**FaGAST2, a Strawberry Ripening-Related Gene, Acts Together with FaGAST1 to Determine Cell Size of the Fruit Receptacle**

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Numerous GAST-like genes have been reported in higher plants, but only one GAST-like gene (FaGAST1) has been described in strawberry so far. Herein, we have identified a novel strawberry FaGAST gene (FaGAST2) whose expression showed an increase throughout fruit receptacle development and ripening, coinciding with those stages where a decrease in fruit expansion processes (G3–W and R–OR stages) occurs. FaGAST2 only shares 31% and 15.7% amino acid and nucleotide sequence homology, respectively, with the previously reported FaGAST1 gene, but both genes contain a signal peptide and a highly conserved GASA domain (cysteine-rich domain) in the C-terminal region. FaGAST2 expression is mainly confined to the fruit receptacle and is not regulated by auxins, GA3 or ABA, but is regulated by ethephon, an intracellular generator of ethylene. In addition, the expression of the FaGAST2 gene also increased under oxidative stress conditions (H2O2 or Colletotrichum acutatum infection), suggesting a direct role for FaGAST2 protein in reactive oxygen species scavenging during fruit growth and ripening and during fungal infection. On the other hand, the overexpression of the FaGAST2 gene in different transgenic lines analyzed caused a delay in the growth of strawberry plants and a reduction in the size of the transgenic fruits. The histological studies performed in these fruits showed that their parenchymal cells were smaller than those of the controls, supporting a relationship between FaGAST2 gene expression, strawberry fruit cell elongation and fruit size. However, transitory silencing of FaGAST2 gene expression through RNA interference approaches revealed an increase in FaGAST1 expression, but no changes in fruit cell size were observed. These results support the hypothesis that both genes must act synergistically to determine fruit cell size during fruit development and ripening.

**Keywords:** Fragaria ×ananassa • Fruit ripening • Fruit size • GAST-like • Oxidative stress • Strawberry.

**Abbreviations:** dABA, deuterated ABA; DMSO, dimethyl sulfoxide; G1, G2, G3, W, R, OR, the six stages of strawberry fruit development; MeJA, methyl jasmonate; MMA, Murashige and Skoog salts, morpholine ethanesulfonic acid and acetasigirosine; NAA, 1-naphthalenacetic acid; NDGA, nordihydroguaiaretic acid; QRT-PCR, quantitative real-time PCR; RNAi, RNA interference; ROS, reactive oxygen species; SA, salicylic acid.

The nucleotide sequence reported in this paper has been submitted to GenBank under accession number GW402389.1.

**Introduction**

In the last years, numerous GAST-like genes have been reported in mono- and dicotyledonous plants. GAST genes code for small proteins with a putative N-terminal signal peptide and a well-conserved C-terminal region (GASA domain) in which 12 cysteines are invariably found in conserved positions. Although most of these genes seem to be related to plant development and gibberellin responses, their exact biological role is not yet clear. In fact, although the name of the family was assigned by the first member identified GAST-1 (Gibberellin Stimulated Transcript 1) from tomato (Shi et al. 1992), some other members subsequently characterized are not affected by gibberellin and are regulated in combination with other plant hormones (Shi et al. 1992, Herzog et al. 1995, Ben-Nissan and Weiss 1996, Berrocal-Lobo et al. 2002, Alonso-Ramírez et al. 2009, Wang et al. 2009). It has been proposed that the GASA domain, highly conserved in all these proteins, could determine their biochemical activities (Ben-Nissan et al. 2004). In this sense, it has been speculated that these proteins may play a role in reactive oxygen species (ROS) scavenging due to the presence in their structure of the capacity for the redox-active cysteines to form catalytic disulfide bonds (Wigoda et al. 2006,
Rubinovich and Weiss 2010). Nevertheless, their specific biological functions are not yet clear, although it is probable that these are determined by their spatial and temporal expression during plant development, and by their variable N-terminal sequences (Ben-Nissan et al. 2004).

GAST1 homologs have been identified in Arabidopsis (GASA genes; Herzog et al. 1995, Roxrud et al. 2007), Petunia hybrida (GIP genes; Ben-Nissan and Weiss 1996), Gerbera hybrida (EGG genes; Kotilainen et al. 1999), potato (SNAKIN genes; Segura et al. 1999, Berrocal-Lobo et al. 2002), tomato (SN genes; Balaji and Smart 2011, Balaji et al. 2011); rice (OsGASR genes; Furukawa et al. 2006), strawberry (FaGAST1 gene; de la Fuente et al. 2006) and maize (ZmGSL genes; Zimmermann et al. 2010). All these genes are expressed during a wide range of developmental processes, including shoot and petal cell elongation, cessation of cell elongation, cell division, transition to flowering, seed, root and fruit development, defense against pathogens, resistance to abiotic stresses and fruit maturation (Ben-Nissan and Weiss 1996, Ikeda et al. 1997, Medina-Escobar et al. 1997, Aubert et al. 1998, Kotilainen et al. 1999, Berrocal-Lobo et al. 2002, Almasia et al. 2008, Ben-Nissan et al. 2004, de la Fuente et al. 2006, Wigoda et al. 2006, Peng et al. 2010, Rubinovich and Weiss 2010, Zimmermann et al. 2010).

In Arabidopsis, 14 GASA genes (GASA1–GASA14) have been reported (Roxrud et al. 2007) although GASA4 and GASA5 are the best characterized so far. Thus, while GASAS seems to control flowering time and stem growth (Zhang et al. 2009), GASA4 appears to promote gibberellin responses such as flowering and seed germination, and it could regulate the redox status of the cell to promote or suppress these responses (Aubert et al. 1998, Rubinovich and Weiss 2010). In P. hybrida, four GIP genes (GIP1, GIP2, GIP4 and GIP5) have been isolated and shown to be induced by gibberellin (Ben-Nissan and Weiss 1996, Ben-Nissan et al. 2004, Wigoda et al. 2006). GIP1 and GIP2 are expressed during cell elongation, whereas GIP4 and GIP5 are expressed during cell division (Wigoda et al. 2006). Moreover, GIP2 reduces the H$_2$O$_2$ level in stressed transgenic tissues, suggesting an antioxidant activity for this gene product (Wigoda et al. 2006). In the same way, the SNAKIN-1 and SNAKIN-2 gene products (StSN1 and StSN2) from potato, both of which present antibacterial and antifungal activity (Segura et al. 1999, Berrocal-Lobo et al. 2002), might be involved in redox regulation to confer plant protection through regulation of the ROS level in the cell (Almasia et al. 2008).

