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PROCERA encodes a DELLA protein that mediates control of dissected leaf form in tomato

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Summary

Leaves of seed plants can be described as simple, where the leaf blade is entire, or dissected, where the blade is divided into distinct leaflets. Mechanisms that define leaflet number and position are poorly understood and their elucidation presents an attractive opportunity to understand mechanisms controlling organ shape in plants. In tomato (*Solanum lycopersicum*), a plant with dissected leaves, KNOTTED1-like homeodomain proteins (KNOX) are positive regulators of leaflet formation. Conversely, the hormone gibberellin (GA) can antagonise the effects of *KNOX* overexpression and reduce leaflet number, suggesting that GA may be a negative regulator of leaflet formation. However, when and how GA acts on leaf development is unknown. The reduced leaflet number phenotype of the tomato mutant *procera* (*pro*) mimics that of plants to which GA has been applied during leaf development, suggesting that PRO may define a GA signalling component required to promote leaflet formation. Here we show that *PRO* encodes a DELLA-type growth repressor that probably mediates GA-reversible growth restraint. We demonstrate that PRO is required to promote leaflet initiation during early stages of growth of leaf primordia and conversely that reduced GA biosynthesis increases the capability of the tomato leaf to produce leaflets in response to elevated KNOX activity. We propose that, in tomato, DELLA activity regulates leaflet number by defining the correct timing for leaflet initiation.

Keywords: leaf shape, DELLA proteins, KNOX proteins.

Introduction

The plant hormone gibberellin (GA) promotes growth by preventing action of the DELLA growth repressors (Silverstone *et al.*, 2001; Sun and Gubler, 2004). Thus, altering GA activity disrupts control of cell and organ size in diverse developmental contexts (Fleet and Sun, 2005). Repression of GA biosynthesis also partially mediates action of KNOTTED1-like homeodomain (KNOX) proteins, which are required for function of the shoot meristem, a pluripotent group of cells that give rise to the aerial parts of the plant (Hay *et al.*, 2002; Sakamoto *et al.*, 2001). The importance of GA homeostasis in mediating KNOX action was further highlighted by observations that KNOX proteins also stimulate GA catabolic gene expression, thereby providing an additional mechanism by which KNOX proteins may antagonise GA activity (Jasinski *et al.*, 2005). While the precise significance of repression of GA biosynthesis for KNOX action is unclear, genetic evidence indicates that GA activity antagonises the action of KNOX proteins in preventing cellular differentiation (Fleet and Sun, 2005; Hay *et al.*, 2002; Sakamoto *et al.*, 2001).

Interactions between GA and KNOX may also have a role in controlling leaf form in tomato (*Solanum lycopersicum*), a plant that has complex subdivided leaves and that expresses *KNOX* genes in leaves, unlike the model systems

Arabidopsis thaliana, snapdragon (*Antirrhinum majus*), maize (*Zea mays*) and rice (*Oryza sativa*) which have simple leaves in which *KNOX* expression is excluded (Piazza *et al.*, 2005). This idea is supported by the finding that exogenous GA application or constitutive GA signalling conditioned by the classical *procera* (*pro*) mutation both simplify the tomato leaf and suppress effects of *KNOX* overexpression, which include increases in leaflet number and repression of GA biosynthesis (Hay *et al.*, 2002). However, while these observations indicate that tomato leaf morphology is sensitive to alterations of GA activity, the signalling pathway via which GA controls dissected leaf form was poorly understood because the molecular identity of PRO was unknown. Additionally, when and how GA acts to regulate leaflet number during development was unclear because the consequences for dissected leaf ontogenesis of altering GA activity had not been investigated.

Here we address these problems by demonstrating that GA modulates tomato leaf form via a canonical GA signalling pathway requiring activity of the DELLA protein PRO. Furthermore, we provide evidence that PRO activity is required at early stages of leaf development, both to promote leaflet formation and to restrict growth of the leaf primordium. We suggest that PRO-mediated growth restraint may influence leaf shape by helping to define the correct timing of leaflet emergence.

Results

PROCERA *encodes a DELLA-type growth repressor expressed in the shoot apical meristem and in developing leaf primordia*

Fully expanded leaves of the cultivated tomato, typically consisting of one terminal leaflet and three to four pairs of lateral, lobed leaflets that bear secondary leaflets, are dissected (Figure 1a,b). Leaves of the *pro* mutant (Jupe *et al.*, 1988) bear fewer leaflets with a smoother margin (Figure 1b,c). To understand how PRO regulates the development of tomato leaves, we determined the molecular identity of *PRO*. Several lines of evidence demonstrated that *PRO* encodes SlGAI (*Solanum lycopersicum* GA insensitive), a DELLA-type growth repressor. Firstly, we showed that *SlGAI* maps to the distal part of chromosome 11, as does *pro* (data not shown, Van Tuinen *et al.*, 1998). Secondly, the *SlGAI* sequence from *pro* plants contained a T905A mutation (this work, Bassel *et al.*, 2008), resulting in a V302E amino acid substitution in the conserved VHV(I/V)D motif in the C-terminal GRAS domain which is thought to be important for DELLA action but has no ascribed biochemical function (Sun and Gubler, 2004; Figure 1e). This SlGAI mutation was recently independently identified as a strong candidate for causing the *pro* phenotype (Bassel *et al.*, 2008). However, in that work it was inconclusive whether a broadly expressed *35S::SlGAI* transgene complemented *pro* and hence whether the single mutant V302E substitution caused the phenotype. In this study, we did not detect recombinants between the T905A mutation and the *pro* phenotype in 86 chromosomes assayed, confirming that the V302E mutant allele is linked to the *pro* locus. Thirdly, transgenic expression of a genomic fragment of *SlGAI* complemented the *pro* phenotype. All 11 primary transgenic plants with intact T-DNA insertions displayed wild-type leaf morphology (Figure S1) and had normal stem elongation (data not shown). Co-segregation of this phenotype with the presence of the T-DNA was shown in three T_1 families. Fourthly, reducing the expression of *SlGAI* using antisense technology resulted in phenotypes similar to *pro*; 10 individual T_1 plants showed a smoother leaf margin phenotype (Figure 1d, Marti *et al.*, 2007) and in addition demonstrated increased stem height, as does *pro* (Jupe *et al.*, 1988). Lastly, to directly test whether the *pro* V-to-E amino acid change in SlGAI is sufficient to perturb the growth repression function of DELLA, we reconstituted this mutation in the *Arabidopsis gibberellin insensitive* (*gai*) dominant mutant allele, which causes dwarfism, because the GAI DELLA-type growth repressor is rendered constitutively active due to deletion of the DELLA domain (Peng and Harberd, 1993, 1997; Peng *et al.*, 2002; Figure 1f). When this synthetic gai^{V273E} allele was expressed in wild-type plants under the broadly expressed *CaMV35S* promoter, the plants were of normal height, unlike the dwarfed *gai* plants (Figure 1f). Thus gai^{V273E} acts as an intragenic suppressor of the gain-of-function *gai* allele, indicating that V273 in the VHV(I/V)D motif of GAI is critical for DELLA-mediated growth repression and that the *pro* mutant is probably a hypomorphic allele.

