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RESEARCH PAPER

The strawberry gene *FaGAST* affects plant growth through inhibition of cell elongation

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Abstract

The strawberry (*Fragaria*×*ananassa*) *FaGAST* gene encodes a small protein with 12 cysteine residues conserved in the C-terminal region similar to a group of proteins identified in other species with diverse assigned functions such as cell division, elongation, or elongation arrest. This gene is expressed in the fruit receptacle, with two peaks during ripening at the white and the red-ripe stages, both coincident with an arrest in the growth pattern. Expression is also high in the roots but confined to the cells at the end of the elongation zone. Exogenous application of gibberellin increased the transcript level of the *FaGAST* gene in strawberry fruits. Ectopic expression of *FaGAST* in transgenic *Fragaria vesca* under the control of the CaMV-35S promoter caused both delayed growth of the plant and fruits with reduced size. The same growth defect was observed in *Arabidopsis thaliana* plants overexpressing *FaGAST*. In addition, the transgenic plants exhibited late flowering and low sensitivity to exogenous gibberellin. Taken together, the expression pattern, the regulation by gibberellin, and the transgenic phenotypes point to a role for FaGAST in arresting cell elongation during strawberry fruit ripening.

Key words: Cell elongation, cysteine-rich protein, *Fragaria*, gibberellin, fruit ripening, fruit size.

Introduction

Plant fruits are specialized organs developed at the end of each life cycle to allow seed dispersal for perpetuation of the species. The high number of agents that participate in this task, from physical agents such as wind to animal vectors such as humans, has produced an evolutionary pressure resulting in the wide range of structures found in nature. Human participation in this process has been critical through the domestication of wild species and manipulation of specific characters such as appearance, texture, size, flavour, and aroma in the course of continuous selection programmes (Seymour *et al*., 1993). The evolution of two of these characters, fruit size and shape, has recently been reviewed (Tanksley, 2004). Progress towards the identification of the loci involved in this process, as well as the characterization of the molecular events associated with these two characters has been made (Tanksley, 2004). However, it seems that only the tip of the iceberg has been revealed and most of the basic information has yet to come.

Fleshy fruits have received particular attention because of their importance in the human diet (Giovannoni, 2004). Some of the mature fruits in these species may result from the development of extracarpellar components, like the receptacle in strawberry. The so-called 'fruit' of strawberry is an aggregation, composed of numerous ovaries, each with a single ovule (Hancock, 1999). The resulting fruits are called achenes and contain one seed each. All the achenes are attached to the surface of the receptacle, which is composed of the epidermal layer, the cortex, and the pith. Cultivated strawberry is a frequent component of the diet of millions of people, and the annual worldwide production is growing steadily (Hancock, 1999). The study of the strawberry ripening process has received special attention among the non-climacteric fruits, resulting in the identification and characterization of numerous ripening-related genes (Medina-Escobar *et al*., 1997; Aharoni and O'Connell, 2002; Agius *et al.*, 2003). The cultivated strawberry (*Fragaria*×*ananassa*) is an octoploid which

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originated in the late 17th century from an accidental hybridization between the two species *Fragaria virginiana* and *Fragaria chiloensis* (Hancock, 1999). The resulting hybrid combined the hardiness, vigour, and productivity of *F. virginiana* with the large fruit size of *F. chiloensis.* Most strawberry breeding programmes have had large fruit size as one of their major objectives (Moore *et al*., 1970).

In the search for genes differentially expressed in strawberry fruits during growth and development a cDNA clone with homology to a gene family known as *GAST* was isolated (Medina-Escobar *et al*., 1997). The name of the family is assigned by the first member identified, *GAST1* (GA Stimulated Transcript 1) from tomato (Shi *et al.*, 1992). Homologues have been identified in *Arabidopsis* (GASA; Herzog *et al*., 1995), *Petunia hybrida* (GIP; Ben-Nissan and Weiss, 1996), *Gerbera hybrida* (GEG; Kotilainen *et al.*, 1999), and potato (SNAKIN; Segura *et al*., 1999), among other species. Features in common among the members of the family are (i) that they encode small proteins with a cysteine-rich C-terminal domain and (ii) they have a putative signal peptide sequence. Despite the high similarity in the C-terminal region with a precisely conserved distance among the 12 cysteines, there is no apparent uniformity in the role played by these proteins at cellular level. They have been associated with events like cell division (Aubert *et al.*, 1998), cell elongation (Ben-Nissan *et al*., 2004), or cell elongation arrest (Kotilainen *et al.*, 1999). Although a feature in common amongst some members of this protein family is the induction by gibberellin (GA), other members are not affected by GA or are regulated by auxin (Shi *et al.*, 1992; Taylor and Scheuring, 1994; Herzog *et al.*, 1995; Ben-Nissan and Weiss, 1996; Kotilainen *et al.*, 1999). Strawberry is considered a non-climacteric fruit whose growth has been attributed to auxin secretion from achenes (Perkins-Veazie, 1995). It was shown long ago that removal of achenes stopped fruit enlargement and that treatment with synthetic auxins caused continued growth (Nitsch, 1950). Free auxin is detected in the achenes 4 d after anthesis, peaks in both receptacle and achenes at about 10 d after anthesis, just before the white stage, and rapidly decreases in the receptacle as the fruit turns red (Lis *et al.*, 1978; Perkins-Veazie, 1995). It has been suggested that GAs may also play a role in the control of strawberry fruit development (Mudge *et al.*, 1981). Although several endogenous GAs have been identified in strawberry fruit (Blake *et al.*, 2000), only one bioassay-based study on their content through growth and ripening has been reported (Lis *et al.*, 1978).

In the present study, the full-length *FaGAST* cDNA was cloned from strawberry, and its expression analysed in different plant tissues and during fruit development and ripening. Ectopic expression of *FaGAST* in wild strawberry (*Fragaria vesca*) affected vegetative growth and produced smaller fruits than control plants. In accordance, overexpression of the gene in *Arabidopsis* gave a similar growth-retarded phenotype and delayed flower induction during both short- and long-day growth conditions. In addition, the transgene reduced GA sensitivity in *Arabidopsis*. All this information points to a role of FaGAST in the control of strawberry fruit size.

Materials and methods

Plant material, growth conditions, and hormone treatments

Strawberry plants (*Fragaria*×*ananassa* Duch.) were grown under field conditions in Huelva, in the south-west of Spain. The cultivar used was a commercial variety registered by the University of California (Davis) as Chandler. Plants of *F. vesca* were provided by the *Fragaria* germplasm collection from the CIFA-Churriana Center (Málaga, Spain). *Fragaria*×*ananassa* fruits were selected at seven previously established developmental stages (Agius *et al.*, 2003): F1, young flower receptacle; F2, mature flower receptacle; F3, green fruit (green receptacle and green achenes); F4, white receptacle and green achenes; F5, white receptacle with some rosy spots and brown achenes; F6, turning receptacle; F7, red receptacle. The different fruit stages are depicted in Fig. 2A. Other tissues were stolons, roots, and expanding leaves. All analyses were performed using tissue that was harvested, immediately frozen in liquid nitrogen, and stored at –80 °C.

