Gibberellin biosynthesis and signalling during development of the strawberry receptacle

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Summary

The enlargement of receptacle cells during strawberry (*Fragaria* × *ananassa*) fruit development is a critical factor determining fruit size, with the increase in cell expansion being one of the most important physiological processes regulated by the phytohormone gibberellin (GA).

Here, we studied the role of GA during strawberry fruit development by analyzing the endogenous content of bioactive GAs and the expression of key components of GA signalling and metabolism. Bioactive GA_1, GA_3 and GA_4 were monitored during fruit development, with the content of GA_4 being extremely high in the receptacle, peaking at the white stage of development.

Genes with high homology to genes encoding GA pathway components, including receptors (*FaGID1(GIBBERELLIN-INSENSITIVE DWARF1)b and FaGID1c*), DELLA (*FaRGA(REPRESSOR OF GA*) and *FaGAI(GA-INSENSITIVE)*)), and enzymes involved in GA biosynthesis (*FaGA3ox*) and catabolism (*FaGA2ox*), were identified, and their expression in different tissues and developmental stages of strawberry fruit was studied in detail. The expression of all of these genes showed a stage-specific pattern during fruit development and was highest in the receptacle. FaGID1c bound GA *in vitro*, interacted with FaRGA *in vitro* and *in vivo*, and increased GA responses when ectopically expressed in Arabidopsis.

This study thus reveals key elements of GA responses in strawberry and points to a critical role for GA in the development of the receptacle.

Introduction

Essential factors controlling the growth and development of strawberry (*Fragaria* × *ananassa*) fruit are poorly characterized at the molecular level. The roles played by different hormones in these processes remain largely unknown (Seeram, 2008). The strawberry achenes (a combination of seed and ovary tissue sited at the base of each pistil) are the true fruits of this species and are embedded in the epidermal layer of the receptacle connected by vascular bundles (Perkins-Veazie, 1995). More than 60 yr ago it was proposed, based on physiological studies, that fertilized achenes govern the fate of the receptacle and, at least during the early stages of fruit development, auxin synthesized in the achenes promotes the growth of the receptacle (Nitsch, 1950; Given *et al.*, 1988). Thus, the removal of achenes from unripe fruits causes the inhibition of growth and expansion, and the induction of a set of ripening-related genes (Manning, 1994; Medina-Escobar *et al.*, 1997; Harpster *et al.*, 1998; Aharoni *et al.*, 2002).

As a consequence of the prominent role of auxin in the development and ripening of strawberry fruit, little attention has been paid to possible roles of other plant hormones in these processes. However, a high number of endogenous gibberellins (GAs) have been identified in strawberry by combined gas chromatography–mass spectrometry in both leaves (Taylor *et al.*, 2000) and immature fruits (Blake *et al.*, 2000), including the bioactive GA_1 and GA_3. It has additionally been reported that growth of the receptacle in the absence of achenes was stimulated by treatment with gibberellic acid (GA_3), although fruits only ripened upon co-application of naphtoxyacetic acid (Thompson, 1969). The application of GA_3

to ripening fruits also caused a significant delay in the development of the red color, probably as a result of a delay in the increase of phenylalanine ammonia-lyase (PAL), chlorophyllase and peroxidase activities in the GA_3-treated fruits (Martínez *et al.*, 1996). More recently, enhanced expression following external application of GA_3 has been reported for several fruit genes, such as *FaGAST* (GA-stimulated transcript), which encodes a protein involved in cell enlargement and final fruit size (de la Fuente *et al.*, 2006), and *FaXyl*, encoding a β-xylosidase (Bustamante *et al.*, 2009).

GAs are tetracyclic diterpenoid compounds involved in the regulation of many developmental processes throughout the life cycle of the plant, including seed germination, cell division and elongation, flower induction and development, and fruit growth (Pharis & King, 1985; Serrani *et al.*, 2007). More than 100 different GAs have been identified in plants; however, only a few of these are biologically active (Olszewski *et al.*, 2002). The accumulation of bioactive GAs is determined by both the rate of bioactive GA biosynthesis and the rate of subsequent conversion of bioactive GAs into inactive forms by hydroxylation reactions (Olszewski *et al.*, 2002; Yamaguchi, 2008). Gibberellin 3-oxidase (GA3ox) catalyzes the final step in the synthesis of bioactive GAs, whereas gibberellin 2-oxidase (GA2ox) catalyzes their deactivation. It is only recently that the receptors and downstream signalling components of the GA pathway have been elucidated. The molecular characterization of various GA response mutants led to the discovery of the GIBBERELLIN-INSENSITIVE DWARF1 (GID1) and DELLA proteins, key components of the molecular GA–GID1–DELLA mechanism that enables the plant response to GA (Harberd *et al.*, 2009). GID1 is localized in the nucleus and acts as a soluble GA receptor, displaying a high affinity for bioactive GAs. GA binding to GID1 results in a conformational change that allows GID1 to interact with the DELLA proteins. DELLA proteins are negative regulators of GA signalling that belong to the GRAS (GAI, RGA and SCARECROW) domain in the carboxy-terminal half family of transcriptional regulators, and can modulate many aspects of GA-induced growth and development in plants (Zentella *et al.*, 2007). The GA–GID1–DELLA interaction promotes its association with an SCF (Skp, Cullin, F-box) complex, resulting in the polyubiquitination and subsequent degradation of DELLA by the proteasome, and the subsequent release of the GA repression (Hedden, 2008; Shimada *et al.*, 2008).

The presence of bioactive GAs in strawberry fruit (Blake *et al.*, 2000), alongside the reported effects of external application in both the development of the receptacle (Thompson, 1969) and the expression of genes associated with fruit ripening (de la Fuente *et al.*, 2006; Bustamante *et al.*, 2009), prompted us to investigate the role played by this hormone in strawberry growth and ripening. We initially observed that concentrations of the bioactive GAs displayed dramatic changes through fruit development. We next studied the GA perception and signalling machinery throughout this process in both the achene and receptacle tissues of commercial strawberry fruit. The functionality of these components both *in vitro* and *in vivo*, in combination with their very specific expression patterns associated closely with developmental events, is consistent with a major role for GAs in the development of the receptacle.

Materials and Methods

Plant material, growth conditions and hormone treatment

Strawberry plants (*Fragaria* × *ananassa* Ducherne, cv Camarosa) were grown under plastic macro-tunnels in the field conditions of Huelva, in the southwest of Spain. Fruits were harvested at five different developmental stages: closed flowers (flowers before anthesis), open flowers (flowers 1-2 d post anthesis (dpa)), green fruit (green achenes and receptacle, *c.* 12 dpa), white fruit (green achenes and white receptacle, *c.* 21 dpa) and red fruit (red achenes and receptacle, *c.* 35 dpa) (de la Fuente *et al.*, 2006). Vegetative tissues utilized were young leaves and roots. For separate sampling of achene and receptacle, the achenes of fruits at green, white and red stages were carefully removed using the tip of a scalpel. All tissues were frozen immediately in liquid nitrogen and stored at −80°C until used.

The experiment in which the achenes were removed was performed in fruits at the green stage, which remained on the growing plant until the end of the experiment. Entire green fruits (achenes and receptacle) that also remained in the plant were used as a control. All fruits, de-achened and control, were harvested 96 h after the treatment, frozen in liquid nitrogen and stored at −80°C.

GA_4 treatment of *Nicotiana benthamiana* plants was performed by applying a solution of 100 µM GA_4 in 0.1% (v/v) ethanol and 0.1% (v/v) Tween 20 to *N. benthamiana* leaves. Samples were taken at 10 min, 30 min and 2 h and frozen in liquid nitrogen.

Arabidopsis plants (*Arabidopsis thaliana*, ecotype Columbia (Col-0)) were grown under short day (SD), 16 : 8 h light (200 µmol m^{-2} s^{-2}) : dark conditions and 24 : 17°C, or long day (LD), 8 : 16 h and 23 : 17°C conditions. *Nicotiana benthamiana* plants were grown in the same conditions as Arabidopsis plants grown in LD.