To understand where PRO acts to repress growth, we analysed the distribution of *PRO* mRNA by RNA *in situ* hybridisation. The *PRO* transcript was detected in both the shoot apical meristem (SAM) and in the developing leaf primordia of 2-week-old wild-type tomato seedlings, as well as in the vasculature of internodes (Figure 1g–i). These findings suggest that *DELLA* gene expression in multiple cell types may underpin the widespread role of these proteins in regulating plant growth and indicate that DELLAs may regulate the growth of lateral organs from very early in their development. Notably, that complementation of *pro* leaf phenotypes was readily observed for the *SlGAI* genomic fragment, unlike the case for expression of *SlGAI* under the broadly active *CaMV35S* promoter (Bassel *et al.*, 2008), suggests that precise spatio-temporal regulation of *PRO* gene expression may be critical for leaf development.

PRO regulates early leaflet morphogenesis and cellular growth in the leaf

To determine when during development PRO acts to promote leaflet outgrowth, we quantified leaflet number over a

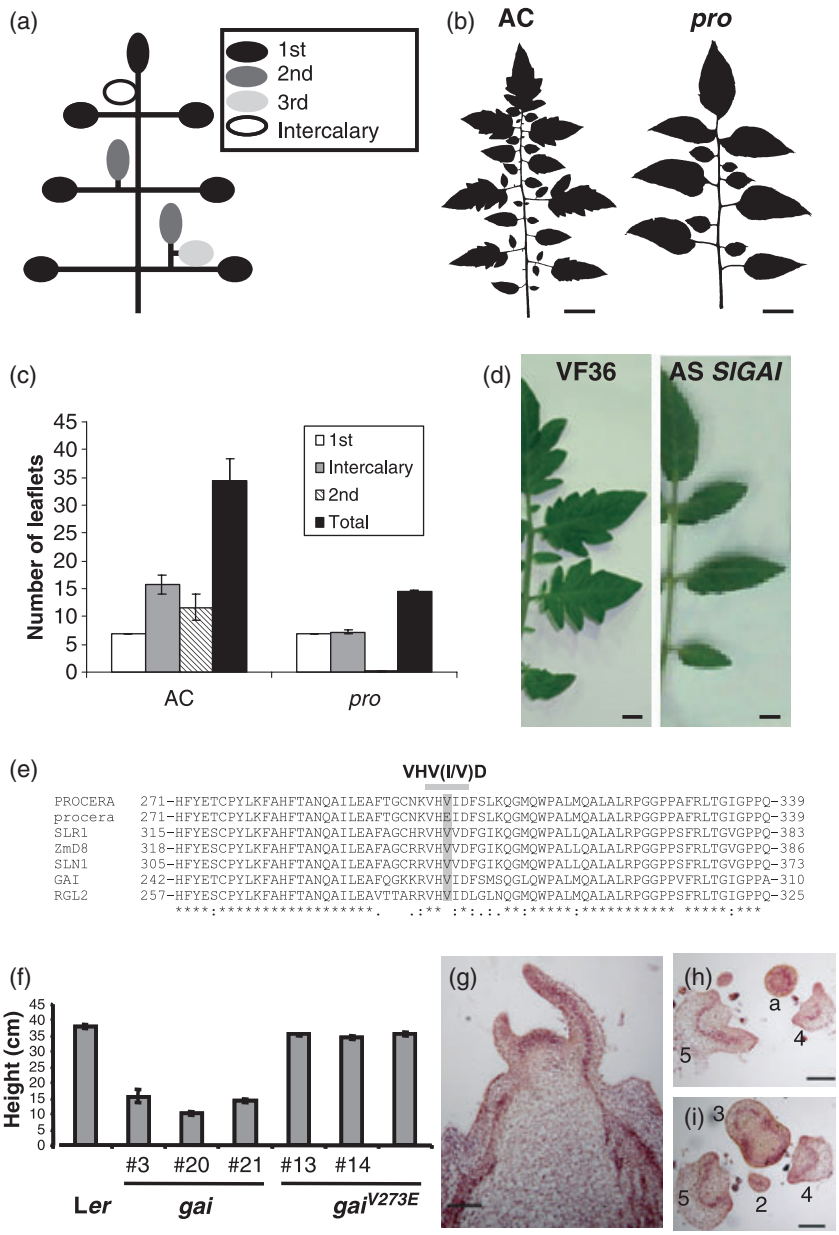

Figure 1. *PRO* encodes SIGAI, a DELLA-type growth repressor.
(a) Diagram defining first, second, third and intercalary leaflets.
(b) Silhouettes of the sixth leaf from wild-type [Ailsa Craig (AC) background, left] and *pro* (right) 8-week-old tomato plants. Note the smoother leaf margin of *pro*. Scale bars: 1 cm.
(c) Graphs showing average leaflet numbers of mature leaves from AC and *pro*. Bars represent standard error (SE, $n = 12$).
(d) Picture of half-leaves (sixth) from a wild-type (VF36, left) and an antisense *SIGAI* plant (AS *SIGAI*, right). Plants are 10-week-old. Scale bars: 2 cm.
(e) Alignment of deduced amino acid sequences of selected angiosperm DELLA proteins, including tomato PROCERA, rice SLENDER RICE 1 (SLR1), maize DWARF8 (ZmD8), barley SLENDER 1 (SLN1) and Arabidopsis GIBBERELLIN insensitive (GAI) and RGA-like 2 (RGL2). The conserved VHV(I/V)D motif is shown. (*) Indicates identical residues, (:) indicates highly conserved residues and (.) indicates weakly conserved residues.
(f) Average height of 6-week-old plants from L*er*, three independent *35S::gai* T_2 lines and three independent *35S::gai*^{V273E} T_2 lines. Bars represent standard deviation ($n \geq 15$).
(g–i) *In situ* hybridisation of 2-week-old wild-type (AC) apices in longitudinal section (g) and transverse section (h, i) probed with *SIGAI*. a, shoot apical meristem, numbers indicate plastochron, i.e. the time interval between successive leaf primordia.

period of 5 weeks (Figure 2a). The *pro* plants ceased leaflet production earlier than wild type, indicating that PRO action is required to determine the time interval in which tomato leaves can produce leaflets. To determine when during development PRO starts acting to sculpt leaf margin morphology, we compared early leaf development in wild-type and *pro* plants using scanning electron microscopy (SEM; Figure 2b–g). We observed that leaf margins are readily distinguishable at plastochron 4 (P4), at which stage *pro* plants had less pronounced marginal outgrowths (Figure 2d,e). Therefore, a requirement for PRO activity in leaf development is evident at the stage when the lamina starts producing marginal outgrowths. Notably, developing leaf primordia of *pro* plants appeared longer at P3, P4 and P5 (Figure 2h), demonstrating that *pro* leaves grow faster at these developmental stages. However, the final size of *pro* leaves was not substantially different from those of the wild type (Figure S2a), hence suggesting a heightened requirement for PRO-dependent growth control at early stages of primordium growth. These findings indicate that the acquisition of final leaf shape in tomato is dependent on precise coordination of the ontogenetic sequence of leaflet emergence with processes controlling organ size, and further suggests that PRO activity may be required for such coordination.

