STUNTED mediates the control of cell proliferation by GA in Arabidopsis

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SUMMARY

Gibberellins (GA) are an important family of plant growth regulators, which are essential for many aspects of plant growth and development. In the GA signaling pathway, the action of GA is opposed by a group of DELLLA family repressors, such as RGA. Although the mechanisms of action of the DELLLA proteins have been studied in great detail, the effectors that act downstream of DELLLA proteins and bring about GA-responsive growth and development remain largely unknown. In this study, we have characterized STUNTED (STU), a receptor-like cytoplasmic kinase (RLCK) VI family gene, which is ubiquitously detectable in all the tissues examined. RGA activity and GA signaling specifically mediate the levels of STU transcripts in shoot apices that contain actively dividing cells. stu-1 loss-of-function mutants exhibit retarded growth in many aspects of plant development. During the vegetative phase, stu-1 seedlings develop smaller leaves and shorter roots than wild-type seedlings, while during the reproductive phase, stu-1 exhibits delayed floral transition and lower fertility. The reduced stature of stu-1 partly results from a reduction in cell proliferation. Furthermore, we present evidence that STU serves as an important regulator mediating the control of cell proliferation by GA possibly through two cyclin-dependent kinase inhibitors, SIM and SMR1. Taken together, our results suggest that STU acts downstream of RGA and promotes cell proliferation in the GA pathway.

KEY WORDS: Gibberellins, DELLLA proteins, Cell proliferation, Kinase

INTRODUCTION

Plants show massive variations in stature and genes involved in the control of plant architecture have recently been categorized into three classes, those affecting hormone metabolism and signaling, transcription and other regulatory factors, and cell cycle regulators (Busov et al., 2008). The ability to manipulate plant stature is a valuable asset in agriculture. For example, dwarf cultivars of wheat and rice that are less prone to lodging in adverse weather conditions and have higher yield were crucial to the success of the Green Revolution. These cultivars have been found to be impaired in gibberellin (GA) biosynthesis or signaling, indicating the importance of GA in determining plant stature (Peng et al., 1999; Monna et al., 2002; Sasaki et al., 2002; Spielmeyer et al., 2002). GA consists of a large family of plant growth regulators that control many developmental processes. The Arabidopsis GA biosynthesis mutant ga1-3 is severely dwarfed (Koornneef and Vanderveen, 1980). In addition, ga1-3 is non-germinating, produces dark-green leaves, and shows reduced apical dominance and defects in flowering and floral organ development (Koornneef and Vanderveen, 1980; Koornneef et al., 1983; Silverstone et al., 1997; Goto and Pharis, 1999).

DELLLA proteins are the major repressors of GA-responsive growth and belong to the plant-specific GRAS protein family of regulatory proteins (Pysh et al., 1999). In Arabidopsis, GA binds to its receptor GID1, which enhances GID1 interaction with DELLA proteins (Griffiths et al., 2006). This interaction inactivates DELLA repressor function in different manners (Schwechheimer and Willige, 2009). In a well-studied proteolytic degradation manner, DELLA proteins are targeted to an SCF-E3 ubiquitin ligase by the F-box protein SLY1, leading to their subsequent ubiquitylation and degradation by the 26S proteasome (McGinnis et al., 2003; Dill et al., 2004; Fu et al., 2004). This lifts the inhibitory effect of DELLA proteins and results in GA-responsive growth and development.

During plant development, final organ size is dependent on growth rate and duration. At the cellular level, growth rate is the result of the interplay between cell proliferation and expansion. Cell expansion directly increases organ size, whereas cell division supplies the cells for expansion. By varying the length and pace of mitotic activity, the total number of cells and therefore organ size is altered. In leaf morphogenesis, growth is divided into two phases. In the initial proliferative phase, cell number increases rapidly, whereas cell size remains relatively constant. In the second phase, cell division rate decreases and eventually ceases accompanied by a dramatic increase in cell size (Donnelly et al., 1999; Beemster et al., 2006). GA regulates cell elongation through stimulating the destruction of DELLA repressors in various tissues, including hypocotyls, stems, and roots (Yang et al., 2004; Cowling and Harberd, 1999; Cheng et al., 2004; Ubeda-Tomas et al., 2008). Recently, a novel function of GA in regulating cell production has also been discovered (Achard et al., 2009; Ubeda-Tomas et al., 2009). GA regulates cell proliferation by removing DELLA proteins in a subset of root meristem cells. Furthermore, GA controls cell cycle activity in the root meristem by modulating mRNA levels of several cell cycle inhibitors via a DELLA-dependent mechanism. Therefore, GA regulates both cell expansion and division by nullifying the negative effects of DELLA repressors.