GA_3 treatment of strawberry fruits was performed by spray application on the whole fruit *in planta* of a 100 μM solution of GA_3 in water containing 0.02% of Tween-20 (v/v) and 0.1% (v/v) dimethylformamide. Control fruits were treated with the same water solution without GA_3.

Arabidopsis thaliana (Columbia) seeds were allowed to imbibe for 4 d at 4 °C to ensure synchronous germination before planting. Plants were then grown in a chamber under 24/17 °C, 16/8 h light (200 μmol m^{-2} s^{-1})/dark conditions for long day (LD), or under a 23/17 °C at 8/16 h light/dark regime for short day (SD) conditions. *Arabidopsis* plants for monitoring root growth were grown in 15 cm Petri dishes in a growth chamber at 25 °C with a photoperiod of 16/8 h light (25 μmol m^{-2} s^{-1})/dark and relative humidity at 60–70%. Before imbibition, seeds were surface-sterilized by washing in 70% ethanol for 1 min, incubation in 20% commercial bleach for 20 min, and five washes for 5 min in sterile water. Seeds were then aligned on the surface of the medium and the Petri dish kept in a vertical position. The medium used was one-quarter-strength MS salts (Murashige and Skoog, 1962) supplemented with 0.6% agar (w/v) and 7.5 g l^{-1} sucrose.

GA_3 treatment of *Arabidopsis* plants for root growth effect was performed by allowing the seedlings to grow in one-quarter-strength MS medium for 5 d and transferring them to new Petri dishes supplemented with 0, 50, or 100 μM GA_3. A 100 mM GA_3 (Sigma) stock solution was made in ethanol and the appropriate volume added to autoclaved medium cooled to 55 °C. Root growth was evaluated after 10 d.

Plant transformation

FaGAST cDNA was excised from the pBS-*FaGAST* (Medina-Escobar *et al*., 1997) by digestion with *Eco*RV and *Sac*I and subcloned in the pUCAP35S-GUS, derived from the pBINPLUS (van Engelen *et al.*, 1995) with the restriction sites *Asc*I and *Pac*I, in the *Sma*I/*Sac*I sites after the excision of the GUS insert. The 35S-*FaGAST* insert was excised from the pUCAP35S-*FaGAST* with the *Asc*I and *Pac*I restriction enzymes and subcloned in the pBINPLUS vector previously digested with the same enzymes. This binary vector was used for transformation of both *F. vesca* and *Arabidopsis*.

Transformation of *F. vesca* plants was performed using *Agrobacterium tumefaciens* according to the protocol previously described (El-Mansouri *et al*., 1996). The *F. vesca* control line was obtained by *in vitro* regeneration in parallel to the transgenic lines, under the same conditions but without kanamycin. All the *F. vesca* lines were vegetatively propagated by stolons every season. *Arabidopsis* plants were transformed using the floral dip transformation protocol (Bechtold *et al*., 1993). Their study was performed in the T_2 generation homozygous plants.

Nucleic acid isolation and analysis

Total RNA from the cultivated strawberry and *F. vesca* was extracted as previously described (Manning, 1991). The extraction of RNA from *Arabidopsis* was performed according to standard procedures (Borsani *et al.*, 2001). For northern analysis 15–20 μg of total RNA was separated in 6% formaldehyde/1.5% agarose gels and blotted onto Hybond-N membrane (Amersham Pharmacia Biotech) using 20× SSC as blotting agent. RNA loading was checked by means of ethidium bromide staining of agarose gels. The blot was hybridized with the *FaGAST* cDNA probe obtained by digestion of the pBS-*FaGAST* plasmid with *Eco*RI and labelled with [α-^{32}P]dCTP, using the Amersham-Pharmacia Oligo prime kit following the manufacturer's instructions. Membranes were prehybridized in [0.3 M $NaPO_4$ pH 7.2, 7% (w/v) SDS, 1 mM EDTA, 1% (w/v) BSA] buffer for 30 min at 60 °C. Labelled probes were added to the prehybridization buffer (1×10^6 cpm ml^{-1} buffer), and hybridization performed overnight at 60 °C. Membranes were washed four times: in 2× SSC containing 0.1% SDS at room temperature; in 2× SSC containing 0.1% SDS at 60 °C; and two washes with 0.5× SSC containing 0.1% SDS at 60 °C. Each wash-time was 15 min. Autoradiographs were prepared by exposing X-ray film (X-Omat AR, Kodak) to the membranes at –80 °C.

In-situ *RNA hybridization*

Roots for *in-situ* hybridization were obtained from strawberry (*F.×ananassa* cv. Chandler) plants maintained in *in-vitro* culture (López-Aranda *et al*., 1994). *In-situ* hybridization with digoxigenin-labelled antisense RNA was performed on 7 μm longitudinal root sections following the procedure described by Coen *et al*. (1990) and Jackson (1991). The antisense *FaGAST* RNA probe was transcribed using T3 polymerase from the pBS-*FaGAST* plasmid (Medina-Escobar *et al*., 1997) after digestion with *Eco*RV. Similarly, the sense (control) RNA probe was generated digesting the same plasmid with *Bam*HI and using T7 polymerase. Signal was detected as a dark blue colour on a light blue background when viewed under the light microscope.

Results

Isolation and characterization of FaGAST *cDNA*

Differential screening of a subtractive strawberry cDNA library identified many genes with higher expression in red than in green fruits (Medina-Escobar *et al*., 1997). One of the cDNAs identified was designated *FaGAST* for its high homology to the tomato *GAST1* (*GA S*timulated *T*ranscript *1*) (Shi *et al*., 1992). *FaGAST* encodes a predicted protein of 106 amino acids with a cleavable N-terminal signal peptide based on computer analysis (von Heijne, 1986). Because the sequence lacks other predicted targeting signals it is likely that this protein is directed to the cell wall or extracellular space. Alignment with other proteins of the same family revealed little sequence conservation at the N-terminal region, but shows a highly homologous C-terminal region, including 12 cysteine residues in identical positions (not shown). These residues define a pattern not related to other known cysteine-rich motifs (Ben-Nissan *et al*., 2004). A phylogenetic tree constructed from this alignment located FaGAST in a cluster of proteins comprising the Lt-COR proteins from *Lavatera thuringiaca*, the *Arabidopsis* GASA1, the potato SNAKIN2, and the GEG protein from *Gerbera hybrida* (Fig. 1). The homology of this group of proteins to FaGAST ranged from 62% to 51% identity at amino acid level. However, when only the C-terminal region is compared, the identity increases to 80–75%.