To determine the cellular basis for PRO action, we compared cell sizes in leaves and petals of wild-type and

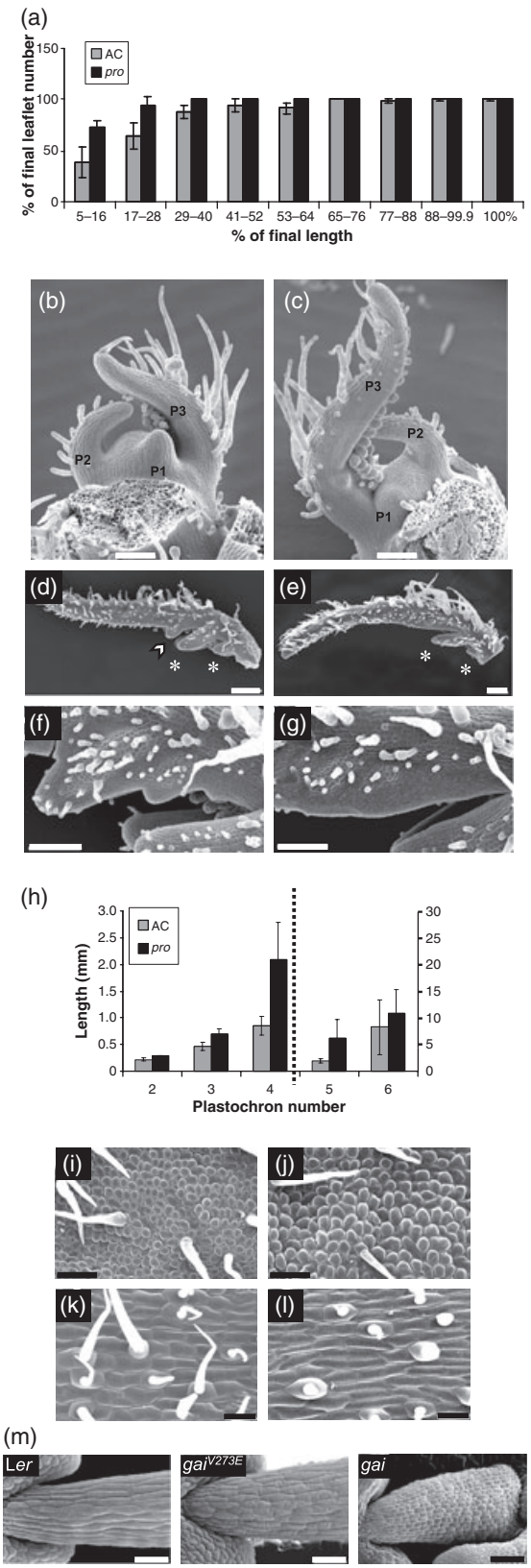

Figure 2. PRO negatively regulates cell growth and is required for the development of wild-type leaf margins.
(a) Graph showing the percentage of final leaflet number (*y*-axis) compared with the percentage of final leaf length (*x*-axis) in wild-type (Ailsa Craig, AC) versus *pro* plants. Bars represent SE (n = 8, two leaves, four plants).
(b, c) Scanning electron micrographs of wild-type (AC background, b) and *pro* (c) apices. Scale bars: 100 μm. P1, P2, P3 are, plastochron 1, 2, 3, respectively, where a plastochron denotes the time interval between successive leaf primordia.
(d–g) Plastochron 4 (d, e) and 5 (f, g) leaves from wild-type (d, f) and *pro* plants (e, g). Scale bars: 200 μm in (d, e) and 100 μm in (f, g). Stars indicate lateral leaflet primordia and arrowheads indicate lobes. Note that the *pro* leaf margin does not produce lobes and hence appears smoother.
(h) Measurement of primordia length from 19-day-old seedlings from wild-type (AC background) and *pro* plants, showing that *pro* primordia are significantly longer from P3 to P5. At P6, the *pro* and WT leaves are more similar. P2–P4 left-hand *y*-axis, P5 and P6 right-hand *y*-axis. Bars represent SE (n = 4).
(i–l) Scanning electron micrographs of petal (i, j) and petiole (k, l) cells from wild-type (i, k) and *pro* (j, l). Scale bars: 50 μm in (i, j) and 100 μm in (k, l).
(m) Scanning electron micrographs of anther filaments from L*er*, *35S::gai*^{273E} and *35S::gai* plants. Scale bars: 50 μm.

pro plants by SEM (Figure 2i–l). The cell area in *pro* leaves and petals was larger (1.3 and 1.8 times, respectively) than that of wild-type leaf and petal cells (Figure 2i–l), consistent with previous observations in *pro* stems (Jupe *et al.*, 1988) and with the idea that the *pro* mutation prevents DELLA-dependent growth restraint. Since petal and leaf area do not differ significantly between *pro* and wild-type plants (Figure S2a,b), these observations suggest that in the developmental context of lateral organs, PRO controls growth predominantly by limiting cell size and not cell number. An alternative possibility would be that PRO activity stimulates cell proliferation and that increased cell size in the *pro* mutant reflects compensation for the reduction in cell numbers observed in lateral organs. These explanations are not mutually exclusive but we favour the former one as more parsimonious, given that in *A. thaliana*, DELLAs regulate floral organ size by regulating cell expansion (Cheng *et al.*, 2004). Notably, the importance of the conserved valine in the VHV(I/V)D motif for DELLA-mediated control of cell size was further confirmed by comparisons of *35S::gai* and *35S::gai*^{V273E} plants by SEM, which showed that the *35S::gai*^{V273E} allele suppressed the reduced cell size conferred by *35S::gai* (Figure 2m).

Reducing GA activity modifies the competence of the leaf to produce leaflets

Our results and previous observations that application of GA reduces leaflet number indicated that elevated signalling through the PRO pathway antagonised leaflet production, perhaps as part of a mechanism that coordinates cellular growth with leaflet emergence. To determine whether, conversely, reducing GA activity was sufficient to increase leaf complexity, we quantified leaflet number in the tomato