The Arabidopsis genome contains more than 600 genes encoding receptor-like kinases (RLKs) (Shiu and Bleecker, 2001), which are generally composed of an extracellular domain, a single
transmembrane span and a cytoplasmic region containing a conserved kinase domain. Receptor-like cytoplasmic kinases (RLCKs), which lack a transmembrane or extracellular domain, are a subfamily of RLKs (Shiu and Bleeker, 2003). They make up ~25% of RLKs and are further divided into eight classes numbered RLCK I to VIII. The subfamily of class VI RLCKs consists of 14 members (Jurca et al., 2008). Although the members in this subfamily from different plant species have been found to interact with a group of plant-specific signaling regulators, the Rho-type GTPases (Molendijk et al., 2008; Dorjgotov et al., 2009), their biological functions are largely unknown.

In this study, we report the characterization of a RLCK VI family protein, STUNTED (STU; meaning stunted plant growth), which was earlier designated as RLCK VI B4 (Jurca et al., 2008). STU expression in seedlings is downregulated by RGA in response to GA. The loss-of-function mutant, stub-1, displays multiple defects, including a smaller plant stature that results from a reduction in cell division. In the absence of STU, the effect of GA signaling on cell division is reduced, whereas overexpression of STU elevates cell cycle activity. GA promotes STU expression via degradation of RGA. STU in turn represses the transcription of two cell cycle inhibitors, SIAMESE (SIM) and SIAMESE-RELATED 1 (SMRI), to enhance cell division, thus promoting GA-mediated growth and development. Our results establish STU as an important regulator that mediates GA regulation of plant stature.

MATERIALS AND METHODS

Plant material and growth conditions

*Arabidopsis thaliana* plants were grown under long days at 22°C. *stu-1* (SALK_093901) and *sim-1* (CS22884) are in Columbia (Col) background, whereas all the other genetic materials used in this study are in Landsberg erecta (Ler) background. *stu-1* (Ler) background was created through backcrossing *stu-1* (Col) to *Ler* three times. Generation of *ga1-3 gai-t6 rgl1-1 rgl2-1 rga-t2* and *ga1-3 rgl2-1 rga-t2 35S:RGA-6HA* was as previously described (Yu et al., 2004a). GA3 was used for all GA treatment in this study.

Expression analysis

Total RNAs were extracted using the FavorPre Plant Total RNA Mini Kit (Favorgen) and reverse transcribed using the SuperScript RT-PCR System (Invitrogen). Quantitative real-time PCR was performed on three independently collected samples using the CFX384 real-time system (Bio-Rad). The relative gene expression was calculated as previously described (Yu et al., 2007). Semi-quantitative PCR was performed as previously described (Yu et al., 2004a). For both quantitative real-time PCR and semi-quantitative PCR, the β-tubulin gene (*TUB2*) was amplified as an internal control. Primers used for gene expression analysis are listed in supplementary material Table S1. GUS staining and quantitative analysis of GUS activity were carried out as previously described (Jefferson et al., 1987). The concentration of dexamethasone, GA, or PAC used for GUS expression analyses was 10 μM.

Plasmid construction

To construct STU:GUS, a 1.91 kb STU 5’ upstream sequence was amplified and cloned into HY107 (Liu et al., 2007). A 4.5 kb STU genomic fragment including the 1.91 kb 5’ upstream sequence was amplified and cloned into pGreen-6HA (Li et al., 2008) to obtain an in-frame fusion of STU:STU-6HA. To construct 35S:GFP-STU, the coding regions of GFP and STU were cloned into pGreen-35S (Yu et al., 2004a). The amir-stu construct was generated according to the instructions on the web-based tool at http://wmd.weigelworld.org (Schwab et al., 2006). 35S:STU was produced by cloning the coding region of STU into pGreen-35S. 35S:RGA-6HA was generated by cloning the RGA-coding region into pGreen-35S:6HA (Liu et al., 2008). All primer sequences used for vector construction are listed in supplementary material Table S1.