However, two different OsGASR genes from rice (OsGASR1 and OsGASR2), strongly expressed in meristem where cell proliferation actively occurs, have been reported recently (Furukawa et al. 2006). Moreover, transgenic rice plants with reduced OsGASR1 expression, showed phenotypes similar to plants deficient in brassinosteroids and reduced sensitivity to gibberellin, suggesting that OsGASR1 could activate brassinosteroid synthesis and be a positive regulator of gibberellin signaling (Wang et al. 2009). In the same way, the PRPGL gene from G. hybrida and ZmGSL genes from maize (ZmGSL1a/b to ZmGSL10) have also been related to proliferation and cell elongation during plant development. Thus, a high level of PRPGL protein has been found in the fast-growing zones of young tissues from petal, leaf and root (Peng et al. 2010). Meanwhile, ZmGSL genes have been shown to play a role in early lateral root development (Zimmermann et al. 2010).

A FaGAST1 gene from strawberry related to the control of fruit size has also been reported (de la Fuente et al. 2006). Strawberry is a non-climacteric fruit, whose growth has been mainly attributed to auxin secretion from achenes (Perkins-Veazie 1995). However, ABA, endogenous gibberellins identified in the fruit, and ethylene have also been described as participating in this process (Blake et al. 2000, Iannetta et al. 2006, Bustamante et al. 2009, Jia et al. 2011). In addition, during the ripening of strawberry fruit, a cell wall relaxation occurs. In other plants, this process is a consequence of both enzyme-mediated alterations (Brummell and Harpster 2001) and non-enzymatic reactions mediated by H$_2$O$_2$ and other ROS (Fry 1998, Rodriguez et al. 2002, Foreman et al. 2003, Liszkay et al. 2004). Like all GAST-like proteins identified so far, the strawberry FaGAST protein contains the well-conserved domain carrying the putative redox-active cysteine residues. So, we have speculated whether this FaGAST1 protein or similar proteins in strawberry could play a role in ROS scavenging during the ripening process.

In the present study, a full-length cDNA corresponding to a novel GAST-like gene (FaGAST2) from strawberry was cloned and its expression analyzed in different plant tissues and during fruit development and ripening. We have explored the expression of this gene under oxidative stress conditions and found that FaGAST2 expression increased significantly after these stress treatments. Also, transgenic plants overexpressing the FaGAST2 gene produced smaller fruits with smaller parenchymal cells than the non-transgenic control plants. This information allows us to suggest a role for the FaGAST2 gene in ripening and elongation of strawberry fruit.

Results

Isolation and sequence analysis of the FaGAST2 gene and protein

Sequencing of a cDNA library obtained from red ripened strawberry (Fragaria × ananassa cv. Camarosa) allowed us to identify and isolate one expressed sequence tag clone (FaGAST2) that showed high sequence homology with genes encoding GASTs from higher plants (ranging from 26.4% to 69%) (Supplementary Fig. S1). The full-length FaGAST2 cDNA isolated contained an open reading frame of 261 bp encoding a polypeptide of 86 amino acid residues with a molecular mass of 9,536 Da. Based on the prediction model of Kyte and Doolittle (1982), the amino acid sequence presented a cleavable N-terminal signal peptide (residues 1–24) and a C-terminal GASA conserved domain (residues 28–86) containing 12 cysteine residues in conserved positions (Aubert et al. 1998) (Supplementary Fig. S2A). Moreover, the complete FaGAST2 cDNA sequence also
contained 84 and 235 bp of the 5’- and 3’-untranslated regions, respectively. The hydrophobic profile of FaGAST2 protein was similar to that of the previously described FaGAST1 protein from strawberry (de la Fuente et al. 2006) and, similarly to FaGAST1 and StSN1, it did not show any amphipathic helical motif (data not shown). However, both a putative cell wall localization and a high probability of excretion (probability of 0.82 and 0.9, respectively) was also predicted for the FaGAST2 protein when the SignalP-HMM program and the iPSORT software were used. Therefore, these later bioinformatics data support the possibility of FaGAST2 protein being located in the extracellular space or within the cell wall.

Amino acid sequence alignment and phylogenetic tree analysis of FaGAST2 protein with other plant proteins of the same family (Supplementary Fig. S1; Fig. 1) revealed a high sequence identity with the strawberry FaGAST1 (31%), but the highest identity was found with the potato StSN1 (69%).

The FaGAST2 and FaGAST1 expression pattern during strawberry fruit growth and ripening indicate that both are ripening-related genes. The analysis of the FaGAST2 gene expression during fruit ripening showed an increase in this transcript throughout fruit receptacle development and ripening. However, a peak of expression was found at the green full-sized stage (G3), and a higher second peak at the full-ripe red stage (R) (Fig. 2). This expression pattern resembles that of the previously described FaGAST1 gene obtained using Northern blot approaches (de la Fuente et al. 2006). However, a significant difference was observed in the over-ripe stage (OR), where FaGAST1 RNA accumulation decreased while FaGAST2 maintained a high expression level (Fig. 2).

Both FaGAST genes were weakly expressed in achenes of all growth and ripening stages analyzed (Fig. 3A). In addition, unlike the FaGAST1 gene, which is also expressed in other vegetative tissues (de la Fuente et al. 2006), FaGAST2 was barely expressed in vegetative tissues (Fig. 3B). These results indicate that the production of FaGAST2 transcripts is mainly confined to the fruit receptacle during its development and ripening (Fig. 2).

**FaGAST2 expression is not regulated by auxins released from the achenes**

Several studies have shown that removing the achenes from immature strawberry fruits induces the expression of ripening-related genes, especially those involved in molecular pathways linked to the organoleptic properties of the fruit. This induction has been related to a decrease in auxin content in the fruit receptacle. However, the expression of the FaGAST2 gene remained unaltered during 5 d after removing the achenes from the strawberry fruit, like the FaGAST1 gene (Supplementary Fig. S3). Therefore, in contrast to other strawberry ripening-related genes, expression of FaGAST1 genes was not negatively regulated by the auxins released by the achenes into the fruit receptacle.

**The expression of both FaGAST genes is not affected by gibberellins and ABA**

It has been demonstrated that GA3 is capable of stimulating the expression of numerous GASA genes. In strawberry fruits, the levels of this hormone change during fruit ripening, coinciding with the maximum amount of auxin at both the G2–G3 stages and the ripe stage (R) (Lis et al. 1978). Therefore, to determine if GA3 could activate FaGAST2 expression, its expression pattern...
FaGAST2 expression increases in strawberry fruit after H₂O₂ treatment and Colletotrichum acutatum infection

To find out whether the expression of FaGAST2 could be responsive to the cell redox status of strawberry receptacles, the expression pattern of this gene was tested in fruit after H₂O₂ treatment. The FaGAST2 transcript level increased at the G2 stage after 8 h of H₂O₂ treatment, while FaGAST1 expression remained constant (Fig. 5). This result agrees with a potential role for FaGAST2 as an antioxidant to protect cells against the damaging effects of ROS.