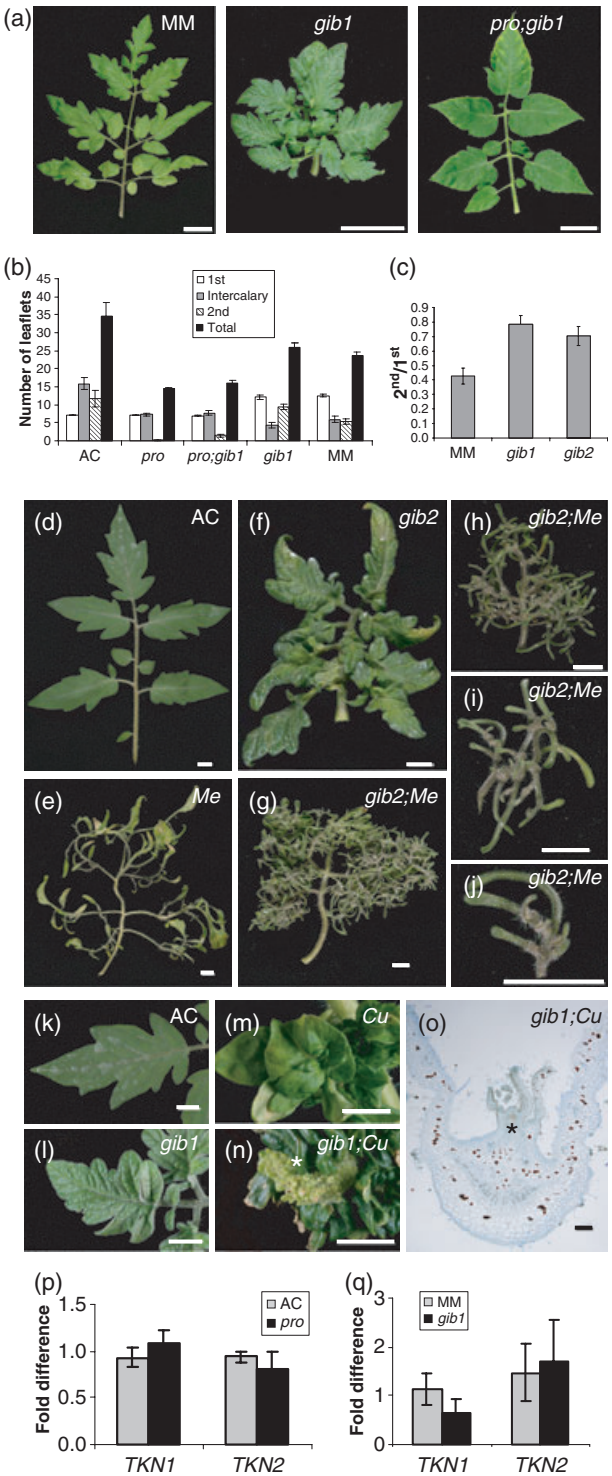

Figure 3. Gibberellin (GA) is required for tomato leaf development.
(a) Photographs of adult leaves from 9-week-old wild-type (MM background), *gib1* and *pro;gib1* plants. Scale bars: 5 cm.
(b) Graphs showing average leaflet number of adult leaves from the genotypes mentioned. Bars represent SE (n = 12 for all genotypes, except for MM and *gib1*, n = 10).
(c) Graph showing the ratio of secondary leaflets number on primary leaflets number. Bars represent SE (n = 10 for MM and *gib1*, n = 14 for *gib2*).
(d–j) Photographs of wild-type (d), *Me* (e), *gib2* (f) and *gib2;Me* (g) mature leaves of 10-week-old plants. Dissection of *gib2;Me* leaf revealed: first (h), second (i) and third (j) order of dissection. Scale bars: 1 cm.
(k–n) Photographs of wild-type (k), *gib1* (l), *Cu* (m) and *gib1;Cu* (n) terminal leaflets from mature leaves of 10-week-old plants showing the midribs. (*) Shows a mass of aberrantly differentiated tissue forming on *gib1;Cu* midrib.
(o) A cross-section in the midrib of *gib1;Cu* showed that this tissue consists of multiple shoot apical meristem-like structures. Scale bars: 1 cm in (k)–(n) and 0.2 mm in (o).
(p, q) Quantitative RT-PCR analysis of *TKN1* and *TKN2* expression in Ailsa Craig (AC) versus *pro* (p) and Moneymaker (MM) versus *gib1* (q) tomato seedlings. The MM and *gib1* plants were germinated on MS plates supplemented (*gib1*) or not (MM) with 50 μM GA for 1 week, then transplanted to soil for 18 days. The first leaf was excluded from the sampling. The AC and *pro* plants were grown on soil for 3 weeks and the aerial parts were used for the RT-PCR analysis. Error bars indicate SE.

GA biosynthesis mutants *gib1* and *gib2* (Figure 3a,b and data not shown). There was no statistically significant increase in total leaflet number in *gib* mutants, relative to wild-type plants. However, *gib* mutants produced more secondary leaflets per primary leaflet than did wild-type plants (Figure 3c). We therefore hypothesized that while reducing GA is itself not sufficient to condition elevated leaflet numbers, reduced GA activity may, under certain circumstances, elevate the competence of leaf tissue to produce leaflets. We predicted that if this was the case, a reduction in GA biosynthesis would be likely to enhance the increased leaflet production phenotype resulting from elevated and ectopic *KNOX* expression in leaves. To test this prediction, we generated double mutants between either *gib1* or *gib2* and the *Curl* (*Cu*) or *Mouse ears* (*Me*) mutants, which both condition *KNOX* misexpression because of regulatory mutations at the *SlT6*/*Tkn2* (*Solanum lycopersicum knotted-like homeobox gene 2*) *KNOX* locus (Chen *et al.*, 1997; Parnis *et al.*, 1997). Both of these double mutants had striking phenotypes (Figure 3d–o). In the *Me* background, *gib2* conditioned substantially increased leaflet number, with plants elaborating multiple orders of dissection over several months (Figure 3g–j). Furthermore, in the *gib1;Cu* double mutant, reduced GA activity resulted in the production of numerous ectopic shoot meristems on leaves (Figure 3n,o), a phenotype we did not observe on *Cu* mutants alone (Figure 3m). Thus, reducing GA increases the sensitivity of the response of tomato leaf tissue to KNOX activity. These observations confirm that GA homeostasis has a role in delimiting the correct degree of leaflet production and that, under certain circumstances, it may safeguard leaf fate by preventing inappropriate meristem formation.

An alternative explanation for the enhanced phenotypes observed after changes in GA activity in the *Me* and *Cu* backgrounds is that KNOX proteins, which are positive regulators of leaflet formation and act at least in part by repressing GA biosynthesis, are themselves sensitive to GA as part of a feedback loop, such that reduced GA activity elevates *KNOX* transcription and, conversely, elevated GA