Particle bombardment

35S:GFP-STU plasmid was mixed with DNAdel gold carrier particles (Seashell Technology) and precipitated following the manufacturer’s instructions. Bombardment was carried out using the Biolistic PDS-1000/He particle gun (Bio-Rad) as previously described (Yu et al., 2000). Prior to bombardment, onion cells were treated with 400 mM sorbitol on MS medium for 4 hours. After bombardment, they were placed on MS medium and incubated overnight in the dark at room temperature before viewing.

Analysis of *Arabidopsis* growth stage

*Arabidopsis* wild-type, *stu-1* and 35S:STU seedlings grown on soil were observed throughout the life of the plants and the growth stages reached were noted every day. Number of days refers to days after sowing (das). Growth stages are defined as previously described (Boyee et al., 2001).

Analysis of leaf growth

Measurements of leaf area, cell area and total cell numbers were carried out on the first true leaf pair of 12 seedlings grown on soil at 10 and 22 das, as previously described (Achard et al., 2009).

ChIP assay

Eight-day-old seedlings of *ga1-3 gai-t6 rgl1-1 rgl2-1 rga-t2 35S:RGA-6HA* were fixed and used for ChIP assays as previously described (Liu et al., 2008). Total chromatin before pull down was used as an input control against DNA recovered after binding to anti-HA antibody (Sigma). Ten pairs of primers (supplementary material Table S1) distributed along the STU genomic region were designed for measuring DNA enrichment.

Flow cytometric analysis

The first true leaves of 3-week-old wild type, *stu-1*, *sim-1* and *sim-1 stub-1* plants were collected for flow cytometry analysis as previously described (Arumuganathan and Earle, 1991). The ploidy levels were analyzed with the CyAn ADP analyzer (Beckman-Coulter, Roissy, France) using Summit v4.3 software.

RESULTS

**STU is downregulated by RGA in the GA signaling pathway**

We performed a microarray analysis to identify immediate targets of RGA in *Arabidopsis* flower development (Hou et al., 2008) and selected STU (At2g16750) from a list of genes whose expression were altered by the induced RGA activity. To understand further the regulation of STU expression in the GA signaling pathway, we examined its expression in seedlings of various mutant backgrounds (Fig. 1A). There was no change in STU transcript levels between wild-type and *rga-2* seedlings, possibly because RGA protein was relatively stabilized. STU transcription was closely controlled by RGA activity. In the absence of RGA in *gal1-3 rga-t2*, STU was significantly upregulated (Fig. 1A). This indicates that RGA activity suppresses STU expression in the whole seedlings, which is similar to the scenario in flower development (Hou et al., 2008). Next, we examined the response of STU expression to GA treatment in wild-type and *gal1-3* seedlings, and found that STU transcript levels were significantly upregulated by GA in *gal1-3* (Fig. 1B). This suggests that GA treatment, which stimulates the degradation of DELLA proteins, promotes STU expression.

To validate the regulation of STU by RGA, we studied STU expression in an established steroid-inducible functional version of RGA (RGA-GR) in *gal1-3 rgl2-1 rga-t2* (Yu et al., 2004b). The time course expression of STU from dexamethasone- and mock-treated *gal1-3 rgl2-1 rga-t2 35S:RGA-GR* seedlings was examined...
To test whether RGA affects STU expression through directly associating with the STU promoter, we created 35S:RGA-6HA lines for chromatin immunoprecipitation (ChIP) analysis. 35S:RGA-6HA was introduced into ga1-3 gai-6 gai-1-1 rgl2-1 rga-t2, and a functional line that mimicked ga1-3 phenotypes was chosen for further ChIP assays. ChIP analyses did not detect the binding of RGA-6HA to the STU locus (supplementary material Fig. S2), suggesting that RGA regulation of STU involves other regulatory factors.

**STU encodes a putative RLCK VI family protein**

The STU gene consists of 11 exons and 10 introns and encodes a putative Ser/Thr kinase, belonging to the subfamily of class VI RLCKs in Arabidopsis (Jurca et al., 2008). In Arabidopsis, the STU protein shares 69% amino acid identity with its closest homolog, RLCK VI_B3 (At4g35030). Multiple sequence alignment revealed a highly conserved kinase domain and an active site among all the RLCKs compared from different plant species (supplementary material Fig. S3). In addition, STU has a UspA domain near its N terminal, which was named after the universal stress protein of E. coli, UspA, the expression of which is enhanced upon stress treatment (Nystrom and Neidhardt, 1994).