In order to identify other family members related to *FaGAST*, a screening of a red-ripe strawberry fruit cDNA library was performed using the full-length cDNA (Medina-Escobar *et al.*, 1997) as a probe. The probe was radiolabelled by random priming using [α-^{32}P]dCTP and the Klenow fragment of DNA polymerase, and the screening performed using standard procedures (Sambrook *et al*., 1989). A total of 30 positive clones was isolated and further sequence analysis determined that all clones corresponded to *FaGAST*, suggesting that at moderate stringency

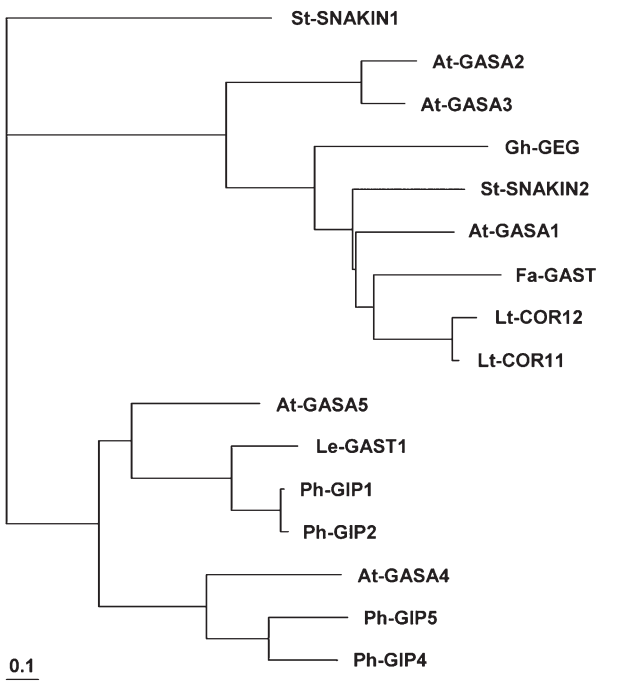

Fig. 1. Phylogenetic tree using the program BioEdit deduced from the multiple sequence alignment of proteins with homology to *FaGAST* from different plant species. Accession numbers are as follows. *Solanum tuberosum*: St-SNAKIN1, AJ320185; St- SNAKIN2, AJ312904; *Gerbera hybrida*: Gh-GEG, AJ005206; F*ragaria×ananassa*: Fa-GAST, AF039183; *Arabidopsis thaliana*: At-GASA1, P46689; At-GASA2, P46688; At-GASA3, P46687; At-GASA4, P46690; At-GASA5: S71371; *Petunia hybrida*: Ph-GIP1, S54832; Ph-GIP2, AJ417389; Ph-GIP4, AJ417391; Ph-GIP5, AJ417392; *Lycopersicon esculentum*: Le-GAST1, P27057; *Lavatera thuringiaca*: Lt-COR11, AF007784; Lt-COR12, AF060569.

conditions only *FaGAST* hybridized and it is the only *GAST*-like gene expressed in red fruits. Southern analysis using the full-length cDNA of *FaGAST* was conducted both to determine the copy number of *FaGAST* in the genome of the octoploid strawberry *F.×ananassa* cv. Chandler and to identify other family members (data not shown). One to three hybridizing bands were obtained for the different restriction enzymes. This low complexity of bands contrasted with those obtained for other *F.× ananassa* genes (Trainotti *et al.*, 1999; Castillejo *et al.*, 2004) and suggested the presence of a single *FaGAST* gene in strawberry. Moreover, Southern analyses in *F. vesca*, a diploid wild relative species, indicated that *FaGAST* is encoded by a single gene and other family members did not hybridize with the *FaGAST* cDNA probe in this species (data not shown).

FaGAST is mainly expressed in ripe fruits and roots

As shown in Fig. 2A, fruit growth of *F.×ananassa* cv. Chandler grown under the conditions present in the south-west of Spain follows a double sigmoidal pattern when measured as fruit length and diameter. Depending upon the temperature regimen of the season, ripening time ranges from 30 to 40 d after anthesis. The growth of the receptacle is due to a combination of cell division and cell enlargement (Havis, 1943). During the first 10 d after anthesis, growth in the strawberry receptacle is primarily due to cell division (Cheng and Breen, 1992). Between 10 d and 15 d after anthesis, when cell division ceased, cell enlargement mostly accounts for fruit growth. Cells of the cortex and the pith are responsible for most of the receptacle growth. However, their development is not synchronized since the cortex cells develop more rapidly than the pith cells (Havis, 1943; Cheng and Breen, 1992). The conjunction of these cellular events in the different cell types of the receptacle describes the growth pattern, which is specific for each cultivar, and is determined by numerous factors (Hancock, 1999). Figure 2B shows pictures of those developmental stages at which the fruits were sampled. The expression of *FaGAST* during strawberry fruit growth and ripening shows two peaks of expression at stages 4 and 7, with low (stages 1, 5, and 6) or undetectable (stages 2 and 3) levels at other stages (Fig. 2B). *FaGAST* expression appears to be high in roots, similar to the expression detected in F7 fruits, and low in leaves and stolons (Fig. 2C).

Within the fruit, the *FaGAST* gene is only expressed in the receptacle tissue, with no expression detected in achenes (Fig. 3A). The expression of the majority of the members of the *GAST* family is induced by GA (Ben-Nissan *et al.*, 2004) and levels of this hormone change during strawberry fruit ripening (Lis *et al.*, 1978). Green F3 fruits on the vine were sprayed with 100 μM GA_3 and harvested after 24 and 48 h. The expression of *FaGAST* was detected only after 48 h, but the mRNA level was higher in GA-treated fruits (Fig. 3B).

