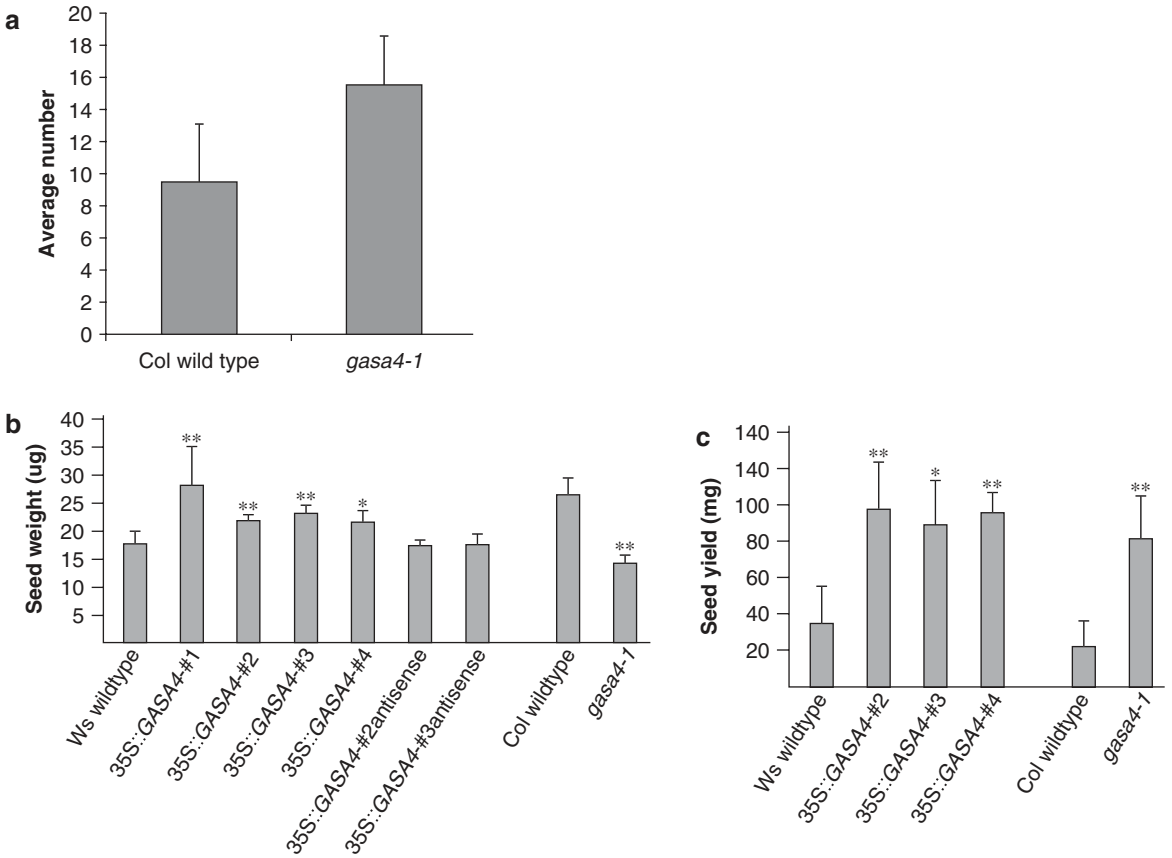

Fig. 6 Phenotypes of the *gasa4-1* null mutant and *35S::GASA4*-overexpressing lines. (a) Mean number of axillary inflorescences in wild-type (Col) and *gasa4-1* plants, with standard deviations shown as error bars. The number of total higher order shoots produced by *gasa4-1* plants ($n = 13$) is significantly higher than that of wild-type Col plants ($n = 13$). The Student *t*-test gives the two-tailed *P*-value of 0.0001. (b) The weight of individual seeds was calculated from 3–5 different seed batches of 100–700 seeds each and is presented as average individual seed weight. (c) Seed yield was calculated as average total seed yield harvested per plant ($n = 5$). Significant differences compared with wild-type control plants are indicated with asterisks ($^{*}P < 0.05$; $^{**}P < 0.01$). Error bars represent the SD.