**Expression pattern of STU**

To gain insights into the biological function of STU, we investigated its temporal and spatial expression during Arabidopsis development. Semi-quantitative RT-PCR was performed with RNAs extracted from different parts of 6-week-old wild-type seedlings (Fig. 2A). STU was expressed in all the tissues examined with the highest expression in roots and lowest expression in open flowers and siliques.

To further examine the detailed expression patterns of STU in Arabidopsis development, we transcriptionally fused a 1.91 kb STU 5’ upstream sequence to the GUS reporter gene, because a STU genomic fragment including this upstream sequence (STU:STU-6HA) was sufficient to rescue stu-1 phenotypes described below (supplementary material Fig. S4). Among 18 independent lines of the transformants harboring STU:GUS, 13 lines displayed similar GUS staining patterns and one representative line was thus selected for further investigation.

At 3 days after germination, GUS activity was detected in shoot apices and roots (Fig. 2B). Intense staining patterns were observed in the same regions of 1-week-old and 2-week-old seedlings with gradually increased staining in the leaf vasculature (Fig. 2C,D). In 3-week-old seedlings, GUS staining remained in main inflorescence apices and was also strongly detected in secondary inflorescence meristems that were subtended by cauline leaves (Fig. 2E). GUS staining was strong in the center of the inflorescence apex and the anthers of floral buds at flower stages 9 to 12 (Fig. 2F). A close examination of longitudinal sections of a stage 10 flower revealed the specific staining in the tapetum (Fig. 2G). At stage 14, GUS staining was mainly detected in ovules and pollen (Fig. 2H). Thus, STU is mainly expressed in various tissues or organs containing actively dividing cells.

To determine the subcellular localization of STU protein, the green fluorescent protein (GFP) was fused to the N terminus of STU under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The 35S:GFP-STU construct was introduced into onion epidermal cells by particle bombardment and the localization of the fusion protein was analyzed. Like 35S:GFP (Fig. 2K,L), GFP-STU signals were observed throughout the cell (Fig. 2J), which is similar to the pattern of several characterized RLCKs (Kong et al., 2007; Molendijk et al., 2008), implying that STU may
exert its function in multiple cell organelles. As 35S:GFP-STU was able to rescue stu-1 (data not shown), GFP-STU protein is probably functional.

RGA mediates regulation of STU by GA in shoot apices

To understand how GA signaling regulates STU expression in tissues containing actively dividing cells in Arabidopsis, we monitored GUS staining patterns in shoot apices of 8-day-old STU:GUS seedlings treated with GA and the GA-biosynthesis inhibitor paclobutrazol (PAC). GA treatment promoted STU:GUS activity in shoot apices when compared with mock treatment, whereas PAC inhibited STU:GUS activity (Fig. 3A,B). These results are consistent with those from gene expression analysis in seedlings (Fig. 1B) and shoot apices (Fig. 3B), suggesting that upregulation of STU by GA occurs in shoot apices of seedlings.

To further confirm that the regulation of STU expression by GA is mediated through RGA, we examined STU:GUS activity and STU transcripts in 8-day-old gal-1 rgl2-1 rga-t2 35S:RGA-GR seedlings mock treated or treated with dexamethasone. Induction of RGA activity at 4 hours after dexamethasone treatment reduced STU:GUS activity and STU expression in shoot apices when compared with those after mock treatment (Fig. 3C,D). This demonstrates the repression of STU expression by RGA in shoot apices, and substantiates that RGA mediates the regulation of STU expression by GA.

stu-1 loss-of-function mutants show multiple developmental defects

To investigate the biological role of STU in Arabidopsis, we identified a STU mutant (SALK_039301) containing a T-DNA insertion in the 6th exon from the Arabidopsis Biological Resource Centre (ABRC) (Fig. 4A). The presence of the T-DNA insertion was confirmed by PCR genotyping (data not shown), and semi-quantitative RT-PCR revealed that STU transcripts in developing seedlings were abolished by the presence of the T-DNA (supplementary material Fig. S5A). As this is the first instance of identification of a STU null allele, we have named this mutant stu-1.