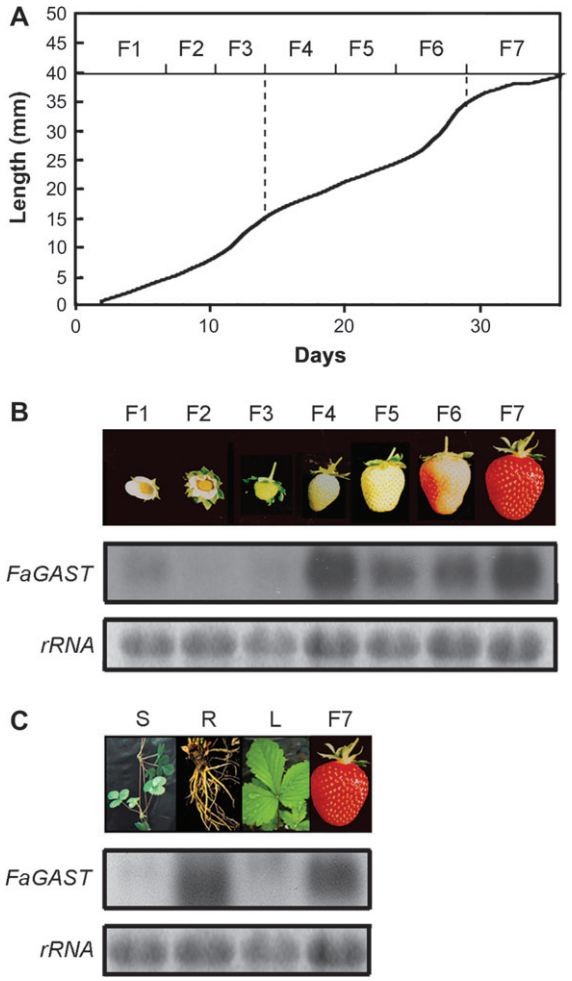

Fig. 2. *FaGAST* expression in different tissues of strawberry and in fruits through growth and ripening. (A) Growth curve of strawberry fruits from anthesis to fruit ripening (35 d) measured as fruit length. Selected sampling stages (F1 to F7) are also indicated. (B) Northern blot analysis of *FaGAST* transcripts in strawberry fruits at different stages. Photographs of fruits representative of each stage (F1 to F7) are shown for illustration. Control of RNA loading is shown by ethidium bromide staining of ribosomal RNA. (C) Northern blot analysis of *FaGAST* transcripts in stolon (S), root (R), leaf (L), and whole fruit at stage F7 (F7).

The spatial distribution of growing zones in the root is well established and it is similar among many species (Dolan and Davies, 2004). Because the expression of *FaGAST* in strawberry roots was high (Fig. 2C), the cell types expressing *FaGAST* in this organ were determined by *in-situ* RNA hybridization. As shown in Fig. 4, *FaGAST* transcripts were detected in cells of the endodermis and cortex of the late-elongation to early differentiation zone (Fig. 4). No expression of *FaGAST* was detected in cells of the meristematic zone.

Fragaria vesca *plants overexpressing* FaGAST *show delayed development and flowering time*

The diploid *F. vesca* is the most common native species of the *Fragaria* genus, and is proposed as the ancestor of the other *Fragaria* species, including the cultivated octoploid

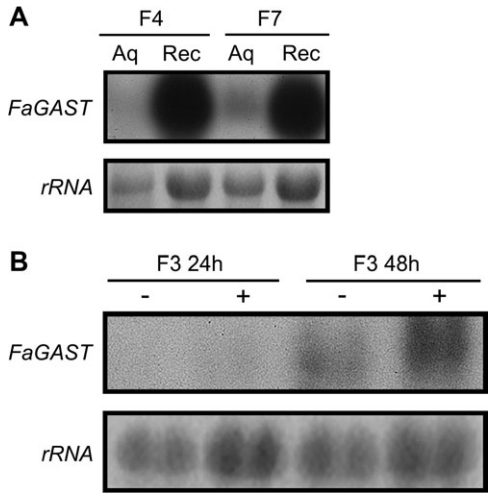

Fig. 3. Expression of *FaGAST* in different fruit organs and in response to the hormone GA. (A) Northern blot analysis of *FaGAST* transcripts in the achenes (Aq) and receptacle tissue (Rec) of strawberry fruits at two stages (F4 and F7). (B) Northern blot analysis of *FaGAST* expression in fruits at the F3 stage 24 h and 48 h after treatment with a solution of 100 μM GA_3 (+) as indicated in Materials and methods and fruits sprayed with water (−). Ethidium bromide staining of ribosomal RNA (rRNA) is shown as control of loading.

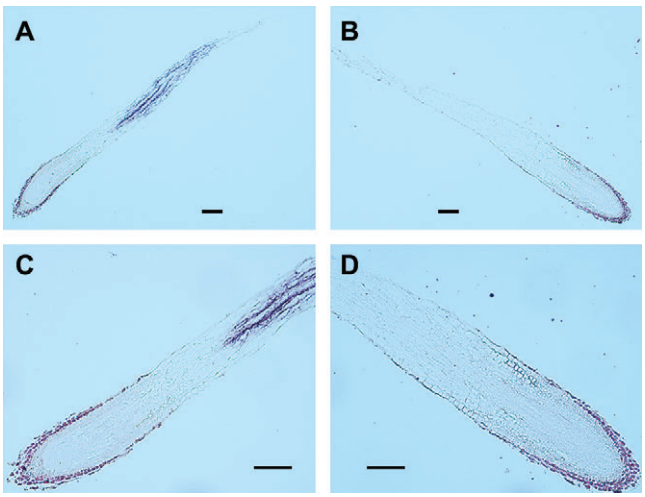

Fig. 4. *FaGAST* expression at the cellular level in the root of strawberry. Localization of *FaGAST* mRNA in strawberry roots by *in-situ* hybridization. Root longitudinal sections were probed with either antisense (A, C) or sense (B, D) digoxigenin-labelled *FaGAST* RNA. Expression is detected as a dark blue colour in the cells. Scale bars=100 μm.

F.×ananassa (Hancock, 1999). Most genes identified in *F.×ananassa* have orthologous genes in *F. vesca* and chromosomes from *F. vesca* are able to pair with most *Fragaria* species (Senanayake and Bringhurst, 1967). For this reason and for the shorter regeneration time after *Agrobacterium*-mediated transformation, it is used as a model for functional analysis of *F.×ananassa* genes (El-Mansouri *et al.*, 1996). In order to gain further insight to the function of *FaGAST*, the *FaGAST* gene was overexpressed under the control of the 35S promoter in *F. vesca*. It was noticed that while the regeneration time of transformed *F. vesca* plants ranged between 6 and 8 weeks (El Mansouri *et al.*, 1996; the present work), this time it was much higher (around 6 months) in plants transformed with the 35S-*FaGAST* construct in independent transformations. This result suggested that overexpression of this protein interfered with the regeneration process of the plant. A total of five kanamycin-resistant independent plants were acclimated and transferred to the greenhouse. After analysing *FaGAST* expression in leaves of the transgenic plants, lines 1 and 3 with high expression and line 4 with lower expression were selected for further characterization (Fig. 5A, B). As shown in Fig. 5A, lines 1 and 3 showed delayed shoot growth and reduced root development. This impairment in shoot growth was visible 30 d and 60 d after their transfer to soil for acclimation (Fig. 5C, D). This dwarfed phenotype becomes less significant as the plants grow. Once the transgenic plants have flowered they are the same size as controls. The delayed growth of the *F. vesca* transgenic lines was also reflected in flower initiation since only transgenic line 4 flowered in the first season, while lines 1 and 3 did not flower until the second season.