expansion, flowering, seed development and germination (e.g. Isabel-LaMoneda et al. 2003). Yet, little is known about how these gibberellin effects are mediated and the genes involved. A search through the promoter regions of the 14 *GASA* genes revealed that they all contain one or more sequence elements putatively associated with gibberellin responses during seed development. This information suggests that many, if not all, of the *GASA* genes may be regulated by gibberellin. Different sensitivity and interactions between different signaling pathways and hormone effects require extensive studies to unravel the different mechanisms during flower and seed development (Swain and Singh 2005). This is illustrated by *GASA4* expression, which is up-regulated by gibberellin in meristematic regions yet down-regulated by gibberellin in cotyledons and leaves (Aubert et al. 1998). *GASA4* is likewise expected to be differentially regulated during flower and seed development. One possible explanation is that *GASA4* may be associated with cell division, in which case it would be expected to be abundant in meristematic tissues with higher cell division rates and reduced in tissues undergoing differentiation (Aubert et al. 1998). Alternatively, the differential effects of gibberellin on *GASA4* transcription may involve as yet unidentified tissue-restricted contributing factors, or may reflect a minor role for gibberellin in regulating *GASA4* gene expression.

GASA4 is a regulator of floral meristem identity and seed development

The localization of p*GASA4::GUS* in meristems, initiating organs and seeds correlates with the abnormal shoot and flower architecture phenotypes as well as the seed size and yield phenotypes observed in the *gasa4-1* null mutants and *35S::GASA4* plants. The *GASA4* promoter is active throughout early stage embryos, the vegetative shoot apical meristem and young leaf primordia. In the reproductive phase, the *GASA4* promoter is active during the early stages of flower development, as well as in specific mature organs such as the style and the stamen filaments.

Analysis of *GASA4* loss-of-function plants indicates that *GASA4* activity is important to promote the transition between axillary shoot meristem formation and flower formation. *gasa4-1* null mutants produce an increased number of axillary shoots before generating flowers, indicating that *GASA4* activity is required to promote floral meristem identity. In contrast, constitutive and ectopic *35S::GASA4* expression does not cause conversion of axillary meristems to a floral meristem fate, revealing that *GASA4* is not sufficient to induce a premature floral transition, or the correct fine tuning of the regulation might be a prerequisite to obtain the floral transition.

The *LFY*, *AP1* and *UNUSUAL FLORAL ORGANS* (*UFO*) genes all promote *Arabidopsis* floral meristem identity, and the increased branching phenotypes of plants with reduced *GASA4* expression are similar to those observed in weak *lfy* mutants (Weigel et al. 1992) and strong *ufo* mutants (Levin and Meyerowitz 1995). In silico searches within the *GASA* upstream promoters for *cis*-regulatory elements using PLACE (Higo et al. 1999) revealed the presence of a conserved sequence element known to be bound by the LFY transcription factor (Lohmann et al. 2001) at two locations in the *GASA4* promoter. The similarity between the *lfy* and *gasa4* phenotypes, and the presence of two putative LFY-binding elements in the *GASA4* promoter, thus suggests that *GASA4* may be regulated by LFY. Indeed, *GASA4* was recently identified as a putative LFY target in a microarray experiment to uncover genes exhibiting significant changes in expression levels upon *LFY* activation (Wagner et al. 2004). Thus, based on the combined genetic and molecular evidence, we propose that *GASA4* is a target of LFY transcriptional regulation and is a part of the physiological output of LFY function.

GASA4-driven GUS is also present in embryos, and *GASA4* plays an important role in seed development. In *Arabidopsis* seeds, the embryo accounts for most of the seed mass, and seed mass is negatively correlated with the number of seeds produced (Ohto et al. 2005). We find that *GASA4* has a direct effect on both seed size and total seed mass. *gasa4-1* plants have reduced seed size and *35S::GASA4* plants have increased seed size, demonstrating that seed size increases quantitatively with increasing *GASA4* expression levels. We do not yet know whether this is caused by an increase in cell size or an increase in cell number due to excess cell division, or possibly both. Both increased and decreased *GASA4* expression significantly increase total seed mass, even though decreased *GASA4* expression results in the production of smaller seeds. This effect can be explained by the increased shoot branching that occurs in *gasa4-1* plants, which increases the total number of flowers that are formed and therefore enhances overall seed set. The *AP2* gene, in addition to affecting floral meristem and floral organ identity (e.g. Jofuku et al. 1994, Bowman et al. 1989) and regulating the stem cell niche in the shoot apical meristem (Würschum et al. 2006), has also been found to regulate seed mass (Jofuku et al. 2005, Ohto et al. 2005). It has been suggested that AP2 acts on seed mass in part by suppressing gibberellin signaling and affecting cell size and cell number during seed growth (Okamuro et al. 1997, Jofuku et al. 2005). Loss-of-function *ap2* plants display increased seed size (Jofuku et al. 2005, Ohto et al. 2005), while the dominant-negative *AP2* allele *l28* causes reduced seed size (Würschum et al. 2006). These phenotypes are opposite to those caused by mutations in *GASA4*, suggesting that *GASA4* and *AP2* may play opposing roles in regulating seed size during plant development.