GA measurements

Extraction of GA from young fruit was carried out as described by Pan *et al.* (2008), with the exception that 10 g of tissue was used. In brief, the liquid nitrogen frozen material was ground into a fine powder. GAs were subsequently extracted by an overnight incubation in 50 ml of 1-propanol/H_2O/concentrated HCL (2 : 1 : 0.002, v/v/v). Then, 50 ml of dichloromethane was added and the mixture was incubated for an additional 30 min, before the two phases were clarified by centrifugation for 5 min at 14 000 ***g***. The lower phase, containing the hormones, was collected and concentrated to dryness in a rotary evaporator. The residue was re-solubilized in 200 µl of methanol. Next, 2 µl of the methanol extract was separated using an ultra performance liquid chromatography (UPLC; Acquity; Waters, Milford, MA, USA) system connected to an LTQ FT-ICR-Ultra mass spectrometer (Thermo-Fisher, Bremen, Germany) (Giavalisco *et al.*, 2009). Separation was performed on an HSS T3 C18 reversed-phase column (100 × 2.1 mm i.d.; 1.8 µm particle size; Waters) at a temperature of 40°C. The mobile phases consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The flow rate of the mobile phase was 400 µl min^{-1} and the following gradient profile was applied: a 1-min isocratic run at 75% A, and a linear 6-min gradient applied to 1% A. This was followed by a 1-min isocratic run at 1% A, before switching back to 75% A. The mass spectra were acquired covering a mass range from m/z 100 to 400. Resolution was set to 100 000 and the transfer capillary temperature was set to 200°C. In preliminary experiments, to evaluate the hormone recovery, 10–50 ng of the internal nonlabeled standards GA_1, GA_3 and GA_4 were added to the extraction buffer.

Sequence analysis

Sequences were obtained from The Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org/) (Arabidopsis sequences) or were identified using BLASTX at the National Centre for Biotechnology Information (NCBI; http://blast.ncbi.nlm.nih.gov/) (other sequences) or in the *Populus* data base (aspen sequences). Sequences were aligned and compared using ClustalW (http://www.ebi.ac.uk/) and Boxshade (http://www.ch.embnet.org/). Dendrograms were generated using the ClustalW (http://align.genome.jp/) program.

RNA extraction and gene expression analysis

Total RNA was extracted from *F.* × *ananassa* samples as previously described (Manning, 1991). Total RNA from Arabidopsis plants was extracted from rosette tissues using TRIzol® Reagent (Invitrogen; http://www.invitrogen.com/), according to the manufacturer's instructions.

RT-PCR reactions were performed starting from 1 µg of total RNA, and using the i-Scrip kit from Bio-Rad (Bio-Rad; http://www.bio-rad.com), according to the manufacturer's instructions.

Gene expression analysis was performed by quantitative RT-PCR (qRT-PCR) using the fluorescent intercalating dye SYBR Green in an iCycler detection system (Bio-Rad) as previously described (Benitez-Burraco *et al.*, 2003). Reactions were performed in triplicate and the absence of primer dimmers was confirmed by examination of dissociation curves. The expression of *FaRib413* (18S–26S ribosomal gene (interspacer)) was used as the normalizer. Relative quantification of expression level was performed using the comparative C_t method (Pfaffl, 2001), where a value of 1 was given to the sample with lower expression. All gene expression analysis was performed on at least two individual biological samples. The primers used for expression analysis are shown in Supporting Information Table S1.

Plasmid construction and plant transformation

A full-length cDNA clone of *FaGID1c* was obtained from a cDNA library of green strawberry fruits. Partial sequences of *FaGID1b*, *FaRGA*, *FaGAI* (*GA-INSENSITIVE*) and *FaGA3ox* were obtained by PCR using gene-specific primers based on the corresponding sequences of *Fragaria vesca* expressed sequence tags (ESTs). Full-length sequences for *FaGID1b* and *FaRGA* were obtained using the Invitrogen 5′RACE system for Rapid Amplification of cDNA Ends, Version 2.0, according to the manufacturer's instructions (Invitrogen). A partial cDNA clone of *FaGA2ox* was cloned by PCR using the primers previously described (Hytonen *et al.*, 2009).

For the generation of the *35S::FaGID1c* and *35S::FaRGA* constructs for expression in Arabidopsis and *N. benthamiana*, full-length sequences of *FaGID1c* and *FaRGA* were PCR-amplified using specific primers described in Table S1, subcloned into the pGEM®-T Easy vector (Promega; http://www.promega.com/) and then introduced into the *Xho*I/*Xba*I sites of the pT70 binary vector. For the expression of the fusion proteins Glutathione S-transferase (GST)-FaGID1c and GST-FaRGA in *Escherichia coli*, the corresponding full-length sequences were PCR-amplified using gene-specific primers (Table S1) and subcloned into the pGEM®-T Easy vector (Promega). *FaGID1c* was then introduced into the *Bam*HI/*Eco*RI sites of the pGEX vector (GE Healthcare; http://www.gehealthcare.com/) and *FaRGA* was introduced into the *Bam*HI/*Hin*dIII sites of the pET32a (+) vector (Merck; http://www.merck-chemicals.com).

Binary vectors were introduced into *Agrobacterium tumefaciens* (LBA4404) by electroporation (Sambrook & Russell, 2001) and then employed for Arabidopsis transformation using the floral dip method (Clough & Bent 1998) or infiltrated into *N. benthamiana* leaves.

GA-binding assay

The GA-binding assay was performed as previously described (Nakajima *et al.*, 2006), using [1,2,16,17-$^{3}H_4$]-16,17-dihydro-GA_4 and protein extracts of *E. coli* that expressed either the fusion protein GST-FaGID1c or GST alone (negative control). An excess amount (50 μl) of unlabeled GA_4 ('cold GA', dissolved in 10% v/v ethanol/DW) or its mock solution was added, and after 15 min at room temperature, a GST-FaRGA protein solution (100 μl) was added to the reaction mixture, which was incubated for another 25 min at room temperature before 100 μl of the mixture was taken for GA-binding activity evaluation using a gel-permeation technique. All data were calculated from triplicate measurements.

Western blot

For strawberry samples, total proteins were extracted from red receptacles with two volumes of 6 × sample buffer (350 mM Tris-HCl, pH 6.8, 5% (v/v) β-mercaptoethanol, 10% SDS (w/v), 0.012% bromophenol blue (p/v) and 36% glycerol (v/v)). Protein samples were separated by 12% SDS-PAGE. Coomassie brilliant blue staining was used to confirm equal loading. The proteins of the gel were transferred to a PVDF membrane and analyzed using rabbit anti-FaGID1 (GenScript; http://www.genscript.com/) primary antibody (1 : 1000) designed against specific peptides of the following sequence: VNSRPWLRSTKDSKC.

Immunolocalization

Cryosections of red strawberry achenes and receptacles were prepared by fixing in paraformaldehyde at 4°C O/N, washed in phosphate-buffered saline (PBS) and then imbibed in agar. The agar-tissue blocks were cryoprotected by immersion in a sucrose solution of increasing concentration (up to 30%) and then frozen by immersion in an isopentane solution at −80°C. The cryosections had a thickness of 10 μm and were kept at −20°C until used. Samples were hybridized with rabbit anti-FaGID1 primary antibody (1 : 1000). For immunohistochemistry, goat biotinilated secondary antibodies (1 : 2000) were used and then detected with ExtrAvidin-peroxidase (Sigma; http://www.sigmaaldrich.com/); the peroxidase–H_2O_2 reaction was histochemically developed using diaminobencidine (DAB) (Beckstead, 1994). For immunofluorescence, the secondary antibodies used were goat anti-rat Alexa Fluor 488 (Molecular Probes, Invitrogen).