In potato plants, the GASA-like genes StSN1 and StSN2 have been described as antimicrobial peptides that may be part of a pre-existing defense barrier against infection due to their capacity in vitro to aggregate and inhibit some bacterial and fungal species (Berrocal-Lobo et al. 2002, Almasia et al. 2008). Also, it is well known that, in plants, ROS are produced after pathogen infection. In order to determine if FaGAST2 gene expression responds to fungal pathogen infections, we analyzed its transcriptional pattern in crown and fruit from strawberry plants challenged with C. acutatum. The results showed that the FaGAST2 gene was up-regulated in both infected tissues analyzed, while FaGAST1 expression did not change (Fig. 6).

**FaGAST2 expression is up-regulated by ethephon but not by SA and MeJa**

Molecules such as salicylic acid (SA), methyl jasmonate (MeJA) and ethylene are well known as plant regulators in response to biotic and abiotic stresses and plant development (Turner et al. 2002, Kunkel and Brooks 2002). These molecules are also involved in plant senescence, although any one of them alone...
is enough to induce the process (Buchanan-Wollaston et al. 2003). In strawberry fruits, exogenous application of these molecules enhanced resistance to fungal pathogens (Amil-Ruiz et al. 2011).

In order to determine whether SA, JA or ethylene signaling pathways control the expression of FaGAST2, we performed experiments using a strawberry cell suspension treated with each of these well known plant regulators. In our study, no significant change in FaGAST2 and FaGAST1 expression was observed after exogenous application of either SA or MeJA, even after 48 h of treatment (data not shown). However, in the case of treatment with ethephon, FaGAST2 expression was strongly up-regulated after 48 h of treatment while FaGAST1 was not significantly induced (Fig. 7). Ethephon is decomposed in the cell releasing ethylene in the cytoplasm (Stotz et al. 2000), and thus mimics the effect of ethylene. The results obtained strongly suggest that ethylene might positively regulate the expression of the FaGAST2 gene.

**In situ expression analysis shows that the FaGAST2 gene is expressed in parenchymal cells of strawberry fruit**

In order to localize the presence of the FaGAST2 transcript within the different cell types, in situ hybridization experiments were carried out in G3 stage strawberry fruit. The hybridization signal was detected exclusively in parenchymal cells of the fruit receptacle (Fig. 8), but no signal was observed in the epidermal...
cell layer, vascular tissue or achenes. These results suggest a putative role for the strawberry FaGAST2 in the physiological processes occurring in parenchymal cells throughout fruit development and ripening.

Overexpression of the FaGAST2 gene in strawberry decreased both the parenchymal cells and the fruit size

To clarify the functional role played by the FaGAST2 gene in strawberry fruit development and ripening, the FaGAST2 gene was overexpressed in *Fragaria × ananassa* cv. Camarosa plants. A significant increase in FaGAST2 gene expression was found in 10 of the transgenic lines generated, while FaGAST1 gene expression remained constant in both transgenic and control fruits. Of these, lines 9, 11 and 14 presented high FaGAST2 expression in transgenic fruits and were selected for a more detailed study (Fig. 9). These transgenic lines were phenotypically shorter, their roots were very poorly developed and they showed a greater difficulty in growing and regenerating than the non-transgenic control lines, suggesting that the overexpression of FaGAST2 protein interferes with the regeneration process of the plant. These results have also been described for transgenic strawberry lines overexpressing the FaGAST1 gene (de la Fuente et al. 2006).

Very interestingly, the fruits produced by the selected FaGAST2 transgenic plant lines were significantly smaller than...
the fruits produced by non-transgenic control lines (Fig. 10A). Although the histological analysis showed a similar cellular organization, the transgenic parenchymal cells were smaller than their control counterparts (Fig. 10B, C, F). However, the vascular bundles, epidermal and pith cells in fruit were identical in both transgenic and control lines (data not shown).

Similar results were obtained in crown cells from control and transgenic lines overexpressing the FaGAST2 gene (Fig. 10D–F). These results suggest that FaGAST2 overexpression promotes a reduction in the transgenic fruit size and plant development probably through the reduction in cell size.

Transitory silencing of FaGAST2 gene expression induces the expression of the FaGAST1 gene but does not affect fruit cell size

To study the biological role of FaGAST2 in strawberry, we tried to obtain knock-down transgenic RNA interference (RNAi) lines. However, despite having tried several times, we never obtained any FaGAST2 silenced transgenic line. Similar results were obtained for the silencing of FaGAST1 gene expression (V. Valpuesta et al. personal communication). These results suggest that the silencing of these genes could be lethal for the plant. For this reason, we have used a transient silencing approach to knock out the expression of the FaGAST2 gene in strawberry fruits (Fragaria x ananassa cv. Elsanta). Thus, we have agroinfiltrated fruit receptacles with the FaGAST2-RNAi construct (a pFRN derive). When compared with the control fruits carrying the empty pFRN vector, the transiently silenced FaGAST2 fruits showed no phenotypic changes. However, while the FaGAST2 gene expression decreased in these fruits, the expression of the FaGAST1 gene was significantly increased (Supplementary Fig. S5A). These results indicate the existence of a putative compensation mechanism in the expression of both genes. Thus, FaGAST1 and FaGAST2 proteins could functionally complement each other in order to maintain the
Fig. 7 Effect of salicylic acid (SA) (A), methyl jasmonate (MeJA) (B) and ethephon (C) treatments on FaGAST2 and FaGAST1 gene expression in strawberry cell cultures. In all experiments, the control sample (0 h) was a cell culture without treatment. Other samples were collected at the times shown in the figure. In (A) and (B), strawberry cinnamyl alcohol dehydrogenase (FaCAD) induced by SA and MeJA (unpublished data) was used as a positive control gene, while in (C) the strawberry ethylene receptor FaEtr2 was used (Trainotti et al. 2005). The increase in mRNA was relative to the control sample in each experiment, which was assigned an arbitrary value equal to unity. Mean values ± SD of three independent experiments are shown. (A and B) Statistical significance with respect to the control samples (0 h) was determined by the Student's t-test. ***P-value ≤ 0.001.
amount of FaGAST protein needed to determine the correct fruit cell size. As stated above, the histological analysis of transgenic fruits with FaGAST2 gene expression transiently silenced did not show any changes in the parenchymal cells size (Supplementary Fig. S5B), strongly supporting this hypothesis.