Compared with wild-type plants, seedling development of stu-1 was retarded, resulting in a smaller plant stature (Fig. 4B). At 4 weeks after germination, corresponding rosette and cauline leaves in stu-1 were more curled and smaller in area than those in wild-type plants (Fig. 4C), but the chlorophyll content of the leaves was unchanged (supplementary material Fig. S6A). stu-1 roots were shorter than those of wild-type plants when they were grown on MS medium (Fig. 4D). At 5 weeks after germination, the first flowers of wild-type plants were usually open, whereas stu-1 had not yet bolted (Fig. 4E). In addition to the above-mentioned retarded growth defects, stu-1 also displayed lower germination rate and reduced fertility, as observed from its short siliques and deformed pollen grains (supplementary material Fig. S6B-H). Taken together, stu-1 demonstrates the similar developmental defects to those of GA-deficient mutants (Koornneef and Vanderveen, 1980).

To verify the phenotypes observed in stu-1, we created amiR-stu knockdown transgenic lines that expressed an artificial microRNA specifically targeting STU (Fig. 4A). A total of 28 independent transgenic lines were subsequently obtained, among which 12 lines were chosen for further analysis of their STU mRNA expression (supplementary material Fig. S5B). STU mRNA levels were downregulated in 11 of these lines, among which eight lines (lines 1, 2, 5, 6, 7, 8, 11 and 12) with the lower STU mRNA levels showed an obvious decrease in plant stature when compared with wild-type plants. Line 2 (hereafter called amiR-stu), which had the lowest STU mRNA expression, exhibited a similar stunted phenotype as stu-1 (Fig. 4B). These results confirm that reduced STU expression causes the retarded plant growth observed. This is further corroborated by a complementation test in which a 4.5 kb STU genomic fragment was able to rescue the stu-1 phenotypes (supplementary material Figs S4, S7).

We also created transgenic lines overexpressing STU under the control of 35S promoter. A total of 29 independent transgenic lines were obtained, among which nine lines were chosen for further analysis of STU expression (supplementary material Fig. S3C). STU mRNA levels increased in eight of these lines, among which six showed a slight increase in plant stature as compared with wild-type plants. Line 1 (hereafter called 35S:STU) with the highest STU mRNA expression was selected for further analysis (Fig. 4B).

To analyze further the plant growth affected by STU, we performed a detailed phenotypic analysis of wild-type, 35S:STU and stu-1 plants at two stages each during vegetative and reproductive development following the system previously
Lower panel shows shoot apices of 8-day-old wild-type seedlings treated as shown in A. Upper panel shows STU mock treated (MOCK), or treated with 10 μM GA or 10 μM PAC for 24 hours. Right panels show enlarged views of the shoot apices shown on the left. Scale bars: 1 mm. (B) Quantitative analysis of STU expression upon GA and PAC treatment. Upper panel shows STU expression in shoot apices of 8-day-old wild-type seedlings treated as shown in A. Lower panel shows GUS activity in shoot apices of 8-day-old STU:GUS seedlings treated as shown in A. (C) GUS expression in shoot apices of 8-day-old ga1-3 rgl2-1 rga-t2 35S:RGA-GR STU:GUS seedlings mock-treated (MOCK) or treated with dexamethasone (DEX) for 4 hours. Right panels show enlarged views of the shoot apices shown on the left. Scale bars: 1 mm. (D) Quantitative analysis of STU expression in response to RGA activity. Upper panel shows STU expression in shoot apices of 8-day-old ga1-3 rgl2-1 rga-t2 35S:RGA-GR seedlings treated as shown in A. Lower panel shows GUS activity in shoot apices of 8-day-old ga1-3 rgl2-1 rga-t2 35S:RGA-GR STU:GUS seedlings treated as shown in A. In B,D, STU expression levels were determined by quantitative real-time PCR analyses and normalized against the expression of TUB2. GUS activity values were scored from at least 15 plants of each treatment. Asterisks indicate significant changes in gene expression or GUS activity when compared with mock treatment (P<0.05, by Student’s t-test). Error bars indicate s.d.

developed (Boytes et al., 2001). As shown in Fig. 4F, the vegetative stages 1.02 and 1.10 indicate the number of days after germination at which the 2nd and 10th rosette leaves are greater than 1 mm in length, respectively. The reproductive stage 5.10 defines the time at which the first floral bud becomes visible, whereas stage 6.00 is the point at which the first flower is open. Our results showed that all wild-type, stu-1 and 35S:STU plants took ~10 days to reach stage 1.02, despite their differences in stature (Fig. 4F). Wild-type, stu-1 and 35S:STU plants showed slight differences in the average number of days taken to reach stage 1.10. The differences in their growth were more obvious at stages 5.10 and 6.00. In wild-type plants, the first flower buds emerged at around 23 days, whereas the corresponding days in stu-1 and 35S:STU were 30 and 20, respectively. The number of days at which the first flowers were open in wild type, stu-1 and 35S:STU were 32, 37 and 30, respectively. Consistently, the differences in rosette radius and plant height of various STU transgenic lines and mutants also support that STU affects plant stature (supplementary material Fig. S7).