Infiltration of *N. benthamiana* leaves

Agrobacterium tumefaciens of the LBA4404 strain containing the appropriate constructs was grown at 28°C for 2 d in LB-agar medium containing 10 μg ml^{-1} tetracycline and 50 μg ml^{-1} kanamycin. A single colony was transferred to 10 ml of LB medium containing 150 μM acetosyringone, 10 μg ml^{-1} tetracycline and 50 μg ml^{-1} kanamycin and grown at 28°C overnight. Liquid cultures were centrifuged and re-suspended in 10 mM $MgCl_2$. The bacterial suspensions were infiltrated into *N. benthamiana* leaves using a needleless syringe. Leaves were kept on the plant for 2 d before harvest.

Co-immunoprecipitation

Nicotiana benthamiana leaves transiently expressing FaGID1c and/or FaRGA fused to HA-Flag and Myc, respectively, were treated with a solution of 100 μM GA_4 in 0.1% (v/v) ethanol and 0.1% (v/v) Tween 20 or the same solvent as a control for 10 min, 30 min and 2 h. Treated leaves were harvested and ground in liquid nitrogen, and the proteins were extracted by adding 500 μl of extraction buffer, containing 150 mM Tris, pH 7.5, 50 mM NaCl, 5 mM EDTA, 5% glycerol, 2% PVPP, 10 mM DTT and a plant protease inhibitor cocktail (Sigma). The extracts were then centrifuged at 10 000 ***g*** for 20 min at 4°C, supernatants were filtered (0.2-μm filter) and 25 μl of Anti-FLAG® M2 Magnetic Beads (Sigma) was added to each pull-down assay. The mixture was rotated for 2 h at 4°C. Flag beads were then washed three times with 1 ml of cold extraction buffer and the bound proteins were eluted using FLAG® Peptide (Sigma) as a competitor. 3 × SDS sample buffer was added to the samples, which were then loaded onto a 10% SDS-PAGE gel. The proteins were transferred to a PVDF membrane and analyzed by western blot using commercial rabbit anti-HA and mouse anti-Myc antibodies. During all procedures, 100 μM GA_4 in water was added to the assay mixture for GA_4-treated samples and was not added to the control samples. Coomassie brilliant blue staining was used to confirm equal loading.

Statistical analysis

Statistical analysis was performed using the StatGraphic Centurion program (Statpoint Technologies, Inc., Warrenton, Virginia, USA: http://www.statgraphics.com). The significance of differences was determined by ANOVA (for three or more samples) or a *t*-test (for two samples).

Results

Analysis of the content of bioactive GAs in strawberry fruit during development

In the strawberry fruit, the development of the achene and that of the receptacle follow highly coordinated programs (Perkins-Veazie, 1995; Fait *et al.*, 2008). Growth and development of the fruit receptacle are characterized by successive and partially overlapping phases, in which the main features are cell division, cell expansion, and metabolic changes associated with fruit ripening (Cheng & Breen, 1992; Perkins-Veazie, 1995). We therefore decided to investigate changes in the concentrations of GAs in the receptacles of strawberry fruits by mass spectrometry, at three stages of development that are representative of these processes; that is, the green stage, where cell division is predominant, the white stage, where the growth of the fruit is mostly attributable to cell expansion, and the red stage, where most metabolic changes take place. This methodology allowed the identification of the three main bioactive GAs (GA_1, GA_3 and GA_4) but their absolute concentrations were highly divergent (Fig. 1a). While GA_1 and GA_3 varied in the range of 3–12 ng g^{-1} fresh weight, GA_4 oscillated between 14 and 151 ng g^{-1} fresh weight. This GA displayed a clear peak at the white stage of development when the content was 151 ng g^{-1} fresh weight. To our knowledge, this is the highest value reported for a bioactive form of GA in any plant tissue. This is clearly suggestive of a major role for GA_4 in the developmental processes underlying the receptacle transition from green to white, and subsequently to red.

The concentration of bioactive GAs is determined by the balance between their rates of biosynthesis and deactivation. The *GA3ox* and *GA2ox* genes encode key enzymes of biosynthesis and inactivation of GAs, respectively (Yamaguchi, 2008). We studied the relative expression of the strawberry genes *FaGA3ox* and *FaGA2ox* by real-time qRT-PCR in vegetative tissues and during the fruit growth and ripening period. The developmental stages selected were closed and open flowers, and fruits at the three ripening stages previously analyzed. The two genes showed somewhat opposite expression patterns (Fig. 1b,c). In

roots, *FaGA3ox* displayed high expression while *FaGA2ox* expression was low. Similarly, *FaGA3ox* expression increased in flowers between the closed and open stages, while *FaGA2ox* expression decreased. More importantly, the expression of *FaGA3ox* in fruits was highest in the green stage, whereas the expression of *FaGA2ox* was highest in the red stage. Expression of the *FaGA3ox* and *FaGA2ox* genes in the achene and receptacle separately (Fig. 1d,e) showed a similar pattern to that in the entire fruit, with the considerable difference that expression of the biosynthetic *FaGA3ox* gene in the green receptacle was *c.* 40 times higher than the expression in the green achene, suggesting that the receptacle is the main source of GA biosynthesis in the strawberry fruit. The *FaGA3ox* and *FaGA2ox* expression patterns were consistent with a continuous accumulation of GAs in the receptacle between the green and white stages but a considerable decline in the bioactive forms of these hormones in the red receptacle, following their inactivation.

Auxin content is high in green fruits, and there are an increasing number of reports on cross-talk between GA and auxin signalling (Weiss & Ori, 2007; Yamaguchi, 2008). Indeed, application of auxin induced *GA3ox* transcription in pea (*Pisum sativum*; O'Neill & Ross, 2002). Therefore, we investigated the effect of reducing the content of endogenous auxin on the expression of the *FaGA3ox* and *FaGA2ox* genes, which in turn are responsible for the steady state of GA. This was done by removing the achenes, the source of auxin, from green fruits and then following the expression of *FaGA3ox* and *FaGA2ox* using qRT-PCR. As shown in Fig. 1(f), *FaGA3ox* expression was reduced 25-fold in the de-achened fruits compared with entire fruits, whereas the expression of *FaGA2ox* was unaffected (Fig. 1g). As a control for the effectiveness of the treatment, we analyzed the expression of the gene encoding pectate lyase A (*FaPLA*), which has previously been shown to be repressed by auxin (Medina-Escobar *et al.*, 1997), and found an 8-fold increase in the receptacle after removing the achenes (Fig. 1h). These data suggest that auxin may have an essential role in controlling the concentrations of GAs in strawberry through regulating the expression of *FaGA3ox*, although we cannot discount the possibility that other factors may also be involved. Changes in the expression level of *FaGA3ox* as a result of changes in auxin content have been reported previously in many species, such as pea (O'Neill & Ross, 2002), tobacco (*Nicotiana tabacum*) (Wolbang & Ross, 2001), barley (*Hordeum vulgare*; Wolbang *et al.*, 2004) and tomato (*Solanum lycopersicum*; Serrani *et al.*, 2008).

It has been reported that, in vegetative tissues, GA is important in promoting cell elongation (Fleet & Sun, 2005), and this role also holds true for tomato fruits, where GA-treated parthenocarpic fruits showed a larger cell size (Serrani *et al.*, 2007). If GA_4 is the main factor regulating cell elongation in the strawberry receptacle, a high content of this hormone should be expected in this tissue. However, in addition to high steady-state concentrations of GA, GA-dependent cell elongation also requires active signalling (Fleet & Sun, 2005). This signalling involves the binding of GAs to their receptors (GID1 proteins), which facilitates the interaction between GID1 and the growth-repressing DELLAs, and their subsequent 26S proteasome-mediated degradation (Yasumura *et al.*, 2007). Although well characterized in other plants, such as rice (*Oryza sativa*) and Arabidopsis (Ueguchi-Tanaka *et al.*, 2005; Griffiths *et al.*, 2006), the GA signalling pathway in strawberry has not been described; we thus decided to investigate this process.