Discussion

A large number of GAST homolog genes have been identified in higher plants, but in strawberry only one GAST-like gene (FaGAST1) has been described so far (de la Fuente et al. 2006). In this study, we have reported on the isolation and functional characterization of a novel strawberry GAST-like gene (FaGAST2), whose expression pattern differs from that of the previously described FaGAST1. Like the FaGAST1 gene, FaGAST2 contains a highly conserved GASA domain and a similar signal peptide. However, both strawberry genes only shared 31% and 15.7% amino acid and nucleotide sequence homology, respectively (Supplementary Fig. S2). This fact could explain the absence of any hybridization signal corresponding to the FaGAST2 gene in the screening reported by de la Fuente et al. (2006), of a cDNA fruit library using a FaGAST1 probe. Both strawberry proteins present the 12 cysteine residues located in exactly the same positions within the protein sequence as those of the GASA gene family in Arabidopsis (Herzog et al. 1995, Roxrud et al. 2007); petunia GIP1, GIP2, GIP4 and GIP5 genes (Ben-Nissan and Weiss 1996, Ben-Nissan et al. 2004); SNAKIN-1 and SNAKIN-2 genes from potato (Segura et al. 1999, Berrocal-Lobo et al. 2002); the GEG gene from Gerbera (Kotilainen et al. 1999); OsGASR1 and OsGASR2 genes from rice (Furukawa et al. 2006); and the ZmGSL1–ZmGSL10 genes from maize (Zimmermann et al. 2010) (Supplementary Fig. S1).

Based on conserved residues inside the conserved domain, in addition to the 12 characteristic cysteines, it has been proposed that proteins with a GASA domain can be grouped into three subfamilies (I–III) (Berrocal-Lobo et al. 2002). According to this classification, FaGAST2 belongs to family I, which also includes StSN1, while FaGAST1 belongs to family II, which includes StSN2 and GhGEG (Supplementary Fig. S2). As was expected, FaGAST2 amino acid sequence comparisons showed the highest sequence identity with the potato StSN1 (69%). It is believed that a conserved distance between these cysteines might be required for the generation of an essential three-dimensional structure and/or for an interaction with other proteins (Ben-Nissan et al. 2004). In addition, recently it has been demonstrated that the loss by mutation of some of these cysteine residues reduces the redox activity of the GASA4...
protein, suggesting that this protein, and all GAST proteins containing these conserved cysteine residues, could participate in determining the redox status of the cell in certain physiological and developmental plant responses (Rubinovich and Weiss 2010).

Fig. 9  Analysis of the phenotype of the transgenic Fragaria × ananassa cv. Camarosa plants transformed with the pK7WG2-FaGAST2 construct: (A) Analysis by QRT-PCR of FaGAST2 gene expression in fruits of control and transgenic lines 9, 11 and 14. (B) General morphology of control (wild type) and transgenic lines 9, 11 and 14 cultivated in vitro. (C) Top view of the control and transgenic lines 60 d after their acclimation in soil. The overexpression level is expressed as a percentage. Statistical significance was determined by the Student’s t-test. **P-value ≤ 0.01 and ***P-value ≤ 0.001. Scale bars = 10 cm.

Both the difference detected between the predicted FaGAST1 and FaGAST2 amino acidic sequences (Supplementary Fig. S1) and the difference observed in their gene expression patterns (Fig. 2) indicate a divergence of both proteins that does not necessarily mean a different physiological
function but rather a spatio-temporal complementarity. Thus, while FaGAST1 showed a high level of expression in roots and other vegetative tissues such as leaves and flowers (de la Fuente et al. 2006), only a high level of expression of FaGAST2 was clearly detected in the fruit receptacle, thus suggesting a very confined functional role for the FaGAST2 protein in this strawberry tissue during fruit development and ripening. Indeed, in situ hybridization indicated that FaGAST2 gene expression was restricted to the external parenchymal cells of the fruit receptacle. Altogether, these results might indicate that the physiological role played by this FaGAST2 protein would be confined to this fruit cell type.

In addition, the FaGAST2 gene showed a similar fruit expression pattern to that of FaGAST1 (de la Fuente et al. 2006), and closely related to the double sigmoidal pattern observed for receptacle growth and ripening. Two peaks of maximum expression level were found for both strawberry genes, which where coincident with the stages of the end of the fruit expansion processes (G3–W and R–OR stages), but the amount of transcripts for both genes were always higher in ripened-red fruit receptacles. In contrast to FaGAST1, the expression of the FaGAST2 gene remained high at the OR stage (Fig. 2) suggesting that this protein might also play an additional role in the fruit senescence process. However, this remains to be addressed. Also, we have observed that transgenic strawberry fruits overexpressing the FaGAST2 gene produced fruit with a decreased size (Fig. 10A). Taken together, these results support a functional relationship between the FaGAST2 gene and the fruit-specific molecular processes leading to control of the expansion of the fruit receptacle. In Fragaria vesca fruit, similar results were found for the overexpression of the FaGAST1 gene (de la Fuente et al. 2006). The histological studies indicated that this phenomenon was, indeed, mainly due to a reduction in parenchymal cell size, and they also support the idea that the FaGAST2 protein could be involved specifically in the cessation of the expansion of these receptacle cells.

On the other hand, we have observed that the transient silencing of FaGAST2 gene expression brings about an increase in the amount of FaGAST1 gene transcript (Supplementary Fig S5A). This phenomenon is intriguing but it suggests a relationship between these two genes in the control of cell size volume. This could occur if both gene products were redundant in

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<td>Control</td>
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Fig. 10 Phenotypic and cellular changes in transgenic fruits overexpressing the FaGAST2 gene. (A) Phenotype of control Fragaria × ananassa cv. Camarosa and transgenic fruits transformed with the pK7WG2-FaGAST2 construct. (B–E) Transversal sections of parenchymal tissue from both control fruits and crown (B and D, respectively) and both transgenic fruits and crown (C and E, respectively). The transgenic tissues were transformed with the FaGAST2-pK7WG2 construct (C and E). (F) Quantitative study of the size and shape of receptacle cells in control and transgenic fruits and also in crown tissues overexpressing the FaGAST2 gene. The cell shape coefficient indicates the shape of cells. Thus, the more similar the value of the index is to 1, the more spherical the cells. Statistical significance was determined by the Student’s t-test. *P-value ≤ 0.001. Scale bars: (B) and (C) = 100 µm; (D) and (F) = 50 µm.
function. Thus, a decrease in FaGAST2 gene expression could be compensated by a concomitant increase in FaGAST1 gene expression, leading in turn to phenotypes which were not obvious. Indeed, the absence of changes in cell size in FaGAST2 transiently silenced fruits was observed. Therefore, a compensatory regulatory system between the expression of both genes probably exists in order to obtain an optimal global gene expression dosage that would allow the adequate production of FaGAST proteins, which is required for the correct cell volume and fruit size. This possibility should be addressed in the future.

Taking into consideration all the results, we propose that both FaGAST proteins act together in strawberry, and this opens up the possibility of modifying the size of fruit in this important cultivated species through biotechnological approaches.