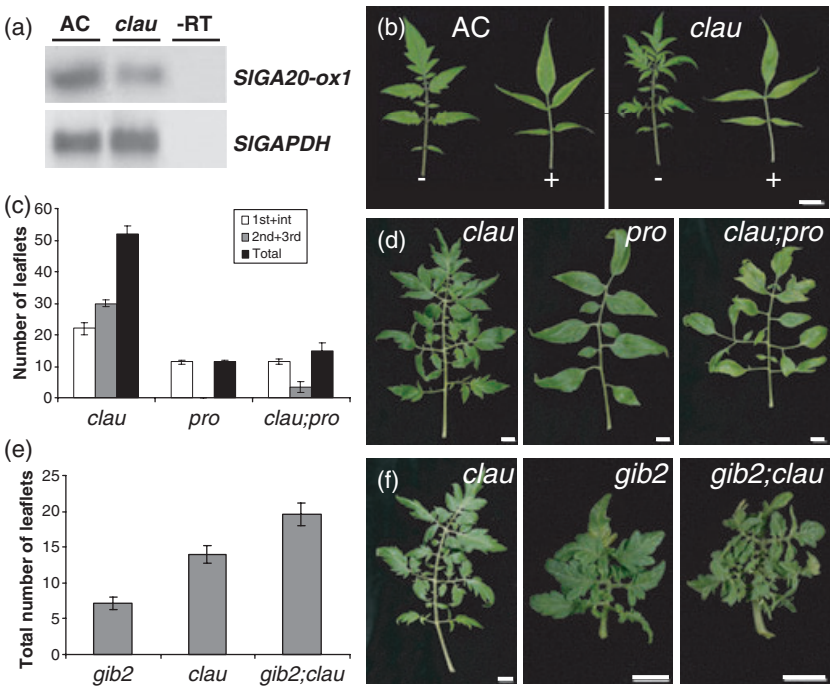

Figure 4. Altered gibberellin (GA) homeostasis contributes to perturbed leaf development in the *clausa* (*clau*) mutant.
(a) A RT-PCR gel blot analysis of *SlGA20-ox1* expression in Ailsa Craig (AC) and *clau*. *SlGAPDH* indicates that equal amounts of cDNA are present in each sample. –RT corresponds to PCR reaction without cDNA.
(b) Photographs of the fourth leaves from AC and *clau* plants treated (+) or not (–) with 100 μM GA. Scale bar: 1 cm.
(c) Graph showing average leaflet numbers of the seventh leaf of 2-month-old plants from the genotypes mentioned. Error bars represent SE ($n = 6$ for *clau*, $n = 5$ for *pro* and $n = 8$ for *clau;pro*).
(d) Seventh leaves of 2-month-old plants from *clau*, *pro* and the *clau;pro* double mutant. Scale bars: 2 cm.
(e) Graphs showing average leaflet numbers of the sixth leaf of 10-week-old plants from the genotypes mentioned. Bars represent SE ($n = 6$ for *gib2*, $n = 4$ for *clau* and $n = 9$ for *gib2;clau*).
(f) Sixth leaves of 10-week-old plants from *clau*, *gib2* and the *gib2;clau* double mutant. Scale bars: 2 cm.

activity antagonises *KNOX* expression. To test this hypothesis, we quantified transcript levels for the *KNOX* genes *TKn1* and *TKn2* in the *gib1* and *pro* mutants. There were no changes in their expression (Figure 3p,q), so it is unlikely that GA or DELLAs regulate *KNOX* transcription in tomato leaves. We therefore concluded that it is more likely that the competence of leaf tissue to respond to KNOX-dependent signals promoting leaflet formation can be modulated by GA in a DELLA-dependent fashion. Because KNOX proteins activate biosynthesis of cytokinin (Jasinski *et al.*, 2005; Sakamoto *et al.*, 2006; Yanai *et al.*, 2005) and GA can antagonize cytokinin activity (Greenboim-Wainberg *et al.*, 2005; Jasinski *et al.*, 2005), it is possible that GA-mediated regulation of leaflet number occurs at least in part by modifying cytokinin activity in the tomato leaf.

Gibberellin homeostasis as a downstream component of endogenous KNOX repressive pathways in tomato

The effects of reducing GA levels on leaf development were shown in the context of dominant gain-of-function mutations that condition elevated and expanded *KNOX* expression (Figure 3d–o). To further validate the significance of these findings, we tested whether pathways acting to delimit *KNOX* expression during wild-type leaf development are also sensitive to perturbations of GA activity, by studying the effects of altering GA activity in the recessive *clausa* (*clau*) mutant, which shows elevated and broadened *KNOX* expression in leaf primordia and increased leaflet number (Avivi *et al.*, 2000; Jasinski *et al.*, 2007). Consistent with their *KNOX* misexpression phenotype, *clau* mutants also show a reduction in expression of a GA biosynthetic gene, *SlGA20ox1* (Figure 4a). It was previously shown that *GA20ox* genes are repressed by KNOX proteins (Hay *et al.*, 2002; Sakamoto *et al.*, 2001). Thus, exogenous GA application should suppress the *clau* phenotype as should the *pro* mutation. This was confirmed (Figure 4b–d), indicating that the reduction of GA levels is a key component of the *clau* phenotype and that *PRO* acts downstream of *CLAU* to regulate leaflet number. Conversely, we observed that the phenotype of *clau* is substantially enhanced in the *gib2;clau* double mutant (Figure 4e,f). These data indicate the presence of a genetic hierarchy in tomato, whereby upstream regulators such as CLAU delimit *KNOX* expression, thus defining a GA homeostasis regime that helps control leaflet numbers in a PRO-dependent fashion. The downstream action of PRO in this hierarchy is further confirmed because *pro*, to a very large extent, masks the *gib1* mutant phenotype (Figure 3a,b; Van Tuinen *et al.*, 1999).

Discussion

Our data suggest that DELLA activity, largely influenced by GA, modulates the timing of leaflet emergence in tomato and the competence to respond to KNOX-dependent signals that direct leaflet formation. It is possible that these two aspects of DELLA action are linked. According to such a scenario, an abnormally low GA regime, for example in *gib* mutants, and consequent failure to relieve

DELLA-mediated growth repression locks the leaf in a state competent to respond to the action of KNOX, resulting in a prolonged period of leaflet production or ectopic shoot formation (Figure 3d–o). Conversely, the rapid growth of *pro* mutants early in development may result in premature reduction in the competence to respond to signals directing leaflet production (Figure 2). An alternative possibility is that *pro* mutants, which grow faster early in development, develop too rapidly to allow elaboration of the full complement of leaflet numbers, but nonetheless can still respond to KNOX activity in the leaf. To distinguish between these possibilities, which are not mutually exclusive, it will be important to understand whether the action of PRO in controlling the timing of leaflet emergence reflects the role of the protein in regulating cellular growth. Because the onset of leaf simplification in *pro* mutants is temporally coincident with premature primordium elongation at P3/4 (Figure 2h), it is tempting to speculate that alterations in cellular growth, in the context of perturbed DELLA activity, contribute to disruption of leaflet emergence. One mechanism that could link the action of DELLA at the cellular and organ levels might be a volume-dependent regulation of the concentration of KNOX-dependent signals that influence leaflet production, similar to what has been previously suggested to explain links between cell size and cell fate in *Volvox* (Kirk *et al.*, 1993). Alternatively, though less parsimoniously, it is possible that GA/DELLAs modulate cellular differentiation independently of their growth-regulating activity.

Further work is needed to evaluate these possibilities, such as characterising the functions of DELLA target genes (Zentella *et al.*, 2007) and studying leaf development in *pro* genetic mosaics. Our work indicates that acquisition of the final shape of tomato leaves cannot be achieved in the absence of PRO activity during early leaf development. Thus PRO, by controlling both cell size during development of leaf primordia and the timing of leaflet emergence, may facilitate coordination of cellular growth with developmental signals that elicit leaflet formation. This suggestion is consistent with the idea that GAs modulate developmental timing in diverse developmental contexts (Gazzarrini *et al.*, 2004; Lawson and Poethig, 1995). Notably, leaflet elaboration was also shown to be sensitive to perturbed activity of LANCEOLATE, a CINCINATA-type TCP (TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR) growth-regulating protein, which controls the time interval for leaflet production by regulating the balance between division and differentiation in the leaf blade (Ori *et al.*, 2007). It remains to be seen how the two different modes of growth regulation by DELLAs and TCPs are integrated during development to define the correct timing and position of leaflet emergence. However, neither reduction of PRO activity nor exogenous GA application can simplify the tomato leaf or condition shoot meristem loss to the extent seen in strong *Lanceolate* dominant mutants, which fail to correctly downregulate LANCEOLATE activity during development (Ori *et al.*, 2007). Thus, it is possible that regulated TCP activity has a more generalized role in determining the balance between division and differentiation in the shoot, while the interaction between GA and DELLA provides an additional layer of regulation, helping to fine-tune cellular growth with leaflet emergence. Finally, it is of note that auxin may also contribute to leaflet formation by polarizing the growth of the leaf margin (Avasarala *et al.*, 1996; Hay *et al.*, 2006; Barkoulas *et al.*, 2008), so an important problem in the future will be to elucidate how the activities of auxin and GA are integrated to sculpt the morphology of the leaf margins.