The strawberry GA-signalling genes *FaGID1b*, *FaGID1c*, *FaRGA* and *FaGAI* show tissue- and developmental-specific expression patterns

Through data mining we identified an *F. vesca* EST (accession number EX666096) and a cDNA from cultivated strawberry (*Fragaria* × *ananassa*) (accession number EX660586) with high homology to previously described *GID1* genes. The corresponding full-length cDNAs were cloned from *F. ananassa* by RT-PCR. The encoded proteins were 69% identical to each other, and showed highest homology to Arabidopsis GID1b and GID1c, and thus were designated FaGID1b and FaGID1c.

An alignment of FaGID1b and FaGID1c with previously described GID1 proteins from dicots such as Arabidopsis (AtGID1a-c), cotton (*Gossypium hirsutum*) (GhGID1a-b) and aspen (*Populus tremula*) (PttGID1.1-4), the monocot rice (OsGID1) and the lycophyte *Selaginella moellendorffii* (SmGID1a-b) (Fig. S1) revealed that the two putative GID1 proteins of strawberry contained the highly conserved HGG and GXSXG motifs of the hormone-sensitive lipase (HSL) family of proteins, as well as the two amino acids (G and R) reported to be essential for GA binding (Ueguchi-Tanaka *et al.*, 2005; Griffiths *et al.*, 2006). The dendrogram generated from the alignment of the GID1 proteins (Fig. 2a) separated the dicot proteins into two clades, each one including a strawberry GID1 protein.

The relative expression of *FaGID1b* and *FaGID1c* was further analyzed using qRT-PCR (Fig. 2b,c). The expression level of *FaGID1b* was similarly low in most tissues with the exception of flowers, where the expression was very high. Also, the expression of *FaGID1c* was similar in most tissues or stages analyzed. However, when the expression of *FaGID1b* and *FaGID1c* was analyzed separately in the receptacle and achene, clear differences in expression patterns were observed (Fig. 2d,e). While *FaGID1b* displayed a similar level of expression in the achene and receptacle at the three developmental stages, the relative expression of *FaGID1c* was over 20-fold higher in the receptacle than in the achene, suggesting that this gene has a prominent role in GA signalling in this tissue. Interestingly, both putative GA receptors showed the highest levels of expression at the white stage (Fig. 2e).

Immunolocalization of FaGID1 was performed in red strawberry fruits in order to investigate its accumulation at the cellular level. Polyclonal antibodies were developed specifically against a selected peptide of the FaGID1c protein sequence (Fig. S2), which recognized a protein of the expected size when analyzed by western blot (data not shown). Because of the high identity between the FaGID1b and FaGID1c sequences, we cannot discount the possibility that the antibody would also recognize FaGID1b. In the achene, FaGID1 was expressed in the embryo and in the pericarp connecting the embryo to the receptacle (Fig. 3a), and was detected in all cells of the receptacle (Fig. 3b). Pre-immune control serum showed a weak background signal in the embryo and external layers of the receptacle (Fig. S2). Despite this, FaGID1 cellular localization could be confirmed by indirect immunofluorescence using the same antibodies, which avoided the peroxidase background in the preparations (Fig. 3c). The extended distribution of FaGID1 in the receptacle compared with the achene again suggests a more prominent role of FaGID1 in this tissue.

Two DELLA-like genes have been reported in strawberry (Hytonen *et al.*, 2009). Their deduced amino acid sequences, FaRGA and FaGAI, were aligned with DELLA proteins from Arabidopsis (AtGAI, AtRGA and AtRGL1-3), tomato (LeGAI), grape (*Vitis vinifera*) (VvGAI) and rice (OsSLR1). The proteins showed high homology throughout their sequences (Fig. S3). In the dendogram derived from this alignment, FaRGA grouped with other fruit-expressed DELLAs such as those from tomato and grape, and the Arabidopsis proteins AtRGA and AtGAI, whereas FaGAI was separated from the other DELLA proteins (Fig. 4a). It should be noted that only FaRGA retained the DELLA and TVHYNP motifs that are essential for the GA response (Achard *et al.*, 2007; Ueguchi-Tanaka *et al.*, 2007; Zentella *et al.*, 2007), whereas the FaGAI sequence showed some amino acid substitutions in these domains (Fig. S3).

The expression of *FaRGA* and *FaGAI* was analyzed using qRT-PCR. *FaGAI* displayed a similar expression in most tissues analyzed, with the highest expression observed in flowers (Fig. 4b). Although *FaRGA* also showed similar expression in vegetative tissues, the expression of this gene decreased sharply (*c*. 30-fold) from green to red fruits (Fig. 4c). Both *FaRGA* and *FaGAI* showed higher expression in the receptacle than in the achene (Fig. 4d,e), revealing a decrease in expression between the green and red stages. Major changes were found for *FaGAI* in the transition from a white to a red receptacle (Fig. 4d), whereas for *FaRGA* the greatest change was found in the transition from a green to a white receptacle (Fig. 4e); therefore, because FaRGA displays the conserved amino acid positions of a genuine DELLA protein (Fig. S3), we can speculate that this protein acts as a true repressor of GA signalling. Following this logic, such repressive activity would be released on the transition of the receptacle from the green to the white/red stages.

FaGID1c and FaRGA are functional components of the GA signalling pathway

Given that GID1 proteins exhibit high binding affinity for biologically active GAs (Ueguchi-Tanaka *et al.*, 2005; Griffiths *et al.*, 2006), we next studied whether FaGID1c, which showed higher expression in the receptacle at all stages (Fig. 2e), displayed GA binding activity. A recombinant fusion GST-FaGID1c protein was generated (Fig. S4) and its binding activity was tested in competition with 16,17-dihydro-GA_4 (labeled GA) and GA_4 (cold GA). GST-FaGID1c bound to 16,17-dihydro-GA_4, and part of the binding was diminished by excess of unlabeled GA_4 (Fig. 5a, left, black and gray bars), indicating that the binding was GA specific. The binding activity of GST-FaGID1c was enhanced in the presence of FaRGA protein (Fig. 5a, left, white bars). Identical binding experiments were performed with GST as a control, and these demonstrated a low background of binding activity (Fig. 5a, right). The specific GA-binding activity of GST-FaGID1c was calculated by subtraction of the values presented in the gray bars from those presented in the black bars. The enhancement of the specific GA-binding activity caused by the presence of FaRGA was calculated by subtracting the values presented in the black bars from those

presented in the white bars (Fig. 5b). These activities were of the same order as those found for other GID1 proteins (Ueguchi-Tanaka *et al.*, 2005; Griffiths *et al.*, 2006; Aleman *et al.*, 2008).

We next analyzed whether FaGID1c and FaRGA interacted *in vivo*. For this purpose, we prepared constructs in a binary vector for each cDNA (*FaGID1c* and *FaRGA*) under the control of the cauliflower mosaic virus *35S* (*CaMV35S*) promoter but fused to sequences encoding different epitope tags (HA-Flag and Myc, respectively) at their respective 3′ ends. The constructs were transiently expressed in *N. benthamiana* leaves and were analyzed for function by co-immunoprecipitation assays to detect GA-dependent interactions between the GID1c and RGA proteins. Leaves were treated with a water solution, with and without a supplement of 100 µM GA_4, and sampled after 10 min, 30 min or 2 h. Western blot analyses of total protein extracts revealed that both FaGID1c-HA-Flag and FaRGA-Myc were expressed as stable proteins, independently of the presence of exogenously applied GA_4 (Fig. 5c, lanes 1–6). From the total protein fractions, FaGID1c-HA-Flag and its interacting partners were precipitated (Fig. 5c, lanes 7–12). The corresponding western blots revealed co-precipitation of FaRGA only in the GA_4-treated samples (Fig. 5c, lines 10–12). These results demonstrate that FaGID1c and FaRGA interact *in vivo* and that this interaction is dependent on the presence of GA_4.