In the strawberry fruit receptacle, auxin levels released from the achenes decline throughout the different stages of fruit development. The absence or reduction of the auxin content in the receptacle appears to control the ripening process of the fruit receptacle (Perkins-Veazie 1995). In this sense, several studies have shown that the expression of many strawberry fruit ripening-related genes, mainly those related to the organoleptic properties of the fruit, are induced in the receptacle in the absence of auxins (Medina-Escobar et al. 1997, Moyano et al. 1998, Blanco-Portales et al. 2002, Benitez-Burraco et al. 2003, Raab et al. 2006, Griesser et al. 2008). On the other hand, it has been hypothesized that the ABA/auxin ratio could be part of the signal that triggers strawberry fruit ripening (Perkins-Veazie 1995). Recently, some studies have provided molecular evidence that ABA is a signal molecule that, at least, can promote the strawberry ripening-related production of anthocyanins (Chai et al. 2011, Jia et al. 2011). In fact, in strawberry fruits, the ABA content gradually increased concomitantly with the ripening process. However, in our experiments, the expression of both FaGAST genes in the fruit receptacle was unchanged under those treatments or physiological conditions where the content of auxins or ABA in this strawberry tissue either decreased or increased, respectively, indicating that none of these hormones regulates the expression of this gene. Similarly, the expression of both FaGAST genes was not induced by GA3, treatment, and it is coincident with the results reported for GAS2 and GAS3 genes whose expression was not induced by this hormone (Herzog et al. 1995). However, our results are different from those previously reported for the FaGAST1 gene, as an increase in expression of this latter gene in fruits after the external application of GA3 has been reported (de la Fuente et al. 2006). These differences could be related to the different methodology used in both studies to determine the amount of transcript (Northern blot vs. QRT-PCR).

Interestingly, the expression of FaGAST2, but not of FaGAST1, was clearly induced in the presence of ethephon, an intracellular generator of ethylene. However, the expression of these genes was not induced by SA or MeJA treatments (Fig. 7). These results strongly suggest that the expression of the FaGAST2 gene is regulated through the ethylene pathway. It is known that, as a non-climacteric fruit, strawberry ripening is not clearly affected by ethylene. However, ethylene has been detected at the G3 stage, preceding the start of ripening, and at the R stage, when maturation has occurred (Perkins-Veazie et al. 1995, Perkins-Veazie et al. 1996, Iannetta et al. 2006). Accordingly, the FaGAST2 expression pattern is higher in both stages of strawberry fruit development where ethylene accumulation has been reported (Abeles and Takeda 1990, Perkins-Veazie et al. 1996). The presence of ethylene, ethylene biosynthetic genes (FaACO1 and FaACO2) and receptors for this hormone (FaER1, FaER2 and FaETR2) in mature strawberry fruits has been previously suggested to be evidence of the involvement of ethylene in some physiological processes during strawberry fruit ripening and senescence (Trainotti et al. 2005). In this context, it has been proposed that the application of ethylene may act in enhancing strawberry fruit senescence (El-Kazzaz et al. 1983), while the inhibition of ethylene perception by 1-methyl cyclopentene delays some ripening processes such as fruit softening (Jiang et al. 2001). Altogether, these results provide information about the relationship between FaGAST2 expression and ethylene, and support an additional role for FaGAST2 in processes related to fruit ripening and senescence.

It is noteworthy that in the strawberry microarray analysis reported by Aharoni et al. (2002), 20 ripening-related genes induced by oxidative stress were identified. Indeed, a positive relationship between the strawberry fruit ripening transcription program and the induced oxidative stress was proposed to be coupled to the development and differentiation of the vascular system in the fruit receptacle (Aharoni et al. 2002). In support of this proposal, López-Serrano and Barceló (2001) had previously reported a basic peroxidase isoenzyme which could control the ROS level in fruit receptacles, and was located around the vascular bundles, and in the vascular connections with the achenes. At this point, the FaGAST2 gene seems to be a component of the ripening transcription program switched on by ROS production as we have observed that FaGAST2 gene expression is induced in strawberry plants after H2O2 treatment. A similar regulation by the oxidative burst has also been reported for GIP2, another GASA-like gene (Wigoda et al. 2006). In fact, there is evidence that the generation of ROS is sufficient to degrade cell wall polysaccharides by cleavage of backbone bonds (Schweikert et al. 2000, Córdoba-Pedregosa et al. 2007, Müller et al. 2009). Thus, it is likely that GAST-like proteins reduce or oxidize specific cell wall proteins as most of them are secreted and/or are associated with the cell wall matrix (Ben-Nissan et al. 2004).

In addition, the expression of the FaGAST2 gene was also found to be induced after challenging strawberry plants with the fungal pathogen C. acutatum. It is well known in other plant systems that ROS production increases after plant pathogen infection. Thus, the expression of FaGAST2 could be related to the antioxidant capacity of its gene product. It has been reported that during the strawberry–C. acutatum interaction, a strong induction of a transcript with sequence homology to an
EREBP transcription factor (an ethylene-response-element binding protein) is induced (Casado-Díaz et al. 2006). This again points to a close relationship between FaGAST2 gene expression and ethylene. Also, ethylene has been described as being involved in specific gene activation during plant defense against pathogens (Wang et al. 2002). Therefore, it remains to be clarified whether the up-regulation of FaGAST2 expression during C. acutatum infection may indicate a direct role for this strawberry FaGAST2 protein in ROS scavenging due to the oxidative burst produced in response to this fungal infection or whether it is a consequence of the putative involvement of ethylene signaling in this plant-pathogen interaction, and so has another different effect within the defense network displayed by the plant against the pathogen. Curiously, it has been recently demonstrated that overexpression in transgenic potato plants of StSN1 confers broad protection against pathogens (Almasia et al. 2008), and also that the silencing of the SN2 gene in Nicotiana benthamiana increases the plant’s susceptibility to Clavibacter michiganensis (Balaji et al. 2011).

In summary, our results indicate that both FaGAST1 and FaGAST2 are ripening-related genes that play an important role during fruit growth and ripening processes, where their protein products seem to act synergistically in order to determine fruit size.

**Materials and Methods**

**Plant material**

Strawberry plants (Fragaria × ananassa Duch. cv. Camarosa, an octoploid cultivar, F. vesca, F. chiloensis and F. virginiana) were grown under field conditions in Huelva (S.W. Spain). Fragaria × ananassa fruits were harvested at different developmental stages: small-sized green fruits (G1, 2–3 g), medium-sized green fruits (G2, 3–5 g), full-sized green fruits (G3, 4–7 g), white fruits (W, 5–8 g), turning stage fruits (T, 6–10 g), fully ripe red fruits (R, 6–10 g) and over-ripe fruits (OR, 6–10 g). Vegetative tissues, such as runners, roots, crowns and expanding leaves, were also harvested. All tissues and fruit samples were immediately frozen in liquid nitrogen and stored at –80°C. Strawberry plants (Fragaria × ananassa Duch. cv. Elsanta) were grown and maintained in a greenhouse, and later used for fruit agroinfiltration experiments in a plant chamber at 25°C, 10,000 lux and 80% humidity.