Experimental procedures

Plant material

All tomato seed stocks were obtained from the Tomato Genetics Resource Center (TGRC) at the University of California, Davis [accession numbers: Ailsa Craig (AC), LA2838A; Moneymaker (MM), LA2706; Condine Red, LA0533; *pro* (AC background), LA3283; *pro* (Condine Red background), LA0565; *gib1* (MM background), LA2893; *gib2* (MM background), LA2894; *clausa* (*clau*, AC background), LA3583; *Mouse ears* (*Me*, AC background), LA3552; *Curl* (*Cu*, AC background), LA3740; *Solanum pennellii* introgression lines in the background of *S. lycopersicum* cv. M-82 IL11-1, LA4092; IL11-2, LA4093]. The *pro* allele exhibits leaf simplification and increased height in both the Condine Red background, where it was isolated (Stubbe, 1957), and in the AC background into which it was repeatedly backcrossed (Smith and Richie, 1983). The *pro;gib1* double mutant was a kind gift from M. Koornneef (Max Planck Institute for Plant Breeding Research, Cologne, Germany) (Van Tuinen *et al.*, 1999). Tomato plants were grown under glasshouse conditions; seeds were germinated in soil and then transplanted to 2.5-L plastic pots, except for *gib* seeds and seeds of family segregating *gib*, which were germinated on solidified MS medium with 50 μM GA and then transferred to soil as soon as the radicle emerged. In summer, plants were grown under natural daylight, at a temperature of 20–25°C. In winter, plants were grown under artificial lighting (16 h light and 8 h dark) at 20°C. The Arabidopsis *gai-1* mutant (accession number CS63, background L*er*) was obtained from the Arabidopsis Biological Resource Center. Arabidopsis plants were grown in a greenhouse under long-day conditions (days 16 h, 20°C; nights 8 h, 16°C).

Construction of double mutants

gib2;Me *and* gib1;Cu. *gib2*/*gib2* or *gib1*/*gib1* plants were crossed to *Me* and *Cu*, respectively, with the *gib* plants as female. All F_1 plants presented the mutant phenotypes as expected. Six F_2 individuals with a *Me* phenotype and eight F_2 individuals with a *Cu* phenotype were self-pollinated and F_3 individuals were planted in order to determine which lines segregated for the *gib* mutation. In the F_3 progeny, in addition to *Me* (or *Cu*), wild-type and *gib* phenotypes, a fourth phenotypic class was identified with phenotypes of both *gib* and either of the dominant mutants. These were presumed to be double mutants, which segregated either at a 1:3:3:9 ratio (*gib*:WT:double mutants:*Me* or *Cu*), indicating those were derived from progenitors heterozygous for both mutants, or at a

ratio of 1:3 (double mutants:*Me* or *Cu*), indicating that the corresponding F_2 progenitor plants were *gib*/+ and homozygous for the dominant mutation. In this experiment we did not attempt to distinguish differential interactions of *gib* with *Me*/*Me* (or *Cu*/*Cu*) versus *Me*/+ (or *Cu*/+) on the basis of their leaf phenotypes.

gib2;clau. A *gib2*/*gib2* plant was crossed to *clau*, with the *gib2* plant as female. All F_1 plants had a wild-type phenotype. Six F_2 individuals displaying the *clau* phenotype were self-pollinated and F_3 individuals were planted and scored for leaf phenotype. In 4 F_3 families, a quarter of the individuals displayed aspects of both *gib2* and *clau* and were presumed to be double mutants.

clau;pro. A *clau/clau* plant was crossed to *pro*, with *clau* as female. Six F_2 individuals displaying the *clau* phenotype were self-pollinated and F_3 individuals were planted and scored for leaf phenotype. In 4 F_3 families, a quarter of the individuals displayed a *pro* phenotype and were presumed to be double mutants.

Measurement of leaflet numbers

On developing leaves. The lengths of the two consecutive smallest leaves readily visible by eye (approximately 1.2 cm) on 4-week-old plants were measured non-destructively with a ruler and the total number of leaflets were also counted. The same leaves were then repeatedly measured at regular intervals over the course of 5 weeks. When length did not increase over a 5-day interval, we considered that the final length and leaflet number was reached and the percentage of final length and numbers of leaflets achieved at each time point was calculated.

On adult leaves. Leaflets were counted in four orders of dissection: 1st, 2nd, 3rd and intercalary (Figure 1a). The *t*-test was used to assess whether the means of two groups were statistically different from each other ($P = 0.05$). The first-formed leaves were small and usually lacked lateral leaflets, the mature form being reached by the fourth node above the cotyledon under our conditions. Therefore, only leaves from node 4 and following were selected for observations.

In situ *RNA localisation*

Fixation and hybridisation were carried out as previously described by Jackson (1992). *SlGAI* cDNA was used for the probe, which was amplified using the following primers: pOX1-5-for4, CCAGCACTTGTCATTCTTACCC; pOX1-5-rev5, CCAACCACAAAATAAACCATAGG.

SEM analysis

SEM analysis was carried out as described in Bowman *et al.* (1991).

Measurement of cell area

The cell area of wild type and *pro* was measured from SEM images, using the ImageTool version 3.00 program developed at the University of Texas Health Science Center at San Antonio, TX (http://ddsdx.uthscsa.edu/dig/itdesc.html). For petals, cells from the distal part were measured. Three hundred and thirty-six cells were measured for wild type and 150 for *pro*. For leaves, the cell area was measured on 120 petiole cells from wild type and 216 petiole cells from *pro*. The *t*-test revealed that the means of two groups were not statistically different from each other ($P = 0.05$).

Measurement of leaf and petal area

Petals and leaves from *pro* and wild type (both in the AC background) were scanned and used to measure the area of individual organs. Twenty-six or 22 petals, and 6 or 5 leaves were measured for wild type and *pro,* respectively. The *t*-test was used to assess whether the means of two groups were statistically different from each other ($P = 0.05$).

Plant transformation

Arabidopsis Landsberg *erecta* (L*er*) plants were transformed by *Agrobacterium tumefaciens* infiltration and phenotypes of the T_1 plants were analysed.

Tomato To generate antisense *SlGAI* plants, the full-length *SlGAI* cDNA sequence was cloned in antisense orientation into the pART27 binary vector, for expression under the control of the *CaMV35S* promoter. Transgenic plants (VF36 background) were generated by *A. tumefaciens*-mediated transformation (McCormick, 1991).