Expression of *FaGID1c* in Arabidopsis demonstrates its involvement in the GA signalling pathway

In Arabidopsis, as in all angiosperms, GA regulates growth via the GID1–DELLA signalling mechanism (Griffiths *et al.*, 2006; Zentella *et al.*, 2007). This involves GA binding to GID1 receptors, thus stimulating interactions between GID1s and the growth-repressing DELLAs. It is known that GID1–DELLA interactions require dual affinities: GID1 affinity for DELLA, and DELLA affinity for GID1, as it has been shown that these affinities have evolved separately during evolution (Yasumura *et al.*, 2007). We therefore investigated a role for FaGID1c in GA signalling *in planta* by generating transgenic Arabidopsis plants expressing *FaGID1c* under the control of the *35S* promoter. A number of independent transgenic lines were obtained and two of them, lines L1 and L2 (Fig. 6a,b), were selected for further studies on the basis of the high expression level of the transgene (Fig. 6c). The transgenic lines presented a phenotype characterized by an increase in vegetative growth in both LD and SD conditions (Fig. 6a,b, respectively). As in rice plants overexpressing *OsGID1* (Ueguchi-Tanaka *et al.*, 2005), transgenic Arabidopsis plants expressing *FaGID1c* were taller than the wild type, consistent with increased GA signalling. The higher expression of the transgene in L2 compared with L1 did not correlate with the severity of the phenotype, as the two transgenic lines displayed similar increases in plant height (Fig. 6a,b). It is likely, however, that increased GA signalling would reach saturation irrespective of further increases in the amount of receptors. We subsequently investigated these putative changes in GA signalling by monitoring the expression levels of *AtGA3ox1* and *AtGA2ox4*. The expression of *AtGA3ox* genes is induced under GA-deficient conditions and down-regulated after GA application (Phillips *et al.*, 1995; Xu *et al.*, 1999), whereas the expression of *AtGA2ox* genes is up-regulated after GA treatment (Thomas *et al.*, 1999). As shown in Fig. 6(d,e), the expression of *AtGA3ox1* was highly reduced in the transgenic lines, while the expression of *AtGA2ox4* was increased.

Discussion

A functional GA signalling pathway is active in the receptacle of the strawberry fruit

To date, only the hormone auxin has been demonstrated to be involved in the developmental program of the strawberry fruit (Nitsch, 1950). However, early studies reported the presence of GAs in strawberry fruits (Perkins-Veazie, 1995), as well as the joint action of auxin and GA in the development of parthenocarpic strawberry fruits (Thompson, 1969). In light of the increasing evidence of cooperation of GA with other hormones in the regulation of different plant development processes (Fleet & Sun, 2005), and particularly with auxin in processes such as tomato fruit set (de Jong *et al.*, 2009), we decided to investigate the possible role of GA in the growth and ripening of strawberry fruit, focusing largely on the receptacle, in an attempt to understand its potential collaboration with auxin in regulating these developmental processes. The GA_4 content of the receptacle was much higher than the contents of GA_1 and GA_3, and peaked at the white stage, although previous studies on green fruit, receptacle and achenes did not detect GA_4 (Blake *et al.*, 2000). Among the various bioactive GA forms, the prevalence of one of

them seems to be species/tissue/developmental stage specific (MacMillan, 2002). For example, GA_1 has been identified to be of particular importance in a wide variety of plant species (Bulley *et al.*, 2005; Serrani *et al.*, 2007), including strawberry, where its role in the runner tips has been demonstrated (Hytonen *et al.*, 2009); however, in Arabidopsis GA_4 is clearly the major bioactive form (Xu *et al.*, 1999). The metabolic regulation which is responsible for controlling the relative concentrations of these two species remains to be clarified via the identification of genes encoding GA 13-oxidase (Yamaguchi, 2008).

We have identified and studied key components of the strawberry GA signalling pathway, including putative GID1 receptors (FaGID1b and FaGID1c) and two DELLA-like proteins (FaRGA and FaGAI). The different expression patterns of the two putative receptors suggest different roles, with FaGID1c probably having a more prominent role in the growth and ripening of the receptacle, whereas FaGID1b may be involved in flower and root development. It is known that the DELLA-like proteins require the highly conserved N-terminal DELLA and VHYNP motifs for the interaction with GID1 (Griffiths *et al.*, 2006). While FaRGA contains all of the conserved amino acids in these motifs, FaGAI contains some divergent residues. The alterations involve nonconservative amino acid substitutions, and it has been reported that a single nonconservative amino acid mutation in a barley DELLA-like gene related to Arabidopsis GAI/RGA was sufficient to produce a mutant phenotype (Chandler *et al.*, 2002). However, whether or not FaGAI has the structural characteristics of a DELLA protein is yet to be investigated, as the protein function is dependent on its amino acid sequence and the genetic background of the species (Hirano *et al.*, 2007). It should be noted, however, that a recent phylogenetic analysis of genes encoding DELLA proteins identified members of the family in two different clades in all species of the *Rosids* genus (Gallego-Bartolome *et al.*, 2010), and data mining in the wild *F. vesca* genome identified the orthologs of FaRGA and FaGAI as the only putative DELLA-like genes (K. Folta, pers. comm.). The expression of *FaRGA* was restricted very specifically to the receptacle tissue, and was very high at the green stage. This suggests that this gene plays a major role in the development of this tissue.

FaGID1c and FaRGA are *bona fide* components of GA signalling given that they display GA-dependent interactions both *in vitro* and *in vivo*, as has been reported for other GID1 receptors (Ueguchi-Tanaka *et al.*, 2005, 2007; Griffiths *et al.*, 2006; Nakajima *et al.*, 2006). This is further supported by the phenotypes of Arabidopsis transgenic plants ectopically expressing *FaGID1c* and their consequent changes in the expression of the endogenous *AtGA3ox1* and *AtGA2ox4* genes. The transcriptional activities of *GA3ox* and *GA2ox* have been reported to be very sensitive to changes in GA, which includes hormone perception and signalling (Yamaguchi, 2008). For instance, in tomato, *GA3ox* transcripts were down-regulated not only after GA treatment, but also when the expression of the *SlDELLA* repressor was reduced in the corresponding anti-sensed plants (Martí*et al.*, 2007). Therefore, the changes in the expression of *AtGA3ox1* and *AtGA2ox4* reported here as a result of the transformation of Arabidopsis with *FaGID1c* seem to be a characteristic plant response to changes in GA sensitivity.

The involvement of GAs in receptacle development is associated with cell expansion

The expression analyses of genes involved in GA perception, signalling and metabolism suggest that the receptacle is mainly responsible for GA biosynthesis within the strawberry fruit, as well as being the site of action of this hormone. Previous studies in Arabidopsis and rice suggest that, in some cases, bioactive GAs are also produced at their site of action (Silverstone *et al.*, 1997; Kaneko *et al.*, 2003). However, there are other cases, such as the seed germination processes, wherein GA-dependent transcriptional events are not restricted to the sites of GA biosynthesis (Ogawa *et al.*, 2003).

Our study revealed extremely high concentrations of the bioactive hormone GA_4 in the receptacle, peaking at the white stage. In terms of gene expression, we noted that the expression of *FaGA3ox*, which is involved in the generation of bioactive GA_4, decreased gradually, while the expression level of *FaGA2ox*, which is involved in the inactivation of GA_4, continued to increase during receptacle development. Therefore, the expression patterns of *FaGA3ox* and *FaGA2ox* suggest a scenario in which GAs accumulate in the receptacle from the green stage, reaching a maximum at the white stage, to be inactivated at the red stage. Not only was the expression of *FaGA2ox* and *FaGA3ox* higher in the receptacle than in the achenes, but the expression of *FaGID1c* and *FaRGA* was also higher, further suggesting that GA may be synthesized at the site of perception.