**Hormone treatments**

For the treatments with auxins, achenes of two sets of 50 medium-sized green fruits (G2) each, still attached to the plant, were carefully removed using the tip of a scalpel blade. One set of deachened G2 stage fruits was treated with the synthetic auxin 1-naphthalenacetic acid (NAA) in lanolin paste (1 ml) with 1 mM NAA in 1% (w/v) dimethylsulfoxide (DMSO). The other set of G2 stage deachened fruits (control group) was treated with the same paste, but without NAA. Both treatments were applied over the whole fruit surface. All the fruits were harvested 5 d after treatment, immediately frozen in liquid nitrogen and stored at –80°C. During the course of the assays, the fruits reached the G3–W developmental stage.

For the GA3 fruit treatments, G2 stage fruits (still attached to the greenhouse plants) were carefully injected with 1 ml of GA3 (0.1 mM) sterile solution or sterile water (control fruits) using a hypodermic syringe. The ABA (0.5 mM) and H2O2 (0.75 mM) treatments were also performed by injection in G2 stage fruits as described above. However, while the fruits treated with GA3 and ABA were harvested 5 d after the corresponding treatment, the fruits injected with H2O2 were harvested at 0, 2, 4 and 8 h after the injection. In all cases, the treated fruits were frozen in liquid nitrogen and stored at –80°C until analysis. Additionally, a second GA3 treatment was performed in G2 stage fruits by spraying with GA3 (0.1 mM) solution at 6 h intervals for 5 d. As control, a set of G2 stage fruits was sprayed with sterile water. Fruits were collected after 5 d of treatment, immediately frozen in liquid nitrogen and stored at –80°C.

**NDGA treatment**

NDGA is an ideal inhibitor of the 9-cis-epoxycarotenoid dioxygenase (NCED) enzyme and was used to block ABA biosynthesis. For this treatment, G2 stage fruits (still attached to the greenhouse plants) were carefully injected with 0.5 ml of NDGA (100 µM) sterile solution or sterile water (control fruits) using a hypodermic syringe. The fruits injected were harvested after 8 d of treatment, when they reached the R developmental stage, frozen in liquid nitrogen and stored at –80°C. These samples were used for measurement of the ABA content and relative expression of the FaGAST2 gene.

**Strawberry cell culture and treatments**

To initiate callus cultures, young leaves from micropropagated strawberry plants were removed and cut into strips (3–4 mm wide) with a sterile scalpel and forceps. These explants were transferred with their lower epidermis downward to Petri dishes with MS solid medium (Murashige and Skoog 1962), supplemented with 2,4-D (2.5 mg). The dishes were sealed with parafilm and incubated at 25°C in a growth chamber under diffuse light. When calli were developed from the explants (4–6 weeks), small pieces (0.3–0.5 cm diameter) were excised and transferred to fresh medium and cultured as before. In this way, callus stocks were maintained indefinitely by subculturing to fresh medium at monthly intervals. To initiate cell suspension cultures, 1 g of friable callus was excised, disaggregated and transferred to 30 ml of MS liquid medium supplemented with 2,4-D (2.5 mg) in a 100 ml Erlenmeyer flask. These cultures were incubated at 25°C and 100 r.p.m. under diffuse light. The cell cultures were maintained by subculturating 1 g of filtered cells in fresh MS liquid medium with 2,4-D every 15 d. For GA3 and ABA treatments, 500 ml of cell culture refreshed for 5 d was divided between two Erlenmeyer flasks and a sample was removed as control at 0 h. Then, GA3 (50 µM) was added to a 250 ml Erlenmeyer flask, and ABA (100 mM) was
added to the other one. Both GA₃ and ABA cell cultures were incubated at 25°C and 100 r.p.m. under diffuse light. Ethephon (2-chloroethylphosphonic acid) 1 mM, which is decomposed in the cell releasing ethylene in the cytoplasm, was used for the ethylene treatment. In this experiment, cells were harvested at 24 and 48 h. Similarly, cell cultures were treated with SA (0.75 mM) and MeJA (1 mM). Cells were harvested by filtering after 2, 4, 8, 24 and 48 h. In all cases, the harvested cells were immediately frozen in liquid nitrogen, and stored at −80°C.

Preparation of deuterated ABA
Deuterated ABA (dABA) was used as an internal standard. In order to prepare the standard, the ring protons of ABA (5 mg) were exchanged over 48 h at room temperature in 10 ml of heavy water (Sigma: isotopic purity 99.96%) in the presence of 1.0 M deuterated sodium hydroxide (Sigma: minimum isotopic purity 99%) according to Rock and Zeevaart (1990). The medium was acidified with hydrochloric acid to pH 3 and the acidic solution was partitioned three times against equal volumes of diethyl ether. Then, the ether phases were combined and dried under vacuum at 35°C and the samples containing ²H₆ were dissolved in methanol. We evaluated the purity of the [²H₆]ABA obtained by HPLC using the conditions indicated below.

ABA extraction procedure
A 1 g aliquot of powdered material was placed in a 50 ml glass beaker and mixed for 5 min with 1.26 nmol of internal standard (40 μl of 31.5 nmol ml⁻¹ intermediate standard solution). The sample was extracted twice with 10 ml of methanol/water pH 5.5 (1:1, v/v) and the mixture was centrifuged at 5,000 × g for 5 min at room temperature. The supernatant was extracted twice with dichloromethane (10 ml), the extracts obtained were centrifuged again at 5,000 × g for 5 min at room temperature, and the lower phase was evaporated under vacuum conditions at 40°C. The residue was dissolved in 100 μl of 100% acetone and 250 μl of water/acetoniitrile (70:30, v/v) (0.1% formic acid). Finally, the sample was centrifuged at full speed for 5 min and the supernatant obtained was used for analysis.

HPLC–mass spectrometry conditions
To determine the amount of ABA in the strawberry fruits treated with NDGA, we used an HPLC–MS system (VARIAN 1200 L Triple Quadrupole) with a column (150 × 2.1 mm i.d. Phenomenex C₁₈ with 3 μm particle size). The injection volume was 8 μl and the mobile phase used was A, water/0.1% formic acid; and B, acetonitrile/methanol (75:25, v/v) with the gradient program: t = 0 min (95/5 A/B), t = 2 min (65/35 A/B), t = 10 min (0/100 A/B) and t = 15 min (0/100 A/B), at a constant flow rate of 0.2 ml min⁻¹. The mass spectrometer was operated in the negative selecting ion monitoring (SIM) mode. The source was operated with N₂ (LCMS quality) at 50 p.s.i. Mass spectrometry parameters were optimized by flow injection analysis (FIA) of the individual solutions of ABA and [²H₆]ABA. These compounds gave a response in the SIM interface in the negative mode but not in the positive mode. The electrospray ionization needle voltage used was −5.5 kV. Other optimized conditions for mass spectrometry: drying gas, 270°C; source temperature, 55°C; capillary voltage, 45 V; shield voltage, 600 V; detector voltage, 1,500 V; and dwelling times of 1 s per scan were chosen. Quadrupole one ion resolution was optimized at 0.5 Da. Work Station software (Varian) was used to process the quantitative data obtained from calibration standards and plant samples.