**STU mediates the control of cell division by GA**

To understand the difference in plant stature between wild-type and stu-1 plants, we investigated cell division and elongation, the two cellular processes responsible for growth, by scanning electron microscopy of the first true leaves at 10 and 22 days after sowing (das) (supplementary material Fig. S8). At 10 das when leaf growth is mainly linked to cell proliferation (Donnelly et al., 1999;
Beemster et al., 2006), leaf area of stu-1 was only 61% of that of wild-type plants (Fig. 5A). Individual cell area in stu-1 remained largely unchanged, whereas cell number in stu-1 was 31% less than in wild-type plants. At 22 days, when cell expansion is the major cause of leaf growth (Donnelly et al., 1999; Beemster et al., 2006), individual cell area of wild-type and stu-1 plants were still similar, whereas leaf area and cell number in stu-1 were reduced by 26% and 25% when compared with wild-type plants, respectively (Fig. 5A). Thus, these results demonstrate that a reduction in cell proliferation is the main reason for the reduced leaf area of stu-1.

We then crossed a Dbox CYCB1;1-GUS expressing line with stu-1 to study the role of STU in cell division. The CYCLIN B1 (CYCB1;1)-GUS reporter is expressed in cells during the G2-M phase of cell cycle, thus allowing us to monitor mitotic activity (Colon-Carmona et al., 1999). The root meristem was examined for the difference in cell division between wild-type and stu-1 plants. Based on the intensity and extent of GUS staining and quantitative analysis of GUS activity, mitotic activity was evidently reduced in root meristems of stu-1 when compared with wild-type plants under mock treatment (Fig. 5B,C). In wild-type root meristems, GA treatment increased the number of dividing cells, whereas PAC treatment significantly decreased mitotic activity (Fig. 5B,C). On the contrary, similar treatments in stu-1 had less effect on the change of cell division (Fig. 5B,C), suggesting that GA mediates plant cell division via STU.

To quantify the observed differences in cell division, we measured the transcript levels of CYCB1;1 and KNOLLE, two genes known to be expressed during mitosis (supplementary material Fig. S9A). At 8 days after germination, transcript levels of CYCB1;1 and KNOLLE in stu-1 were reduced by approximately two-thirds and one-half, respectively, when compared with wild-type plants, substantiating that STU mediates cell proliferation rate. Consistent with the change of CYCB1;1 and KNOLLE expression in stu-1, the root meristem size, which was measured as the number of cells between the quiescent center and the first elongating cell, was reduced in stu-1 at both 3 and 5 days after germination (supplementary material Fig. S9B,C).

To confirm that STU mediates the control of plant stature by GA, we examined whether increased STU activity is able to rescue GA-deficient phenotypes. Hence, we created 35S:STU in the Ler
background and found increased plant stature in 35S:STU lines (Ler background) as those in the Col background (supplementary material Fig. S5C). One representative 35S:STU line was selected for genetic crossing with ga1-3 (Ler background). As expected, 35S:STU was able to partially rescue the reduced stature of ga1-3 (Fig. 5D,E). We further created ga1-3 stu-1 double mutants, in which stu-1 (Col) had been backcrossed to Ler background for three times. ga1-3 stu-1 showed comparable stature to ga1-3, suggesting that STU acts downstream of the GA biosynthetic pathway (Fig. 5D,E). Taken together, these results substantiate that STU acts in the GA pathway to control plant stature. Interestingly, GA dose-response assays of the maximum rosette radius and time to bolting and flowering showed that although stu-1 always grew slower than wild-type plants, stu-1 still responded to GA treatment (supplementary material Fig. S10). This implies that GA also affects plant stature in an STU-independent manner.