The expression of *FaRGA* showed a sharp decline in fruits during the transition from a green to a white receptacle, corresponding to a switch from repressed GA signalling in the green receptacle to a stage of active GA signalling in the white receptacle. At the cellular level, this transition correlates with change from a stage of prevalent cell division to one in which cellular expansion prevails. Interestingly, the expression of the *FaGID1c* receptor reached its highest level in the white receptacle. Taking these data together, it is likely that GA_4 is involved directly in the development of the receptacle by promoting cell expansion mainly during the white stage. The effect of GAs on cell expansion has been widely reported (Lemaire-Chamley *et al.*, 2005; Ubeda-Tomas *et al.*, 2008). In tomato, exogenous GA_3 induced parthenocarpy, in which the fruits were smaller than seeded fruits because of a lower number of cells, but these cells were also smaller (Serrani *et al.*, 2007).

We propose that in the strawberry receptacle the actions of auxin and GA are not independent of one another but are coordinated, as has been proposed previously in other plant systems (Serrani *et al.*, 2007). This hypothesis is supported by early studies in strawberry, which showed that, in the absence of pollination, only an application of 2-naphtoxyacetic acid in combination with GA_3 was able to maintain growth rate throughout the whole developmental period (Thompson, 1969). Whereas each compound was able to promote parthenocarpic growth, neither of them alone was able to maintain the growth rate to the end of the ripening process. A similar situation has been reported in the growth of parthenocarpic tomato fruits, where auxin application accelerated the rate of cell division, and GA treatment enhanced cell expansion (de Jong *et al.*, 2009), but only the joint application of both hormones resulted in parthenocarpic fruits similar to those obtained by pollination (Serrani *et al.*, 2007). It was proposed that, in the growth of the tomato fruit pericarp, cell elongation and cell division activity are coordinated by a delicate balance between the two phytohormones (de Jong *et al.*, 2009), and this could also be the case for the strawberry fruit receptacle. There are an increasing number of reports on cross-talk between GA and auxin (Weiss & Ori, 2007; Yamaguchi, 2008). In tomato fruits, auxin-induced fruit set was proposed to be mediated by GAs in light of the finding that application of auxin to unpollinated ovaries is followed by the up-regulation of genes encoding GA biosynthesis enzymes such as *GA3ox*, and down-regulation of *GA2ox*, which is involved in GA inactivation (Serrani *et al.*, 2008). In the green receptacle of strawberry, the auxin content, supplied by the achenes, is high, as is the expression of *FaGA3ox*, while the expression of *FaGA2ox* is low. Removal of the achenes, the source of auxin, diminishes the transcriptional activity of *FaGA3ox* in the receptacle. We therefore suggest that the receptacle, which is a very important part of the fruit as it is responsible for fruit quality, also represents a likely site where two hormones could act in a coordinated and sequential program, with GA_4 playing a prominent role.

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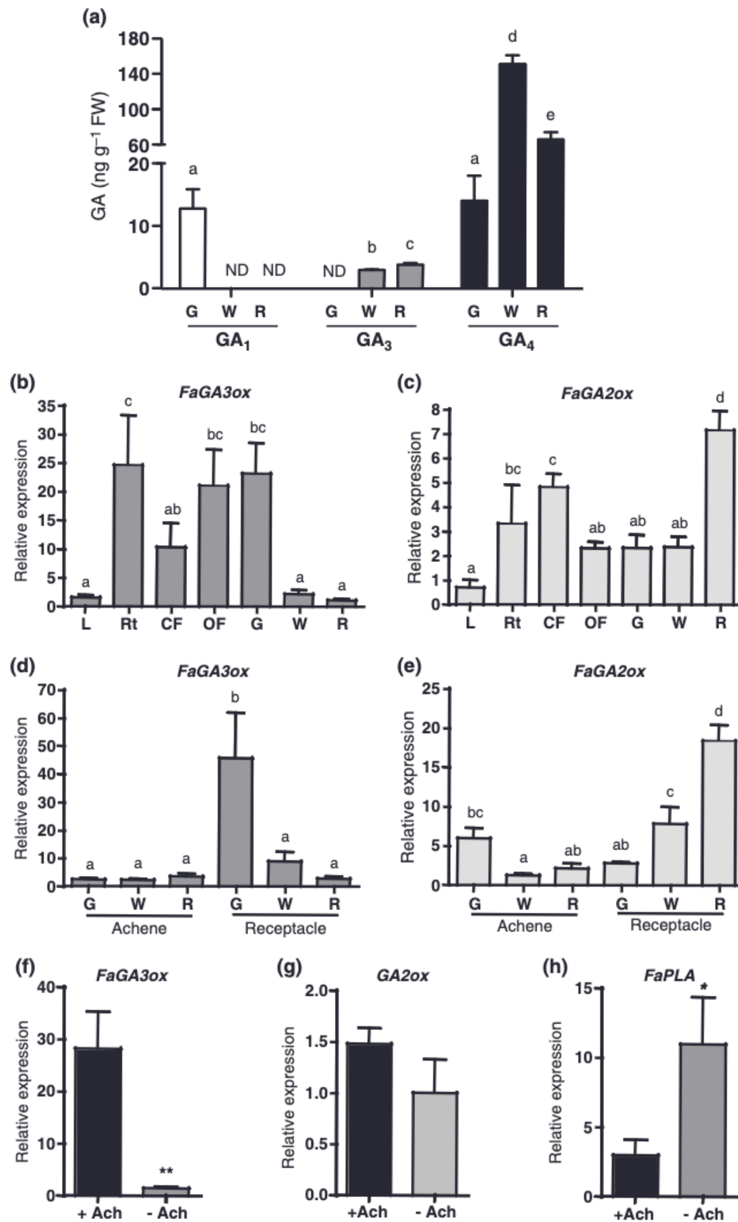

Fig. 1 Changes in endogenous concentrations of gibberellin (GA) and in the expression of GA biosynthetic genes during fruit development and ripening in different strawberry tissues.
(a) Endogenous concentrations of bioactive GAs in green (G), white (W), and red (R) receptacles. GA1, white bars; GA3, gray bars; GA4, black bars. GA concentrations were determined using the ultra-performance liquid chromatography Fourier transform cyclotron resonance mass spectrometry technique. Bars represent the mean of three independent biological samples ± SD. Different letters indicate a significant difference between samples according to the corresponding ANOVA ($P < 0.05$). ND, not detected.
(b–e) qRT-PCR analysis of strawberry GA biosynthetic genes. Total RNA was extracted from different strawberry tissues and parts, and the relative transcript levels of FaGA3ox and FaGA2ox were determined by qRT-PCR.
(b, c) FaGA3ox (b) and FaGA2ox (c) genes in different plant tissues. CF, closed flower; OF, open flower; G, green fruit; W, white fruit; R, red fruit; L, leaf; Rt, root.
(d, e) FaGA3ox (d) and FaGA2ox (e) genes in different fruit parts at green (G), white (W), and red (R) stages. Bars represent the mean of two independent biological samples ± SE. Different letters indicate a significant difference between samples according to the corresponding ANOVA ($P < 0.05$).
(f–h) qRT-PCR analysis of FaGA3ox and FaGA2ox in de-achened green fruits and entire fruits. Achenes were removed from green strawberry fruits and total RNA was extracted after 96 h of treatment. The relative transcript levels of FaGA3ox (f) and FaGA2ox (g) were determined by qRT-PCR (–Ach; gray bars). Entire fruits were used as a control (+Ach; black bars). The pectate lyase A (FaPLA) gene (h) was used as a positive control of the treatment. Bars represent the mean of two biological replicates ± SE. Asterisks indicate a significant difference between samples, as determined by t-tests: *, $P < 0.05$; **, $P < 0.01$.