In the following season, plants were vegetatively propagated by stolons. It was confirmed that *FaGAST* was also expressed in fruits of the transgenic lines (Fig. 6A), which was expected since it is known that the 35S promoter is active in strawberry fruits (Agius *et al.*, 2005). Because size variability within the fruits of a strawberry plant is highly dependent upon the position of the fruit in the inflorescence (Forney and Breen, 1985), primary fruits from inflorescences of transgenic plants and controls were compared. As shown in Fig. 6B fruits from lines 3 and 4 were smaller than fruits produced by control plants. This phenotype was maintained in a third season of vegetatively propagated plants (data not shown).

Arabidopsis *overexpressing* FaGAST *showed delayed shoot development and flowering time*

Next it was determined whether overexpressing *FaGAST* in *Arabidopsis* plants caused a similar phenotype to that in *F. vesca*. For this purpose, transgenic *Arabidopsis* plants overexpressing the same construct previously used for *F. vesca* transformation were generated. Four homozygous transgenic lines that expressed *FaGAST* were selected for further analysis (Fig. 7A). All four lines constitutively expressing *FaGAST* showed reduced growth. As an example, in Fig. 7B–F, the growth of *Arabidopsis* line 2 is depicted 15, 23, and 34 d after germination. Differences in rosette leaf size were clearly visible in young plants (Fig. 7F), but became hardly noticeable as plants reached the adult phase. A phenotype also observed in all transgenic lines was a delay in flowering time (Fig. 7E). While bolting in wild-type plants grown under long days occurred ~30 d after sowing, bolting was clearly delayed in transgenic lines, as shown for line 2 in Fig. 7E.

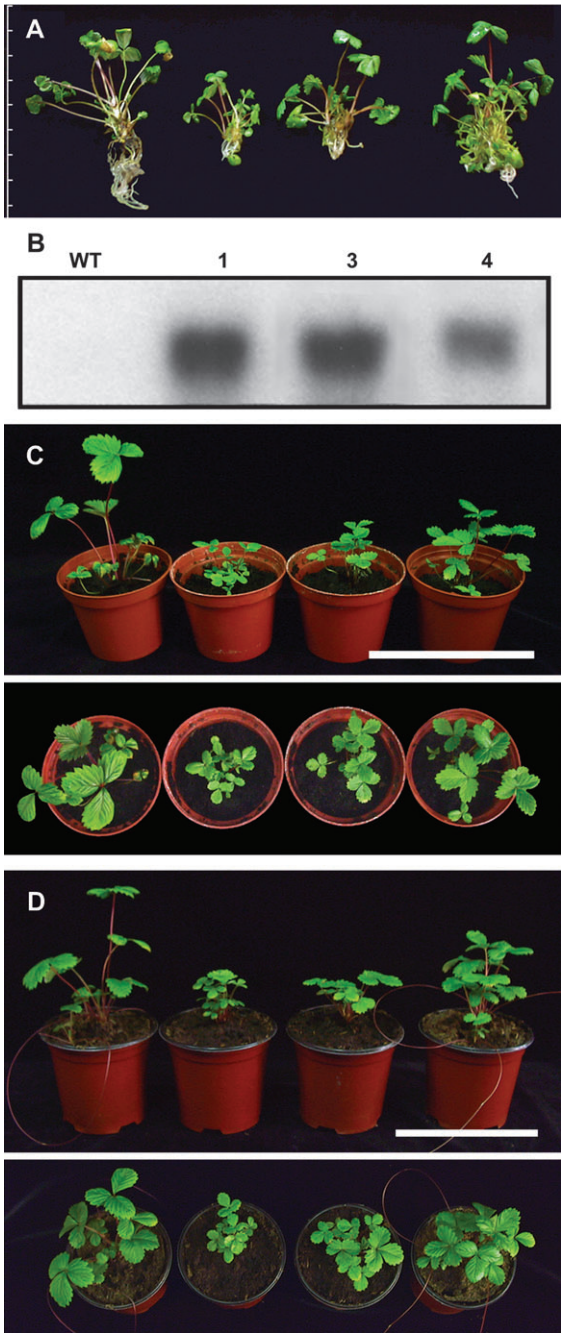

Fig. 5. Analysis of the phenotype of the transgenic *F. vesca* plants transformed with the 35S-*FaGAST* construct. (A) General morphology of the wild type (WT) and transgenic lines 1, 3, and 4, after their regeneration and prior to their transfer to soil. (B) Analysis by northern blot of *FaGAST* expression in leaves of wild type and transgenic lines 1, 3, and 4. (C) Top- and side-view of the same wild type and transgenic lines 30 d after their transfer to soil for acclimation. (D) Top- and side-view of the same wild type and transgenic lines 60 d after their transfer to soil for acclimation. Scale bars=12 cm.

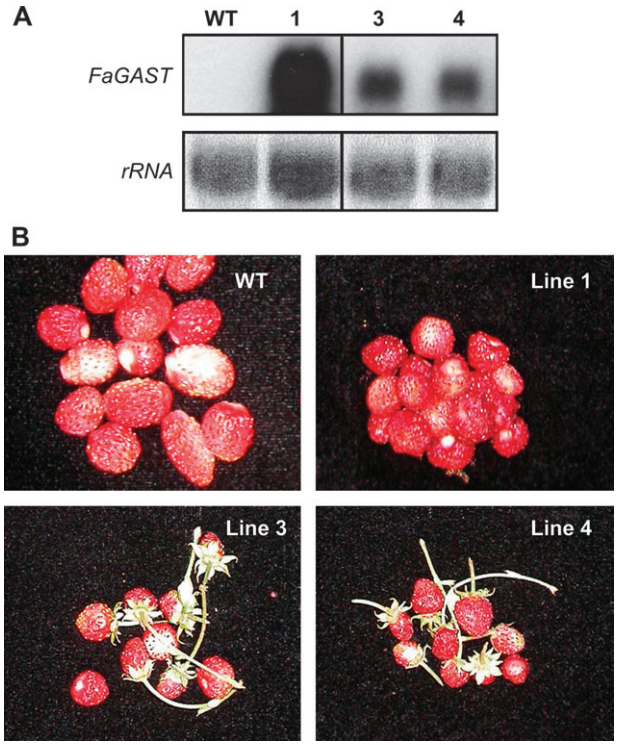

Fig. 6. Effect of *FaGAST* overexpression on fruits of *F. vesca*. (A) Analysis of *FaGAST* expression in fruits of the wild type (WT) and transgenic lines 1, 3, and 4 by northern blot. Ethidium bromide staining of ribosomal RNA (rRNA) is shown as control of loading. (B) Phenotype of primary fruits representative of the wild type and transgenic lines 1, 3, and 4.