**STU affects cell division partly through SMR1 and SIM**

A recent study has found that DELLA restrain of cell proliferation results from the accumulation of transcript levels of cyclin-dependent kinase inhibitors, a group of proteins that bind to cyclin/cyclin-dependent kinase complexes (CYC/CDK) and inhibit cell cycle (Achard et al., 2009). Hence, we further examined the link between the expression of several cyclin-dependent kinase inhibitor genes and STU activity in the GA pathway. Quantitative real-time PCR revealed that two of the cyclin-dependent kinase inhibitor genes, SMR1 and SIM, were regulated through STU in the GA signaling pathway (Fig. 5F). In Ler wild-type plants, GA treatment reduced the expression of SMR1 and SIM, which is consistent with the previously published data (Achard et al., 2009), indicating that both genes are regulated in the GA pathway. Without GA treatment, both SMR1 and SIM were downregulated in 35S:STU, but upregulated in stu-1, indicating that STU inhibits the expression of SMR1 and SIM. As GA treatment triggers the degradation of RGA and increases STU levels, 35S:STU seems to mimic the effect of GA treatment on cell proliferation, thus inhibiting the expression of SMR1 and SIM. Furthermore, GA treatment of 35S:STU and stu-1 (Ler) had less pronounced effects on reducing the expression of SMR1 and SIM than GA treatment of wild-type plants (Fig. 5F). The similar attenuated effect of GA treatment on regulating the expression of SMR1 and SIM was also observed in ga1-3 35S:STU and ga1-3 stu-1 when compared with ga1-3 (Fig. 5F). These results corroborate that STU acts in the GA pathway to regulate SMR1 and SIM expression. Consistently, loss of SIM function in sim-1 significantly suppressed the defects in stu-1, including the stunted stature (Fig. 6A), reduced cell number (Fig. 6B) and higher ploidy levels (Fig. 6C), suggesting that SIM indeed acts downstream of STU in the control of cell division.

In contrast to SMR1 and SIM, the expression of other two cyclin-dependent kinase inhibitor genes, SMR2 and Kip-related protein 2 (KRP2), was not similarly affected by STU activity and GA treatment (supplementary material Fig. S11), implying that they might be differentially regulated.

**DISCUSSION**

**STU encodes an RLCK that functions in maintaining normal plant stature**

In this study, we characterized the biological function of STU, an RLCK VI family protein, in Arabidopsis. Although STU is ubiquitously detectable in all the tissues examined, it is highly expressed in shoot apices and roots, which contain actively dividing cells, indicating that STU activity is relevant to active cell proliferation. stu-1 mutants exhibit retarded growth in many aspects of plant development during vegetative and reproductive stages. stu-1 seedlings develop smaller leaves and shorter roots compared with wild-type seedlings at the vegetative phase, while at the reproductive phase, stu-1 exhibits delayed floral transition and lower fertility, and produces deformed pollen grains. A detailed analysis of cell division and expansion in leaves has revealed that the reduced stature of stu-1 at least partly results from a reduction in cell proliferation (Fig. 5). These observations suggest that STU functions in the control of cell proliferation to maintain normal plant stature.

There are 14 members in the subfamily of class VI RLCKs in Arabidopsis (Jurca et al., 2008). A few of them have been shown to interact with and be specifically activated by Rop GTPases (Molendijk et al., 2008), implying that RLCKs may function as effectors of Rop GTPases, which are plant-specific Rho small GTPases involved in signaling pathways and act as molecular switches to control the transmission of extracellular signals in plants (Zheng and Yang, 2000; Li et al., 2001; Yang, 2002; Agrawal et al., 2003). Although the diverse tissue expression of STU and ubiquitous subcellular localization of the protein imply a possible role of STU as an effector of Rop in cellular signaling, whether STU affects plant stature through its interaction with Rop GTPases needs to be further investigated.

**RGA negatively regulates STU in the GA pathway**

The phytohormone GA plays an important role in controlling many aspects of plant development throughout the plant life cycle. DELLA proteins serve as the major repressors of GA-
responsive growth and null mutations of different combinations of DELLA proteins rescue different aspects of GA-deficient phenotypes (Dill and Sun, 2001; Cheng et al., 2004; Yu et al., 2004b; Cao et al., 2006). Although several microarray analyses have been performed to identify genes regulated by DELLA proteins or GA in *Arabidopsis*, detailed analyses on the effectors that function downstream of DELLA proteins and bring about GA-responsive growth and development remain largely unknown. Our studies have shown that *STU* expression is repressed by RGA activity and is an immediate target of RGA regulation, because induced RGA activity represses *STU* expression within 4 hours and independently of protein synthesis. On the contrary, GA treatment stimulates the degradation of RGA, and thus promotes *STU* expression. In particular, *STU* expression in shoot apices and roots where cell division actively occurs is specifically mediated by RGA activity and GA signaling. These observations strongly suggest that *STU* is a downstream effector of the GA signaling pathway, and that GA promotes *STU* expression through degradation of RGA, which might be relevant to the regulation of cell proliferation.