Cloning of PRO

A PCR-based strategy (using degenerate primers DGF1 (5′-GTIGCICARAARYTIGARCA-3′) and DGR1 (5′-RTTIGCIGTRAARTGIGCRAAYTT-3′) followed by rapid amplification of cDNA ends (RACE; using the SMARTTM RACE cDNA Amplification Kit, BD Biosciences, http://www.bdbiosciences.com/) was used to clone cDNAs corresponding to tomato DELLAs expressed in leaves. Subsequent sequencing of both genomic and cDNA *SlGAI* fragments from *pro* and wild type demonstrated the presence of a T905A nucleotide change in *pro*. Four independent PCR reactions for each genotype were performed, two using cDNA and two using genomic DNA as templates.

pro *mutant genotyping*

Amplification of genomic DNA with primers proF, 5′-TCTTGCGGTTTCACAATCTG-3′ and proR2, 5′-CGCATCAAGATCTGCTAACG-3′, yielded a 500-bp product that was digested once by *Bsp*hI in *pro* mutant DNA but not in wild-type DNA. Co-segregation analysis was performed in an F_2 population derived by crossing *pro* plants into the AC background.

Complementation of pro *mutant*

A Cosmid clone with the *PRO* gene was isolated from a genomic library prepared from *S. lycopersicum* cv. Moneymaker as described in Schumacher *et al.* (1999) and the region from −5603 to +8137 relative to the start codon was sequenced. A 6115-bp *Avr*II/*Afl*II fragment (−3470 to +2651) was subcloned into an *Xba*I/*Afl*II-cut plant transformation vector pGPTV-Kan (Becker *et al.*, 1992). This construct was transformed into *pro* mutant plants (LA3283) as described in Schmitz *et al.* (2002).

Site-directed mutagenesis of gai

The QuickChange® Site-Directed Mutagenesis Kit from Stratagene (http://www.stratagene.com/) was used to mutate T^{818} into A in the Arabidopsis *gai* nucleotide sequence. The PCR primers were: Atgai_mut1, 5′-GGGAAGAAAAGAGTTCATGACATTGATTTCTCTATGAGTCAAGG-3′; Atgai_mut2, 5′-CCTTGACTCATAGAGAAATCAATGTCATGAACTCTTTTCTTCCC-3′.

The mutation was verified by sequencing. This synthetic gai^{273E} allele was cloned into the binary vector pART27, which allows expression under the control of the *CaMV35S* promoter, to yield the construct $35S::gai^{273E}$. None of the 45 independent T_1 plants analysed were dwarfed, even though northern blot analysis indicated that the transgene was expressed. In contrast, 54.5% (6 out of 11) of the *35S::gai* plants showed a dwarf phenotype.

Quantitative RT-PCR

Complementary DNA from Ailsa Craig, *procera*, Moneymaker and *gib1* were amplified on the ABI PRISM 7300 Sequence Detection System (Applied Biosystems, http://www3.appliedbiosystems.com/). Primer pairs were designed with Primer Express 2.0 (Applied Biosystems) to obtain a PCR product of 50–100 bp. Amplification reactions were prepared with the SYBR-Green PCR Master kit (Applied Biosystems) according to the manufacturer's specifications with 0.4 μM of primers and with 1 μl of cDNA per reaction. Data are mean values of three independent biological replicates, each including at least two individuals. The efficiency of each set of primers and calculation of the level of induction was determined according to Pfaffl (2001). The error bar represents the standard error calculated from experiment repetitions. Expression levels were normalized with the values obtained for the housekeeping *SlUBIQUITIN3* (*SlUBI3*) gene, which was used as an internal reference gene as described in Hoffman *et al.* (1991). Primers were: qRT-TKN1-F, TGATCACTTGGTGGGAGTTGC; qRT-TKN1-R, CCGACTCCGAAGGGTATGG; qRT-TKN2-F, AGGCATTGGAAACCATCAGAA; qRT-TKN2-R, TGAGCAGCATCCATCACAACA; qRT-SlUbi3-F, TCCTCCAGACGAAGATGCAGA; qRT-SlUbi3-R, TCGTCTTCCCCGTTAGGGTT. All primers are given in the 5′–3′ direction.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Complementation of the *pro* mutant.

Figure S2. *pro* does not affect final organ area.

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References

Avasarala, S., Yang, J. and Caruso, J.L. (1996) Production of phenocopies of the *lanceolate* mutant in tomato using polar auxin transport inhibitors. *J. Exp. Bot.* **47**, 709–712.

Avivi, Y., Lev-Yadun, S., Morozova, N., Libs, L., Williams, L., Zhao, J., Varghese, G. and Grafi, G. (2000) Clausa, a tomato mutant with a wide range of phenotypic perturbations, displays a cell type-dependent expression of the homeobox gene LeT6/TKn2. *Plant Physiol.* **124**, 541–552.

Barkoulas, M., Hay, A., Kougioumoutzi, E. and Tsiantis, M. (2008) A developmental framework for dissected leaf formation in the Arabidopsis relative Cardamine hirsuta. *Nature Genetics*, doi: 10.1038/ng.189.

Bassel, G.W., Mullen, R.T. and Bewley, J.D. (2008) *procera* is a putative DELLA mutant in tomato (*Solanum lycopersicum*): effects on the seed and vegetative plant. *J Exp Bot.* **59**, 585–593.

Becker, D., Kemper, E., Schell, J. and Masterson, R. (1992) New plant binary vectors with selectable markers located proximal to the left T-DNA border. *Plant Mol. Biol.* **20**, 1195–1197.

Bowman, J.L., Smyth, D.R. and Meyerowitz, E.M. (1991) Genetic interactions among floral homeotic genes of Arabidopsis. *Development*, **112**, 1–20.

Chen, J.-J., Janssen, B.-J., Williams, A. and Sinha, N. (1997) A gene fusion at a homeobox locus: alterations in leaf shape and implications for morphological evolution. *Plant Cell*, **9**, 1289–1304.

Cheng, H., Qin, L., Lee, S., Fu, X., Richards, D.E., Cao, D., Luo, D., Harberd, N.P. and Peng, J. (2004) Gibberellin regulates Arabidopsis floral development via suppression of DELLA protein function. *Development*, **131**, 1055–1064.

Fleet, C.M. and Sun, T.P. (2005) A DELLAcate balance: the role of gibberellin in plant morphogenesis. *Curr. Opin. Plant Biol.* **8**, 77–85.

Gazzarrini, S., Tsuchiya, Y., Lumba, S., Okamoto, M. and McCourt, P. (2004) The transcription factor FUSCA3 controls developmental timing in Arabidopsis through the hormones gibberellin and abscisic acid. *Dev. Cell*, **7**, 373–385.

Greenboim-Wainberg, Y., Maymon, I., Borochov, R., Alvarez, J., Olszewski, N., Ori, N., Eshed, Y. and Weiss, D. (2005) Cross talk between gibberellin and cytokinin: the Arabidopsis GA response inhibitor SPINDLY plays a positive role in cytokinin signaling. *Plant Cell*, **17**, 92–102.