GASA proteins as putative regulatory molecules

The *GASA*-like gene *GEG* has been proposed to encode a cell wall-localized protein that plays a structural role during cell elongation in *G. hybrida*, based on its putative secretion, the relative abundance of its transcripts and its induction by gibberellin, which is typically involved in cell wall properties such as division and extension (Kotilainen et al. 1999, Ben-Nissan et al. 2004). Our results suggest that at least one member of the GASA family plays a regulatory rather than a structural role. We have shown that *GASA4* regulates seed development, plant architecture and the flowering transition, and based on their tissue- and stage-specific promoter activation patterns it appears that additional *GASA* gene products may also regulate aspects of development. The regulatory aspect introduced by the observed *GASA4* phenotypes suggests that it and the other GASA proteins might function by participating in signaling pathways, e.g. as extracellular signaling molecules. Thus, a future goal is to determine whether the GASA proteins act as small secreted ligands in developmental signal transduction pathways.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana seeds were surface sterilized for 5 min in a 2% sodium hypochlorite solution and plated on Murashige and Skoog (1962) agar plates with 20 g liter^{-1} sucrose and 80 mg liter^{-1} kanamycin for selection of transgenic plants. Seeds of ecotype Ws were surface sterilized and then directly transferred to growth chambers holding 20°C and long-day conditions (16 h light, 8 h dark) or, alternatively, constant light conditions. Seeds of ecotype Colombia (Col) were cold treated at 4°C for 4 d before being transferred to the growth chambers. At 10–14 d after germination, the seedlings were transferred to soil, and placed in a greenhouse complex with 20°C and long-day conditions, or to growth chambers holding 20°C and constant light. The T-DNA mutant allele *gasa4-1* (N042431), containing a T-DNA insertion in the first exon of *GASA4*, was obtained from the T-DNA Express Collection at the Salk Institute (http://signal.salk.edu/cgi-bin/tdnaexpress).

Phylogenetic analysis

Putative GASA protein sequences were obtained from public databases (http://www.ncbi.nlm.nih.gov/, http://www.arabidopsis.org, http://mips.gsf.de/proj/thal/db/index.html) and from our cloning results. Multiple sequence alignments were constructed using ClustalW (Thompson et al. 1994) and displayed with GeneDoc (www.psc.edu/biomed/genedoc). On the basis of the multiple sequence alignment, a neighbor-joining phylogenetic tree was obtained using the PAUP v.4.0 software (http://www.paup.csit.fsu.edu), and statistical confidence was calculated by bootstrap analysis with 1,000 resamplings.

PCR amplification, cloning and transformation

The coding regions corresponding to the *GASA5*, *GASA9*, *GASA10* and *GASA11* genes were amplified by PCR from poly(A)-primed reverse-transcribed total RNA isolated from Ws flowers, while Ws genomic DNA was used as a template to amplify *GASA8*, *GASA12* and *GASA13*. The seven remaining *GASA* genes *GASA1*, *GASA2*, *GASA3*, *GASA4*, *GASA6*, *GASA7* and *GASA14* were amplified from expressed sequence tag (EST) clones in the vector Lambda ZAP II Reference pBluescript, provided by The Arabidopsis Biological Resource Center (http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm). Gene-specific primers were used in all amplifications from *Arabidopsis* genomic DNA and cDNA, while amplifications of *GASA* genes from EST clones were performed using Sp6, gatttaggtgacactatag; T3, attaaccctcactaaaggga; and T7, taatacgactcactataggg primers. The gene-specific primers used to amplify *GASA* coding regions from cDNA or genomic DNA were: *GASA9* f, B1-tcaaaatcactact caag; *GASA9* r, B2-rcttttaaggacatttgag; *GASA11* f, B1-cctcttttgtt ttctctg; *GASA11* r, B2-ttttaaggacactttctgc; *GASA12* f, B1-gaaata tatcataagaaaatc; *GASA12* r, B2-ttttcatggacattttggtc; *GASA8* f, B1-caaactctttcgagaaaacaa; *GASA8* r, B2-tcaaggacacttggatcc; *GASA5* f, B1-taagaatcattcattggcaatc; *GASA5* r, B2-tttaaggacatttt ggacg; *GASA10* f, B1-ccaaacagattattattaaaac; *GASA10* r, B2-ctataaggataatcttaagaac; *GASA13* f, B1-acctgagcttcattacaatc; and *GASA13* r, B2-aattaagaggagaaactaag. B1 and B2 refer to the flanking 25 bp recombination sites necessary for GATEWAY™ recombination (Invitrogen).