**STU mediates the control of cell division by GA**

GA has been shown to modulate growth by promoting cell expansion (Yang et al., 1996; Cowling and Harberd, 1999; Cheng et al., 2004; Ubeda-Tomas et al., 2008) and cell division (Achard et al., 2009; Ubeda-Tomas et al., 2009). Our study has provided several pieces of evidence that supports a role for *STU* in mediating the control of cell division by GA. First, *stu-1* loss-of-function mutants exhibit a decrease in cell proliferation in leaves, whereas cell expansion is unaffected (Fig. 5A). This result, together with the observation that the expression of *STU* transcripts is mainly in the tissues where active cell division takes place (Fig. 2), suggests that *STU* is involved in the control of cell division. Second, staining and quantitative analysis of Dbox CYCB1;1-GUS in root tips (Fig. 5B,C) and gene expression analyses of *CYCB1;1* and *KNOLLE* in seedlings (supplementary material Fig. S9A) have shown that mitotic activity is obviously reduced in *stu-1* when compared with wild-type plants. This further substantiates that *STU* controls cell division. Third, in wild-type roots, GA treatment promotes cell proliferation, whereas PAC treatment reduces cell proliferation (Fig. 5B,C). However, these effects are impaired or almost completely abolished in *stu-1* roots, demonstrating that, in the absence of *STU*, the effect of GA signaling on cell proliferation is compromised. Last, increased *STU* activity is able to rescue the reduced plant stature in *ga1-3*, whereas loss of *STU* function in *ga1-3* mimics the phenotypes of *ga1-3* (Fig. 5D,E). These results all support that *STU* serves as an important regulator that mediates the control of cell proliferation by GA.

It has been found that DELLA proteins restrain cell production in leaves and root meristems by enhancing the transcript levels of the cyclin-dependent kinase inhibitors KRP2, SIM, SMR1 and SMR2 (Achard et al., 2009). These inhibitors prevent cell cycle progression by interacting with D-type CYC and A-type CDK subunits (Inze, 2005). Our results suggest that two of the cyclin-dependent kinase inhibitor genes, *SMR1* and *SIM*, are regulated through *STU* in the GA signaling pathway (Fig. 5F). Further genetic and phenotypic analyses have shown that loss of *SIM* function rescues *stu-1* mutant phenotypes (Fig. 6). These results propose a role for *STU* in conveying the signal for growth from GA through RGA to the repression of cyclin-dependent kinase inhibitors, which in turn mediate cell division.

![Fig. 7. *STU* mediates the control of *Arabidopsis* plant stature by GA. GA binds to its receptor GID1, which enhances the interaction with RGA. This inactivates RGA activity partially through SLY1-mediated degradation of RGA by the 26S proteasome. Inactivation of RGA upregulates *STU* expression, which in turn decreases the expression of two cyclin-dependent kinase inhibitors, *SIM* and *SMR1*. Downregulation of these inhibitors drives cell proliferation and normal plant growth.](image)

**An updated model for GA-mediated control of plant stature**

Our results have led to an updated model that includes a new player, *STU*, in GA-mediated control of plant stature (Fig. 7). In this model, the presence of GA is perceived by the GA receptor GID1. The GA-GID1 complex interacts with RGA and inactivates RGA activity partially through SLY1-mediated degradation of RGA by the 26S proteasome. *STU* expression is repressed by RGA in the default state and inactivation of RGA causes an increase in *STU* transcript levels. *STU* in turn decreases the levels of *SIM* and *SMR1*, thus driving cell proliferation and promoting GA-responsive growth and development.

The exact mode of action of *STU* is an interesting topic for future studies as it will reveal how plant growth is executed in its entirety, from hormone perception to cell proliferation. Several recent publications have shown that members of RLCK VI family in *Arabidopsis* and *Medicago truncatula* can interact with and be specifically activated by Rop GTPases (Molendijk et al., 2008; Dorjgotov et al., 2009). Although we have not found any interaction between STU and Rop GTPases by yeast two-hybrid analyses, it is still possible that STU participates in cellular signaling through its interaction with other families of GTPases, such as ARF, RAN and RAB. Further elucidation of the regulatory cascade and substrate(s) of STU will greatly advance our understanding of the intricate details of GA-responsive growth.

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