Hay, A., Kaur, H., Phillips, A., Hedden, P., Hake, S. and Tsiantis, M. (2002) The gibberellin pathway mediates KNOTTED1-type homeobox function in plants with different body plans. *Curr. Biol.* **12**, 1557–1565.

Hay, A., Barkoulas, M. and Tsiantis, M. (2006) ASYMMETRIC LEAVES1 and auxin activities converge to repress BREVIPEDICELLUS expression and promote leaf development in Arabidopsis. *Development*, **133**, 3955–3961.

Hoffman, N.E., Ko, K., Milkowski, D. and Pichersky, E. (1991) Isolation and characterization of tomato cDNA and genomic clones encoding the ubiquitin gene ubi3. *Plant Mol. Biol.* **17**, 1189–1201.

Jackson, D. (1992) *In situ* hybridization in plants. In *Plant Molecular Pathology: A Practical Approach* (Gurr, S.J., McPherson, M.J. and Bowles, D.J., eds). Oxford: Oxford University Press, pp. 163–174.

Jasinski, S., Piazza, P., Craft, J., Hay, A., Woolley, L., Rieu, I., Phillips, A., Hedden, P. and Tsiantis, M. (2005) KNOX action in Arabidopsis

is mediated by coordinate regulation of cytokinin and gibberellin activities. *Curr. Biol.* **15**, 1560–1565.

Jasinski, S., Kaur, H., Tattersall, A. and Tsiantis, M. (2007) Negative regulation of KNOX expression in tomato leaves. *Planta*, **226**, 1255–1263.

Jupe, S.C., Causton, D.R. and Scott, Y.M. (1988) Cellular basis of the effects of gibberellin and the pro gene on stem growth in tomato. *Planta*, **174**, 106–111.

Kirk, M.M., Ransick, A., McRae, S.E. and Kirk, D.L. (1993) The relationship between cell size and cell fate in *Volvox carteri*. *J. Cell Biol.* **123**, 191–208.

Lawson, E.J. and Poethig, R.S. (1995) Shoot development in plants: time for a change. *Trends Genet.* **11**, 263–268.

Marti, C., Orzaez, D., Ellul, P., Moreno, V., Carbonell, J. and Granell, A. (2007) Silencing of DELLA induces facultative parthenocarpy in tomato fruits. *Plant J.* **52**, 865–876.

McCormick, S. (1991) Transformation of tomato with *Agrobacterium tumefaciens*. In *Plant Tissue Culture Manual*, Vol. B6 (**Lindsey, K.**, ed.). Dordrecht, The Netherlands: Kluwer Academic Publishers, pp. 1–9.

Ori, N., Cohen, A.R., Etzioni, A. *et al.* (2007) Regulation of *LANCEOLATE* by miR319 is required for compound-leaf development in tomato. *Nat. Genet.* **39**, 787–791.

Parnis, A., Cohen, O., Gutfinger, T., Hareven, D., Zamir, D. and Lifschitz, E. (1997) The dominant developmental mutants of tomato, *Mouse-Ear* and *Curl*, are associated with distinct modes of abnormal transcriptional regulation of a *Knotted* gene. *Plant Cell*, **9**, 2143–2158.

Peng, J. and Harberd, N.P. (1993) Derivative alleles of the Arabidopsis *gibberellin-insensitive* (*gai*) mutation confer a wild-type phenotype. *Plant Cell*, **5**, 351–360.

Peng, J. and Harberd, N.P. (1997) Gibberellin deficiency and response mutations suppress the stem elongation phenotype of phytochrome-deficient mutants of Arabidopsis. *Plant Physiol.* **113**, 1051–1058.

Peng, J., Richards, D.E., Moritz, T., Ezura, H., Carol, P. and Harberd, N.P. (2002) Molecular and physiological characterization of Arabidopsis GAI alleles obtained in targeted Ds-tagging experiments. *Planta*, **214**, 591–596.

Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45.

Piazza, P., Jasinski, S. and Tsiantis, M. (2005) Evolution of leaf developmental mechanisms. *New Phytol.* **167**, 693–710.

Sakamoto, T., Kamiya, N., Ueguchi-Tanaka, M., Iwahori, S. and Matsuoka, M. (2001) KNOX homeodomain protein directly suppresses the expression of a gibberellin biosynthetic gene in the tobacco shoot apical meristem. *Genes Dev.* **15**, 581–590.

Sakamoto, T., Sakakibara, H., Kojima, M., Yamamoto, Y., Nagasaki, H., Inukai, Y., Sato, Y. and Matsuoka, M. (2006) Ectopic expression of KNOTTED1-like homeobox protein induces expression of cytokinin biosynthesis genes in rice. *Plant Physiol.* **142**, 54–62.

Schmitz, G., Tillmann, E., Carriero, F., Fiore, C., Cellini, F. and Theres, K. (2002) The tomato *Blind* gene encodes a MYB transcription factor that controls the formation of lateral meristems. *Proc. Natl Acad. Sci. USA*, **99**, 1064–1069.

Schumacher, K., Schmitt, T., Rossberg, M., Schmitz, G. and Theres, K. (1999) The *Lateral suppressor* (*Ls*) gene of tomato encodes a new member of the VHIID protein family. *Proc. Natl Acad. Sci. USA*, **96**, 290–295.

Silverstone, A.L., Jung, H.S., Dill, A., Kawaide, H., Kamiya, Y. and Sun, T.P. (2001) Repressing a repressor: gibberellin-induced rapid reduction of the RGA protein in Arabidopsis. *Plant Cell*, **13**, 1555–1566.

Smith, M.J.W. and Ritchie, D.B. (1983) A collection of near-isogenic lines of tomato: Research tool of the future? *Plant Mol. Biol. Rep.* **1**, 41–45.

Stubbe, H. (1957) Mutanten der Kulturtomate Lycopersicon esculentum Miller I. *Die Kulturpflanze*, **5**, 190–220.

Sun, T.P. and Gubler, F. (2004) Molecular mechanism of gibberellin signaling in plants. *Annu. Rev. Plant Biol.* **55**, 197–223.

Van Tuinen, A., Peters, A.H.L.J. and Koornneef, M. (1998) Mapping of the *pro* gene and revision of the classical map of chromosome 11. Tomato. *Tomato Genet. Coop. Rep.* **48**, 62–70.

Van Tuinen, A., Peters, A.H.L.J., Kendrick, R.E., Zeevaart, J.A.D. and Koornneef, M. (1999) Characterisation of the *procera* mutant of tomato and the interaction of gibberellins with end-of-day far-red light treatments. *Physiol. Plant*, **106**, 121–128.

Yanai, O., Shani, E., Dolezal, K., Tarkowski, P., Sablowski, R., Sandberg, G., Samach, A. and Ori, N. (2005) Arabidopsis KNOXI proteins activate cytokinin biosynthesis. *Curr. Biol.* **15**, 1566–1571.

Zentella, R., Zhang, Z.L., Park, M. *et al.* (2007) Global analysis of DELLA direct targets in early gibberellin signaling in Arabidopsis. *Plant Cell*, **19**, 3037–3057.