GASA promoter regions extending between 1.0–2.0 kb upstream of the expected translation start site were amplified for the *GASA1*, *GASA3*, *GASA4*, *GASA8*, *GASA10* and *GASA14* genes using genomic *Arabidopsis* DNA as a template. The precise amplified regions were as follows: for *GASA1*, a fragment from −1,298 to −15 upstream of the expected translation start site, for *GASA3* from −1,218 to −5, for *GASA4* from −1,717 to −20, for *GASA14* from −1,075 to −3, for *GASA8* from −1,574 to −6, and for *GASA10* from −1,669 to −59. The primers used to amplify these fragments were *GASA1* fw, cataactcataagaagaagtgc; *GASA1* r, gtttatgatgagagaacttagc; *GASA3* fw, tttgttccaagtttgg caacc; *GASA3* r, aaaaagcatgcagtttcgtgc; *GASA4* fw, gataatgaaat gatccaactgg; *GASA4* r, atccaaagaaccaaacactcc; *GASA14* fw, gagattgtggagtactgtgg; *GASA14* r, tgtgggagatgagaaagtgg; *GASA8* fw, B1-gtgctgatgaggaccacc; *GASA8* r, B2-ttctcgaaagagtttgctacg; *GASA10* fw, B1-gaaccggagaccgtttacc; and *GASA10* r, B2-agaa gatgaagagaaaactcg. All PCRs were performed with the thermal cycler GeneAmp PCR system 9600 provided by Applied Biosystems.

Amplified PCR fragments were cloned into appropriate constructs using GATEWAY™ cloning technology (Invitrogen) as described by the manufacturer. The binary GATEWAY™ destination vectors (Karimi et al. 2002) used were PKGWFS7 for obtaining a GASA::*GUS-GFP* fusion construct and PK7 WG2D giving the CaMV35S::*GASA* construct for overexpression. The destination vectors were provided by Flanders Interuniversity, Institute for Biotechnology (http://www.psb.rug.ac.be/gateway). Standard *Agrobacterium tumefasciens*-mediated transformation of binary constructs was performed with ecotype Ws (Clough and Bent 1998).

RNA isolation, reverse transcription and PCR

Total RNA was isolated from inflorescence tissues containing developing flower buds using the RNeasy RNA isolation kit (Qiagen) according to the manufacturer's instructions. Single-stranded cDNA was synthesized from 2 μg of total RNA with the Superscript™ III first-strand synthesis system (Invitrogen). A 80 ng aliquot of cDNA was used in a semi-quantitative PCR, using the primers *GASA4*f, gaggagtgtttggttctttgg; *GASA4*r, taaaaagggaacgaagggag; EF1αf, caggctgattgtgctgttcttatcat; and EF1αr, cttgtagacatcctgaagtggaaga, in a 20 μl reaction using *Taq* polymerase (Promega). In order to rule out possible contaminating genomic DNA templates, each primer set was designed to span one or more introns. The PCR (94°C, 1 min; followed by cycles of 94°C, 30 s; 55°C, 30 s; 72°C, 1 min; followed by 72°C, 7 min) was performed for 26 cycles.

Histology and microscopy

For promoter::GUS analysis, plant material was stained in GUS staining solution (100 mM $NaPO_4$ pH 7.2, 1 mg ml^{-1} X-Gluc in dimethylformamide (DMF), 5 mM potassium hexacyanoferrate (II), 0.5 nM potassium hexacyanoferrate (III), 0.1% Triton X-100) and incubated overnight at 37°C. The material was washed repeatedly in 70% ethanol followed by 100% ethanol, and seeds were further cleared in clearing solution (8 g of chloral hydrate, 1 ml of glycerol, 2 ml of H_2O). The tissue was analyzed under a LEICA DM LB microscope, and photographed with a Nikon Coolpix 995 digital camera.

Tissue was prepared for sectioning by fixation in 1.25% glutaraldehyde and 2% paraformaldehyde, dehydrated through a graded ethanol series, and infiltrated and embedded in LR White Hard Grade Acrylic Resin (Electron Microscopy Sciences) according to the manufacturer's instructions. Sections of 1 μm were prepared using an ultramicrotome and a diamond knife, the sections mounted on slides, and GUS staining visualized in dark-field mode.

Seed mass and seed yield analyses

Average seed weight was determined by weighing mature dry seeds in batches of 100–700 seeds. The weight of 3–5 batches was further measured for each seed lot. Total seed yield from individual plants was collected using the Aracon system (Aracon containers, BetaTech, Gent, Belgium).

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