The delayed flowering phenotype was observed under both long and short days. A detailed analysis in long days showed that transgenic lines flowered between 7 d and 10 d later than control plants (Fig. 8A). *Arabidopsis*, like many annual plants, stops to produce true leaves after bolting. Thus, generally a plant that fails to flower but continues to produce new nodes at a normal rate will accumulate more leaves than one that flowers early (Martínez-Zapater and Somerville, 1990). The number of rosette leaves produced by control and transgenic lines was recorded until flowering. Plants that constitutively expressed *FaGAST* averaged one leaf less than the control at early stages of development, indicative of a slow vegetative growth (Fig. 8B). However, transgenic lines produced one or two rosette leaves more than the control as a result of the delayed flowering time of transgenic lines (Fig. 8B).

The 35S-*FaGAST* lines showed a slight dwarf phenotype and delayed flowering, features which resemble *Arabidopsis* mutants affected in GA biosynthesis (such as *ga1-3*) and GA signalling (such as *gai*) (Richards *et al*., 2001). GAs play an essential role in the control of flower induction in *Arabidopsis*, particularly under short days (Richards *et al*., 2001; Simpson, 2004). To investigate whether the overexpression of *FaGAST* altered the concentration of active GAs or the GA response pathway, the effect of exogenous application of GA_3 on flowering time in both long and short days was analysed in control and transgenic lines. If GA concentration was reduced in transgenic lines, external application of GA would rescue the observed phenotype as

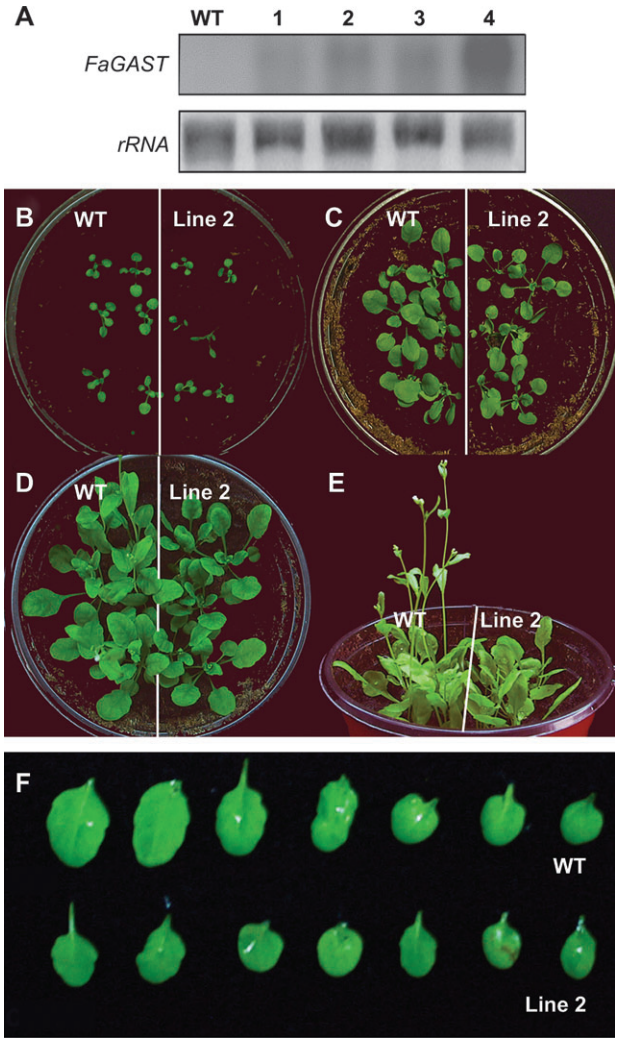

Fig. 7. Analysis of *Arabidopsis* lines transformed with the 35S-*FaGAST* construct. (A) Northern blot analysis of *FaGAST* expression in wild type (WT) and transgenic *Arabidopsis* lines 1, 2, 3, and 4. Ethidium bromide staining of ribosomal RNA (rRNA) is shown as control of loading. (B–D) Top view of wild-type and 35S-*FaGAST Arabidopsis* (line 2) plants 15 d (B), 23 d (C), and 34 d (D) after germination. (E) Side view of wild-type and 35S-*FaGAST Arabidopsis* (line 2) plants 34 d after germination. (F) Photograph of the individual rosette leaves of wild-type and 35S-*FaGAST Arabidopsis* (line 2) plants 23 d after germination.

in *ga1-3* mutants. Alternatively, if GA signalling is affected by *FaGAST* overexpression, the application of GA_3 would have reduced or no effect on reverting to the phenotype, as occurs in *gai* mutants. Flowering time was delayed in the transgenic lines to similar extents in both long and short days, as observed for line 2 in Fig. 9A, B. This result suggests that GA-deficiency is not the main cause of the delayed flowering phenotype because GA biosynthetic or signalling mutants are mostly affected in flowering under short days (Richards *et al.*, 2001). Furthermore, when transgenic and control plants were sprayed with 100 μM GA_3, the phenotype of the transgenic plants did not revert either in short or long days, excluding a defect in GA biosynthesis as a result of *FaGAST* overexpression (Fig. 9A, B). However, these results do not exclude the possibility that overexpression of *FaGAST* partially reduces GA sensitivity.

It is also known that GA promotes cell expansion in roots (Fu and Harberd, 2003). Thus, the effect of exogenous application of GA in roots of both wild-type and transgenic lines was examined. Similar results were obtained for all transgenic lines and, as an example, results for line 2 are shown in Fig. 9C. Roots of transgenic lines were, in general, slightly shorter than the control in MS medium, although the difference was not statistically significant (Fig. 9C). When plants were transferred to MS medium supplemented with 50 and 100 μM GA_3, root growth of both control and line 2 was stimulated, as previously reported (Fu and Harberd, 2003). However, the enhancement of root growth in line 2 was significantly lower than in wild type indicating that sensing or signalling of GA is affected due to *FaGAST* overexpression. This root growth response to external GA treatment was similar in all the *FaGAST* transgenic lines (results not shown).

Discussion

Cloning and expression analysis of the *FaGAST* gene of strawberry is reported. *FaGAST* belongs to a family of genes encoding small plant-specific proteins with highly conserved structural features. In strawberry, only one *GAST*-like gene expressed in fruits and roots has been detected. The presence of other members of the family in this species cannot be excluded, as it has been reported for other species (Herzog *et al.*, 1995; Ben-Nissan *et al.*, 2004). However, other family members may be specifically expressed in vegetative tissues or/and the similarity to *FaGAST* must be low since the present screening of a strawberry fruit cDNA library using low stringency did not identify any other *GAST*-like genes in this organ. The most relevant similarity among the members of the *GAST* genes is found in the C-terminal domain of the encoded proteins, with 12 cysteines conserved in exactly the same positions. Genes of this family have been characterized in distant species like the *GAST1* (Shi *et al.*, 1992) and *RSI-1* genes in tomato (Taylor and Scheuring, 1994), the GASA family in *Arabidopsis* (Herzog *et al.*, 1995), the petunia *GIP1*, *GIP2*, *GIP4*, and *GIP5* genes (Ben-Nissan *et al.*, 2004), *SNAKIN1-2* from potato (Segura *et al.*, 1999, Berrocal-Lobo *et al.*, 2002), and the *GEG* gene in *Gerbera* (Kotilainen *et al.*, 1999). Their expression has been associated with reproductive organs like flower buds (*GASA1* and *GASA4* from *Arabidopsis*), petals (*GEG* from *Gerbera*), siliques (*GASA1*), and seeds (*GASA2* and *GASA3*), or vegetative tissues like roots (*GASA4* and *RSL-1* from tomato), tubers (*SNAKIN-1* and *SNAKIN-2* from potato), and shoots (*GASA4*). Although the functions of these genes are not yet clear, it has been suggested