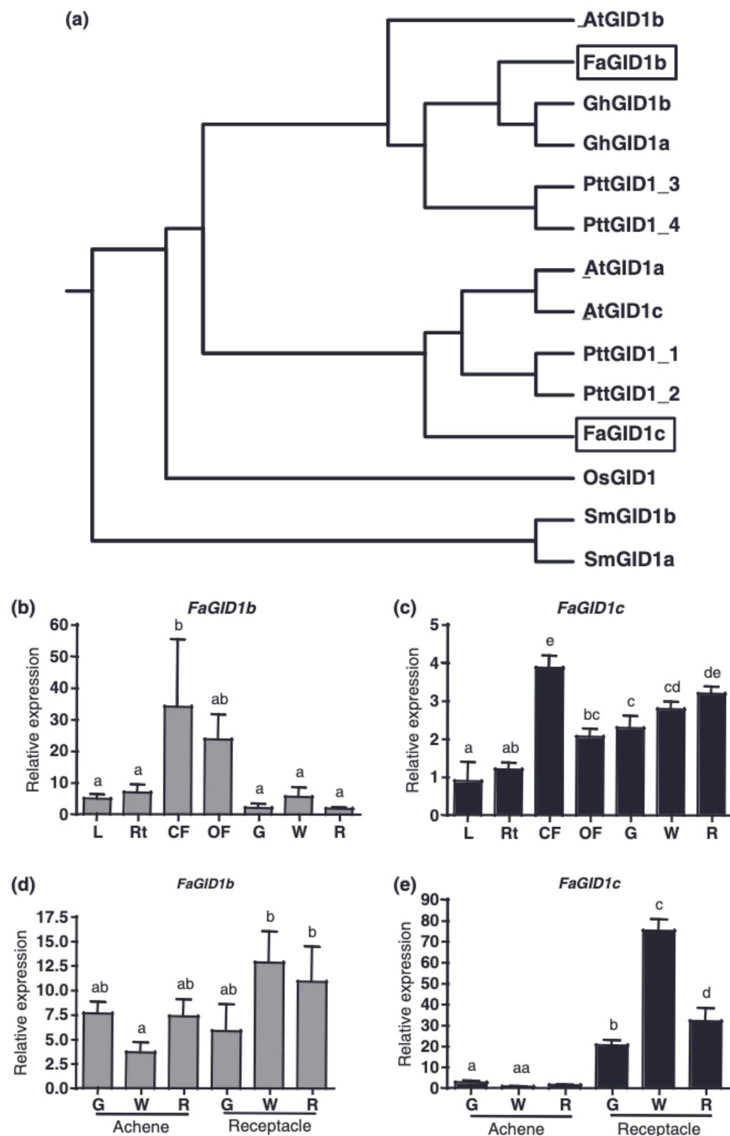

Fig. 2 Dendrogram of the alignment of FaGID1b with other GIBBERELLIN-INSENSITIVE DWARF1 (GID1) proteins, and expression patterns of FaGID1b and FaGID1c in different strawberry plant tissues, and different fruit parts at different stages.

(a) Neighbor-joining (NJ) tree for GID1 proteins from different plant species. The NJ tree was created using CLUSTAL W. Strawberry proteins are marked with black squares. At, Arabidopsis thaliana; Os, Oryza sativa; Sm, Selaginella moellendorfii; Pt, Populus tremula; Gh, Gossypium hirsutum; Fa, Fragaria × ananassa.

(b–e) Expression analysis of strawberry GID1 genes. Total RNA was extracted from different strawberry tissues and parts, and the relative transcript levels of FaGID1b and FaGID1c were determined by qRT-PCR.
(b, c) FaGID1b (b) and FaGID1c (c) in different plant tissues: L, leaf; Rt, root; OF, open flower; CF, closed flower; G, green fruit; W, white fruit; R, red fruit.
(d, e) FaGID1b (d) and FaGID1c (e) in achene or receptacle tissues corresponding to the green (G), white (W), or red (R) ripening stage. Bars represent the mean of two independent biological samples ± SE. Different letters indicate a significant difference between samples according to the corresponding ANOVA ($P < 0.05$).

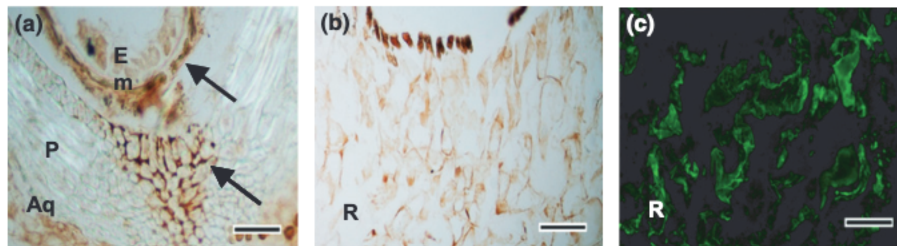

Fig. 3 Immunolocalization of FaGID1 (GIBBERELLIN-INSENSITIVE DWARF1) in the achene and receptacle of the strawberry fruit.

(a, b) Cellular localization of FaGID1 was determined by immunohistochemistry in sections of red achene (a) and red receptacle (b). Tissue sections were labeled with rabbit anti-FaGID1 antibody followed by biotinylated goat anti-rabbit secondary antibody.

(c) Immunofluorescence was used for the red receptacle, where sections were labeled with rabbit anti-FaGID1 antibody followed by goat anti-rat Alexa Fluor 488.

Bars: (a) 200 µm; (b) 400 µm; (c) 400 µm. Aq, achene; R, receptacle; Em, embryo; P, pericarp. Arrows indicate sites with high signal in the achene.

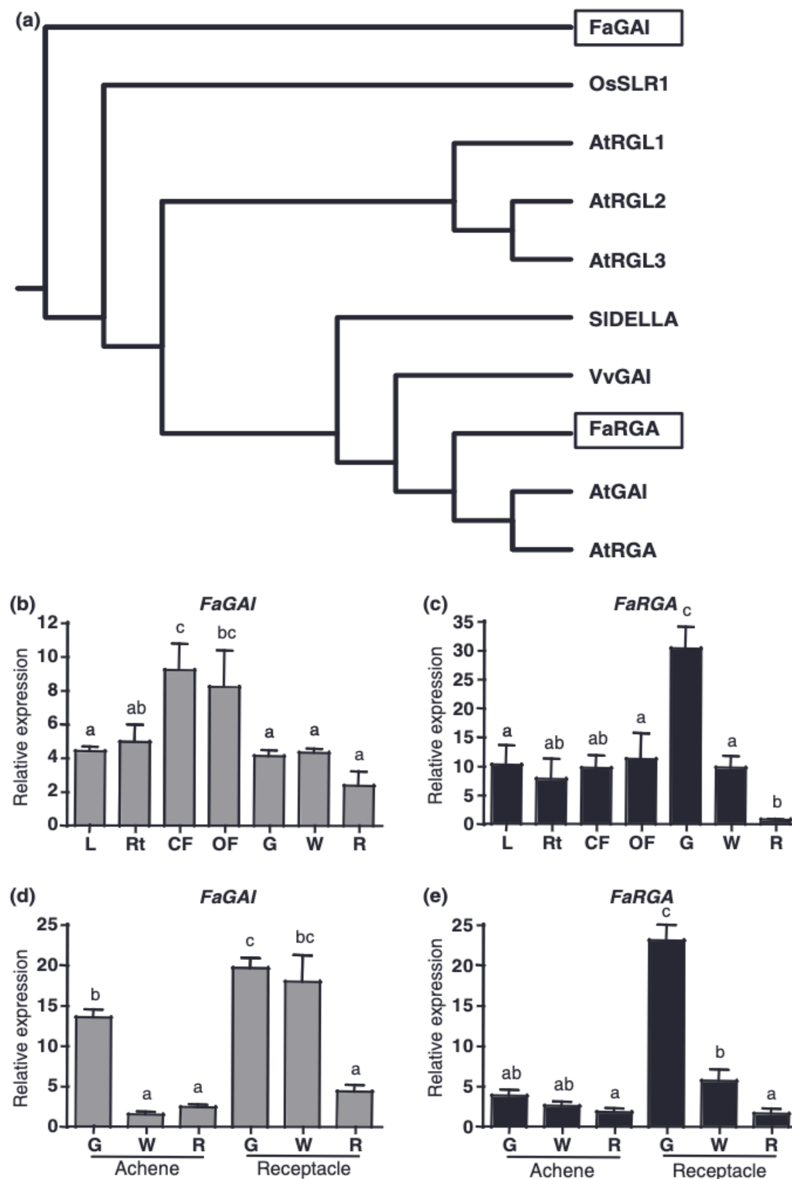

Fig. 4 Dendrogram of the alignment of FaGA-INSENSITIVE (GAI) and FaRGA (REPRESSOR OF GA) with various DELLA-like proteins, and relative expression of FaGAI and FaRGA in different strawberry tissues at different developmental stages.