Cloning and sequence analysis of full-length cDNA of FaGAST2
The full-length cDNA corresponding to the FaGAST2 gene (GW402389.1) was isolated from a Fragaria × ananassa R stage fruit cDNA library (Medina-Escobar et al. 1997). The nucleotide and deduced amino acid sequences and the phylogenetic tree construction were analyzed using the ‘Lasergene’ software package (DNASTAR). A similarity analysis was performed using the alignment tool (BLAST, National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/BLAST). The prediction of N-terminal sequences was performed with the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP) and iPSORT (http://hypothesiscreator.net/iPSORT/) software.

RNA isolation
Total RNA was isolated from independent pools of strawberry fruits at different growth and ripening stages and from vegetative tissues according to Asif et al. (2000). Achenes were always removed from fruit samples, and only receptacle RNA was extracted and purified. The total RNA obtained was treated with DNase I (RNase free) (Invitrogen) according to the manufacturer’s instructions to remove genomic DNA contamination, and purified by using the RNeasy Mini kit (Qiagen). RNA samples were considered DNA free when no amplicons corresponding to the analyzed genes were observed using RNA as template in a standard PCR.

Expression analysis by QRT-PCR
Gene expression analysis of FaGAST2 was performed by QRT-PCR using the iCycler (BioRad) system as previously described (Benitez-Burraco et al. 2003).

First-strand cDNA was obtained using 2 μg of total RNA and the iScript kit (BioRad) according to the manufacturer’s instructions. FaGAST2 gene primer sequences for quantitative amplification were 5’-CAG GCT TCT TCA TCT GTA CGT GGT GTT GGT-3’ and 5’-ACC CCA CAA GAC TGA AAC AGT GAA ATT CAG-3’, and for the FaGAST1 gene were 5’-CAG AAG AAA TTA-3’ and 5’-CAG ACC ACC AGG ACC AAT GGA ATT CAG-3’. Each reaction was performed at least in triplicate and the corresponding C_threshold values were normalized using the C_threshold value corresponding to an interspace 26S-18S strawberry RNA gene (housekeeping gene) (Benitez-Burraco et al. 2003, Raab et al. 2006, Encinas-Villarejo et al. 2009).
All these values were then used to determine the relative increase or decrease in FaGAST2 gene expression in the samples with respect to the control according to Pedersen (2001). The interspacer 26S-18S gene (primers: 5'-ACC GTT GAT TCG CAC AAT TGG TCA TG-3' and 5'-TAC TGC GGG TCG GCA ATC GGA CG-3') was selected as a control for its constitutive expression throughout the different experimental conditions tested. The efficiency of each particular QRT-PCR and the melting curves of the products were also analyzed to ensure the existence of a single amplification peak corresponding to a unique molecular species. All analyses performed by QRT-PCR included positive control genes to check the effectiveness of experiments. The primer sequences of the control genes used were: 5'-TGG GGA GGT GAG AGG ACG ACC CC-3' and 5'-TGG CAA GCA TAC TGG CAC CAA GAT TTC-3' (Cumplido-Laso et al. 2012) for the FaAAT2 gene; 5'-TTA TTA TTA GTA TGC GAG AAC A-3' and 5'-GAC GGT GCT GAA GAA CAA TG-3' (Encinas-Villarejo et al. 2009) for Fawky-1; 5'-ATT CGA GCC GCT TGA GGA GG-3' and 5'-CTT GCC AGG TCC CCT GAG TG-3' (Aharoni et al. 2002) for Ferritin; 5'-TCC CAG ACA ACT TGC CTC ATG GTG TTG-3' and 5'-ACT CCC ATA GCC TTG GCA AAC TCG ACC-3' for FaCAD; 5'-GGT CGG TGT TGT TTT TAC-3' and 5'-AGC TTC CCT CGT CCT CAC-3' (Trainotti et al. 2005) for FaEtr2; 5'-GAG CTT GTA TGC TGT GGC GTA AA-3' and 5'-CCG TTC AGC AAT GGC GAC ATA AGC ATA-3' for FaNCED1; 5'-TGA GGC GTA GCC GTC CAT TT-3' and 5'-GCT GCC GCT GGT TGG ATC TT-3' for FaHVA22; and 5'-TGA AGG GGT TGT TGG CAG TG-3' and 5'-GCC AAC GGT TAT CCA ACC AA-3' (Csukasi et al. 2011) for FaGID1c.

**In situ hybridization experiments**

G3 stage fruit samples were fixed in 4% formaldehyde in phosphate-buffered saline overnight at 4°C, dehydrated in an increasing ethanol series, and embedded in paraffin wax (Paraplast Plus; Sherwood Med. Co.). Tissue sections (8 μm) were taken using a rotary microtome and mounted on slides covered with poly-l-lysine. Probes were made from FaGAST2 cDNA using T7 and T3 promoters to generate sense or antisense RNA, respectively. The methods used for digoxigenin labeling of RNA probes, tissue preparation and in situ hybridization are as described by Jackson (1991) and Coen et al. (1990) with the modifications of Yubero-Serrano et al. (2003). Prior to hybridization, samples were incubated with 3 μg ml⁻¹ proteinase K for 30 min, and 5 min with 0.2% glycine to block the protease. Hybridization of the samples was performed at 50°C for 12–14 h in hybridization solution [50% formamide, 6 × SSC, 3% SDS, 100 μg ml⁻¹ tRNA, 100 μg ml⁻¹ poly(A)]. Afterwards, the samples were washed with 2 × SSC, 50% formamide, at 50°C for 30 min, and twice with the same wash solution for 1 h 30 min. Then, the samples were rinsed twice for 5 min with NTE solution (10 mM Tris–HCl pH 7.5, 500 mM NaCl and 1 mM EDTA) at 37°C and incubated in a pre-warmed NTE solution containing RNase A (20 μg ml⁻¹) at 37°C for 30 min. Finally, samples were rinsed again for 5 min with NTE solution and 1 × SSC for 20 min twice respectively, and once with 0.1 × SSC for 1 h at room temperature. The hybridized probes were detected using an alkaline phosphatase antibody conjugate. After final color development, slides were dehydrated through an ethanol series, dried, mounted with Entellan (Merck) and viewed using brightfield microscopy.