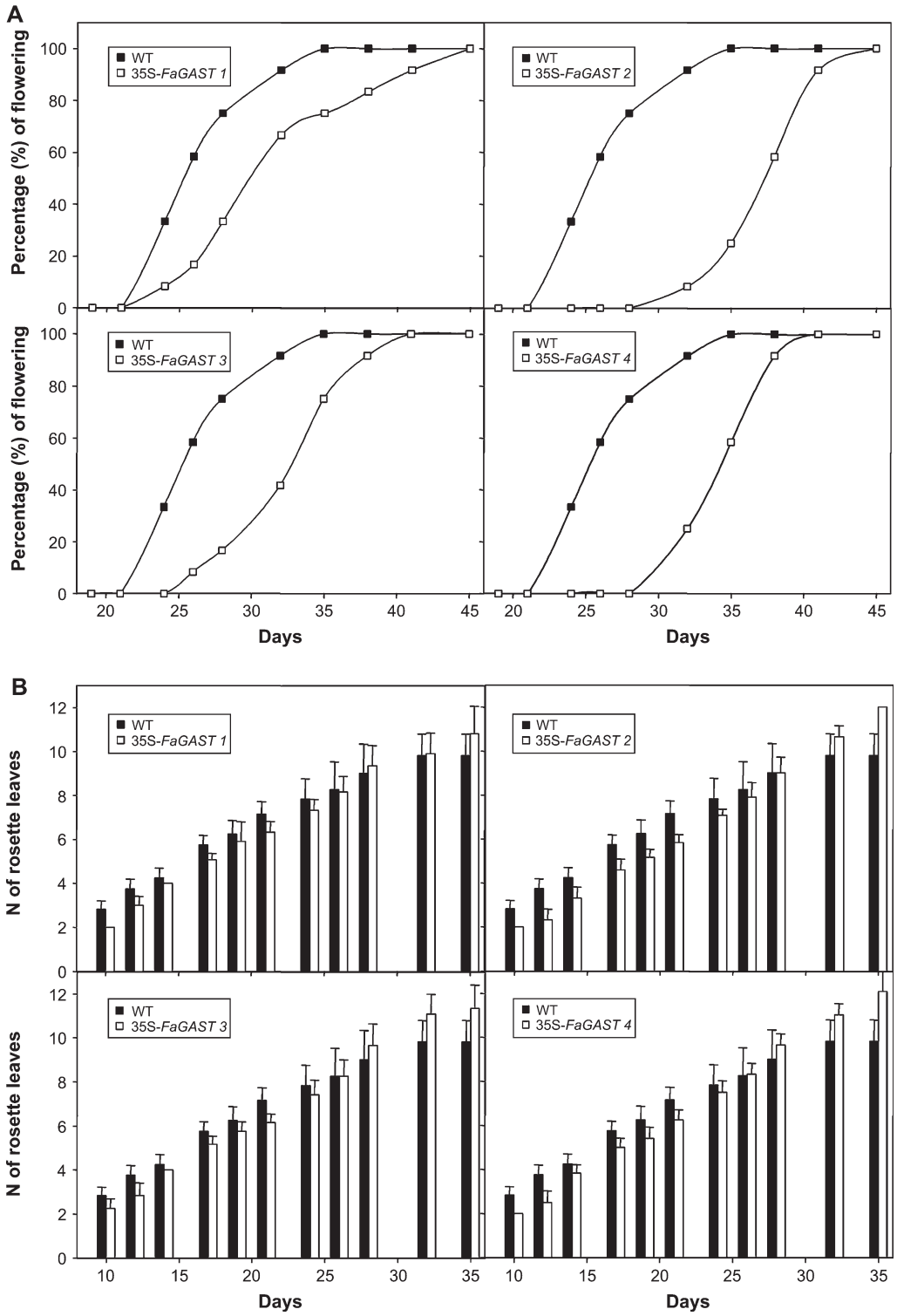

Fig. 8. Flowering time and number of rosette leaves in wild-type (WT) and 35S-*FaGAST Arabidopsis* plants grown in long days. (A) Percentage of flowered plants over time (days after germination) for the wild-type and 35S-*FaGAST* lines (1–4). (B) Number of rosette leaves produced over time for the wild type and 35S-*FaGAST* lines (1–4) from germination to 35 d after, when 100% of wild-type plants had flowered. Ten to twenty plants of each line were tested and error bars represent ±standard error.

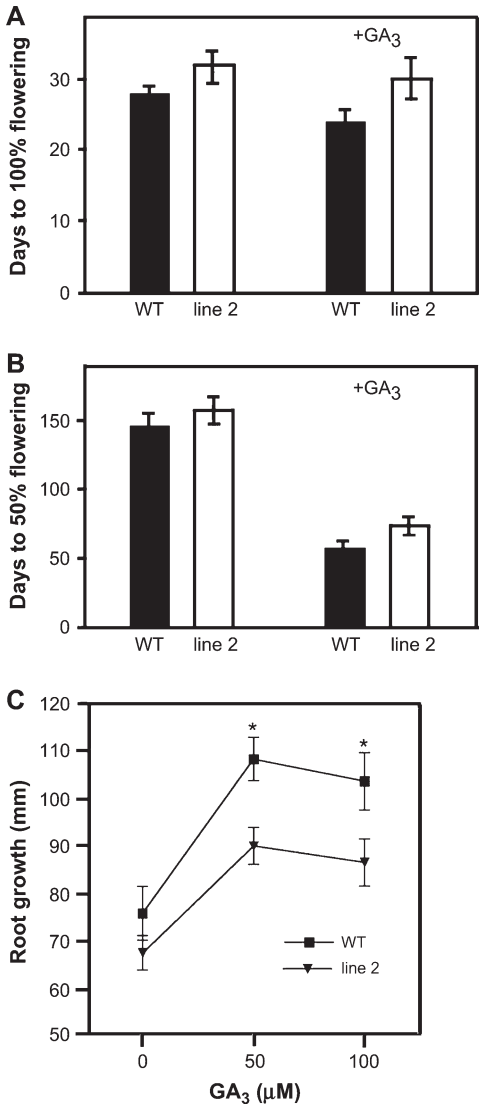

Fig. 9. Effect of GA on flowering time and root growth in wild-type (WT) and 35S-*FaGAST* line 2 *Arabidopsis* plants. (A) Effect of exogenous GA_3 on flowering time in long days, measured as the time at which 100% of the plants have produced visible flowers. (B) Effect of exogenous GA_3 on flowering time in short days, measured as the time at which 50% of the plants have produced visible flowers. Results are presented as means with error bars representing ±standard error (n=15–20). (C) Root growth was measured after 10 d growth in Petri dishes supplemented with different GA_3 concentrations, as indicated in the Materials and methods. Error bars represented ±standard error (n=10–15). Asterisks indicate that values are significantly different (P <0.0001) by the χ^2 test.