(a) Neighbor-joining (NJ) tree generated with CLUSTAL W after the alignment of DELLA-like proteins from different plant species. Putative strawberry DELLA proteins are marked with black squares. At, Arabidopsis thaliana; Os, Oryza sativa; Sl, Solanum lycopersicum; Vv, Vitis vinifera; Fa, Fragaria · ananassa.

(b–e) Relative expression levels of FaRGA and FaGAI determined by qRT-PCR in different plant tissues (b, c) or in achene and receptacle tissues (d, e) corresponding to the green (G), white (W), or red (R) ripening stage. L, leaf; Rt, root; OF, open flower; CF, closed flower; G, green fruit; W, white fruit; R, red fruit.

Values correspond to the means of two independent biological samples ± SE. Different letters indicate a significant difference between samples according to the corresponding ANOVA ($P < 0.05$).

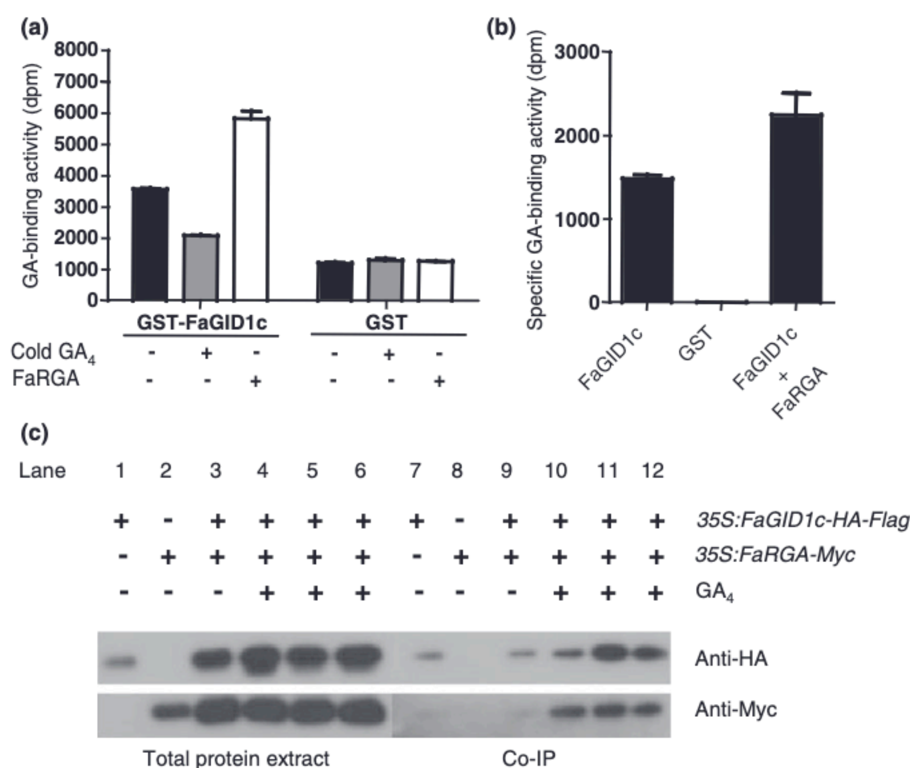

Fig. 5 GA4-binding activity of FaGID1c and gibberellin (GA)-dependent interaction of FaGID1 (GIBBERELLIN-INSENSITIVE DWARF1)c and FaRGA (REPRESSOR OF GA).

(a) GA4-labeled binding activity of FaGID1c in the absence or presence of cold GA4, and FaRGA. GST-FaGID1c was incubated with [1,2,16,17-3 H4]-16,17-dihydro-GA4, and an excess amount of cold GA4 was added, and finally Glutathione S-transferase (GST)-FaRGA was also added to the mixture. The same binding activity was measured using GST as a negative control.

(b) Specific GA4-binding activity calculated from (a). The SE was determined from three independent measurements.

(c) Total protein extracts from transgenic Nicotiana benthamiana plants expressing 35S:FaGID1c-HA-Flag plus 35S:FaRGA-Myc were immunoprecipitated with anti-Flag beads. Samples were loaded onto a 10% SDS-PAGE gel and immunoblotted using anti-HA or anti-Myc antibodies. Total protein extracts (lanes 1–6), and proteins after anti-Flag precipitation (Co-IP; lanes 7–12) are shown. In lanes 4–6 and 10–12, 100 µM GA4 was applied for 10 min, 30 min and 2 h, respectively.

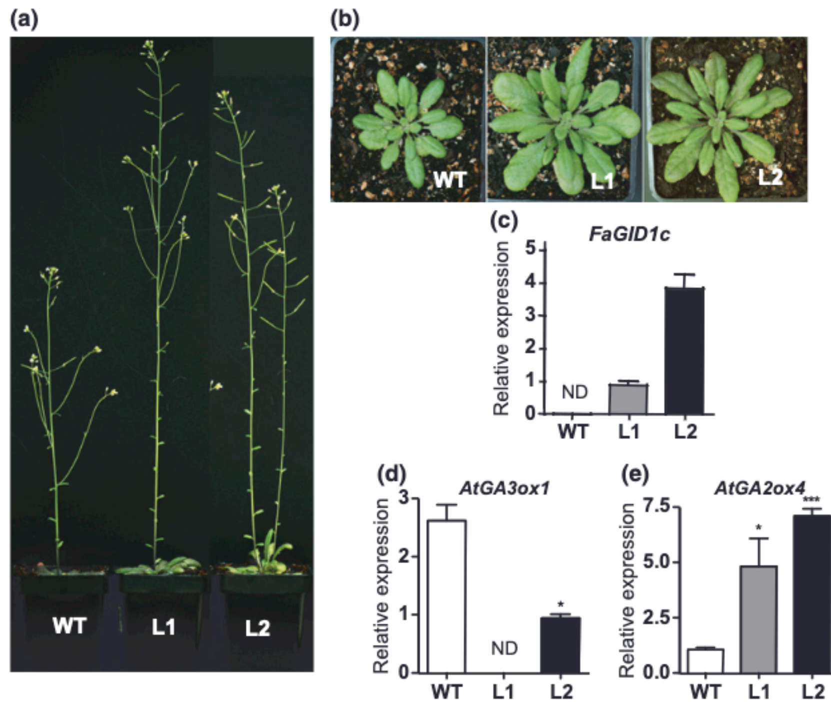

Fig. 6 Ectopic expression of FaGID1 (GIBBERELLIN-INSENSITIVE DWARF1) in Arabidopsis.

(a, b) Phenotypes in long day (a) or short day (b) conditions of Arabidopsis wild-type (WT) and L1 and L2 transgenic lines expressing FaGID1c under the control of the 35S promoter.

(c–e) Relative expression levels of the FaGID1c transgene (c), Arabidopsis GA3ox (d) and GA2ox (e) as determined by qRT-PCR in the same lines as in (a). Bars represent the mean of two biological replicates ± SE. Asterisks indicate a significant difference between transgenic lines compared with WT, as determined by t-tests: *, $P < 0.05$; ***, $P < 0.001$.