**Controlled inoculation of strawberry plants with Colletotrichum acutatum**

The controlled infection of Fragaria × ananassa cv. Camarosa plants was performed as previously described by Casado-Díaz et al. (2006). Briefly, the C. acutatum isolate CECT 20240 was grown on Difco potato dextrose agarplus 2 g l⁻¹ yeast extract (PDAY) under continuous fluorescent light (Osram L 18 W/21-840 Hellweiss Lumilux Cool White, 75 mE m⁻² s⁻¹) for 7 d at 25°C. A conidial suspension was prepared by flooding the surface of culture plates with sterile distilled water, scraping the surface with a scalpel and filtering the suspension through cheesecloth. The concentration was adjusted to 104 conidia ml⁻¹ by dilution and counting with a hemocytometer. Strawberry plants were artificially inoculated with a hand-pump sprayer and the conidial suspension was applied until run-off (Denoyes-Rothan and Baudry 1995). Inoculated plants were enclosed in light plastic bags to maintain high relative humidity and were incubated in a growth chamber at 25°C, exposed to a 16 h photoperiod under fluorescent light (Sylvania Luxline Plus F58W/840 Cool White DE Luxe, 100.5 mE m⁻² s⁻¹). Control plants were sprayed with sterile distilled water and incubated as described above. Isolating the pathogen from the strawberry plants after the same inoculation time tested the efficacy of the inoculation procedure: crown sections were surface-sterilized and plated on PDAY for re-isolation of the pathogen.

**Overexpression of the FaGAST2 gene in strawberry plants**

The FaGAST2 cDNA was cloned into the pK7WG2 binary vector using Gateway technology (Invitrogen). The coding region was PCR amplified using high fidelity Taq polymerase (BioRad) and the forward primer 5'-GGC TCT TGG CAT CAT CTC CAT CCA-3' and reverse primer 5'-ACT GAA ACT ACT GAA GCA CCC CAC A-3'. The resulting fragment was first cloned into pCR8/GW/TOPO vector and then transferred to pK7WG2 vector by specific recombination between both plasmids using LR clonase (Invitrogen). The overexpression construct (pK7WG2-FaGAST2) was tested by sequencing and restriction analyses prior to transformation of strawberry plants. The binary vector used is listed at http://www. psb.ugent.be/gateway/index. php?_app=vector&_act=construct_list_plant&.

The overexpression construct (pK7WG2-FaGAST2) was used for transformation of Fragaria × ananassa cv. Camarosa using Agrobacterium tumefaciens according to the protocol described previously (El-Mansouri et al. 1996). The Fragaria × ananassa cv. Camarosa control lines were obtained by in vitro...
regeneration in parallel to the transgenic lines, under the same conditions, but without kanamycin. In general, all lines obtained were vegetatively propagated by stolons every season.

The percentage of FaGAST2 overexpression was determined by comparing by QRT-PCR the amount of FaGAST2 transcripts in transgenic fruit receptacles with those observed in fruit receptacles of untransformed plants and plants transformed with the empty pK7WG2 vector (control fruits).

**Generation of a FaGAST2 RNAi construct and transfection of strawberry fruit by agroinfiltration**

The FaGAST2 gene was cloned into the pFRN binary vector (courtesy of Dr. Marten Denekamp from the Department of Molecular Cell Biology, University of Utrecht, The Netherlands) using Gateway technology (Invitrogen). A 529 bp region from the FaGAST2 CDNA (cv. Camarosa) was PCR amplified using it as the RNAi fragment in the silencing construct. The forward primer 5’-TGA CCT CTT CCG TTC ATC TCC ATC CAA C-3’ and reverse primer 5’-ACC CCA CAA GAC TGA AAC AGT GAA ATT CGT-3’ were used. The resulting fragment was cloned into pCR8/GW/TOPO (Invitrogen) and subsequently transferred to the Gateway pFRN vector by specific recombination using LR clonase (Invitrogen). The generated RNAi construct (pFRN-FaGAST2) was tested by sequencing and restriction analyses prior to transformation of strawberry plants. The pFRN-FaGAST2 construct generates RNAi directed against the endogenous FaGAST2 gene.

The A. tumefaciens strain AGL0 (Lazo et al. 1991) containing the pFRN-FaGAST2 or empty pFRN (control) was grown at 28 °C in fresh Luria–Bertani medium supplemented with appropriate antibiotics. When the culture reached an OD600 of about 0.8, Agrobacterium cells were harvested and resuspended in a modified MMA medium (Murashige and Skoog salts, 10 mM morpholine ethanesulfonic acid pH 5.6, 20 g l-1 sucrose and 200 μM acetosyringone) according to Spolaore et al. (2001). Both Agrobacterium suspensions were evenly and independently injected using a sterile 1 ml hypodermic syringe into the base of the entire fruits once (Fragaria × ananassa cv. Elsanta) while they were still attached to the plant 14 d after pollination (Hoffmann et al. 2006, G3 stage). About 10–14 d after treatment, the fruits infiltrated with the silencing or the control constructs were harvested and analyzed by QRT-PCR. A total of 15–25 strawberry plants and 30–40 agroinjected fruits were inoculated and analyzed respectively. The transitory silencing technique was used as an alternative approach to the stable and permanent transformation (transgenic plants).

The percentage of silencing was determined by comparing the amount of FaGAST2 transcripts in pFRN-FaGAST2 agroinjected fruits with those observed in pFRN vector agroinfiltrated fruits.

**Histological analyses**

Pieces of fruit receptacle and crown were dissected from control and transgenic plants and fruits overexpressing the FaGAST2 gene, and then fixed in ethanol: acetic acid (3:1, v/v), dehydrated through an ethanol–tertiary butanol series and embedded in paraffin wax (Paraplast Plus; Sherwood Med. Co.). Sections of about 5–8 μm were cut with a rotary microtome, mounted on slides covered with gelatine, deparaffinized in xylene and rehydrated through an ethanol series. To perform a quantitative study of cells number and their size in the tissues analyzed, transversal sections from the samples were stained with periodic acid-Schiff's reagent (PAS) (Horobin et al. 1988), dehydrated through a graded ethanol series, cleared in xylene and mounted in Entellan New (Merck). About 1,000 cells were scored for both control and transgenic fruits and crown with the ImageJ version 1.40 G software (NIH). A PALM MicroBeam photomicroscope (Zeiss) was utilized for sample visualization and photography.

Pieces of both FaGAST2 transiently silenced strawberry fruits and control fruits were dissected, treated and analyzed as above.


Ikeda, A., Yamguchi, J. and Futsuhara, Y. (1997) Characterization and
Iannetta, P.P.M., Laarhoven, L.-J., Medina-Escobar, N., James, E.K.,
In
Lis, E.K., Borkowska, B. and Antoszewski, R. (1978) Growth regulators
in
Kotilainen, M., Helariutta, Y., Mehto, M., Pollanen, E., Albert, V.A.,
Elomaa, P. et al. (1999) GEG participiles in the regulation of cell and
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