that they are involved in diverse processes based on their sequences and different expression patterns. In some cases, a cleavable signal peptide targets the protein to the cell wall or the extracellular space. This might determine that the protein participates either in the cell elongation process, as the tomato *GAST1* and the petunia *GIP2*, or the cessation of elongation, as is the case of *GEG* from *Gerbera*. The presence of a putative non-cleavable signal peptide would target the proteins to the endoplasmic reticulum. *GAST*-like genes with this putative signal peptide include the *GIP1* gene from petunia, which is similar to the petunia *GIP4*, and the *Arabidopsis GASA4* and *GASA5* genes. For these proteins, a participation in the cell division process has been proposed. Based on computer analysis, the strawberry *FaGAST* product is predicted to be in the cell wall or extracellular space, where a direct role in cell division is unlikely. However, it should be noted that function assignment in all cases has been mainly based on the expression pattern of the genes rather than on the cellular location.

It has been shown that *FaGAST* is differentially expressed in the receptacle of strawberry fruits and in roots. Expression in fruit is higher at two ripening stages, F4 and F7. Fruit growth of the strawberry cultivar Chandler shows a double sigmoidal pattern in the growing conditions used in the present study. Interestingly, changes in the slope of this growth curve take place at stages 4 and 7, when *FaGAST* expression peaks. At these two time-points of the curve, fruit growth slows down, suggesting a role of *FaGAST* in decreasing growth rate. The final size of the strawberry fruit is highly variable in *Fragaria* species and is dependent on many factors, being the combined result of both cell division and elongation (Moore *et al.*, 1970; Cheng and Breen, 1992). Cell division ceases 10–15 d after anthesis, which corresponds to the end of stage 3. Between stage 4 and stage 7 it is mainly cell expansion that takes place. Because FaGAST protein is putatively localized to the cell wall and its expression does not coincide with the cell division phase, a role of this protein in cell division can be excluded.

Auxin concentration is highest in the receptacle and achenes at the end of stage F3, at about 10 d after anthesis, and rapidly decreases in the receptacle as fruit ripen (Archbold and Dennis, 1984). The highest GA content coincides with the maximum amount of auxin, then decreases with fruit development, rising again in the ripe receptacle at F7 (Lis *et al.*, 1978). Maximum expression of *FaGAST* in fruits occurs in F4, just after the peak of GA, suggesting that this gene might be regulated by this hormone. This was further supported by the finding that exogenous application of GA_3 increased *FaGAST* expression (Fig. 3B).

The strawberry gene *FaGAST* is also highly expressed in roots (Fig. 2). *In-situ* hybridization indicated that the expression of *FaGAST* in roots is limited to cells at the end of the elongation zone prior to differentiation (Fig. 4), where root growth rate is highly reduced (Dolan and Davies, 2004). Because expression in roots was coincident to the final stage of cell elongation, *FaGAST* may be involved in the growth arrest of root cells. A map of gene expression for >22000 genes has been performed in *Arabidopsis* roots, where up to 15 different zones of expression have been studied separately (Birnbaum *et al.*, 2003). Analysis of the *GASA* genes reveals that *GASA4*

had the highest expression level, as expected according to previous studies (Herzog *et al.*, 1995), with a peak in the endodermis of the early elongation zone, followed by *GASA1* with highest values in the stele, endodermis, and cortex of the mid-elongation to early differentiation zone. *GASA2* and *GASA3* expression is very low in roots, but highest values were always in the mid-elongation to early differentiation zones, i.e. when cells stop to elongate. Interestingly, the *Arabidopsis GASA1* gene clusters close to *FaGAST* in the phylogenetic analysis, with GASA2 and 3 also in the same related group.

Overexpression of *FaGAST* in transgenic plants of both *F. vesca* and *Arabidopsis* further supports a role of FaGAST in cell elongation arrest. The phenotypic changes observed in transgenic lines of both species were similar, with plants being retarded in their growth compared with the wild type. During vegetative growth, transgenic lines were slightly smaller than wild type, with fewer leaves that were smaller. In *Arabidopsis*, the transgene also affected flowering time. The same effect occurred in most transgenic lines of *F. vesca*, which failed to flower the first season while all control plants (wild type from tissue culture and plants transformed with an unrelated gene) produced flowers. Repressing the expression of *GIP2* by RNA interference showed a function of this gene in cell elongation (Ben-Nissan *et al.*, 2004). Also, these transgenic plants exhibited late flowering. Interestingly, inactivation of *GIP2*, a cell elongation gene, and overexpression of *FaGAST*, an elongation arrest gene, produced the same effect on flowering time.

Overexpression of *FaGAST* in *F. vesca* reduced the size of strawberry fruits. This result is similar to that previously reported in *Gerbera* plants overexpressing the *GEG* gene, where flowers had shorter corollas (Kotilainen *et al.*, 1999). Interestingly, *GEG* clusters in the group of proteins with highest homology to *FaGAST* (Fig. 1). Similar to *GEG* in *Gerbera* corollas, the present results suggest a role for *FaGAST* in the inhibition of cell elongation in both strawberry fruit and root cells. In *Arabidopsis*, overexpression of *FaGAST* not only affected vegetative growth and flowering time, resembling the phenotype of GA mutants, but also reduced sensitivity of roots to GA. How a predicted cell wall protein such as FaGAST is able to delay the growth of strawberry and *Arabidopsis* plants and how it may interfere with the GA response needs further investigation.

The biochemical role played by the proteins encoded by this gene family, which have been suggested to participate in different cellular events such as cell division, cell elongation, or its detention, is still intriguing. GAST-like proteins have been shown to be present in distant species, various organs, different developmental processes, and even separated cell compartments. However, all of them maintain structural features, like the 12 cysteines of the C-terminus, in highly conserved positions of the amino acid sequence. This probably points to a protein structure in common that is essential for their biochemical role. The presence of a gene family within species sets up barriers for a reverse genetics approach. Therefore, biochemical studies, especially in the interaction with other macromolecules or cell polymers, would provide some hints as to the function of these proteins, followed by structural studies of this possible interaction.

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