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FEATURED ARTICLE

Characterization of temperature-sensitive mutants reveals a role for receptor-like kinase SCRAMBLED/STRUBBELIG in coordinating cell proliferation and differentiation during Arabidopsis leaf development

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SUMMARY

The balance between cell proliferation and cell differentiation is essential for leaf patterning. However, identification of the factors coordinating leaf patterning and cell growth behavior is challenging. Here, we characterized a temperature-sensitive Arabidopsis mutant with leaf blade and venation defects. We mapped the mutation to the *sub-2* allele of the *SCRAMBLED/STRUBBELIG* (*SCM/SUB*) receptor-like kinase gene whose functions in leaf development have not been demonstrated. The *sub-2* mutant displayed impaired blade development, asymmetric leaf shape and altered venation patterning under high ambient temperature (30°C), but these defects were less pronounced at normal growth temperature (22°C). Loss of *SCM/SUB* function results in reduced cell proliferation and abnormal cell expansion, as well as altered auxin patterning. *SCM/SUB* is initially expressed throughout leaf primordia and becomes restricted to the vascular cells, coinciding with its roles in early leaf patterning and venation formation. Furthermore, constitutive expression of the *SCM/SUB* gene also restricts organ growth by inhibiting the transition from cell proliferation to expansion. We propose the existence of a SCM/SUB-mediated developmental stage-specific signal for leaf patterning, and highlight the importance of the balance between cell proliferation and differentiation for leaf morphogenesis.

Keywords: Arabidopsis, receptor-like kinase, leaf development, venation patterning, cell proliferation, temperature sensitivity.

INTRODUCTION

Plants rely on temporal and spatial coordination of cell proliferation and differentiation to generate new tissues and organs from meristematic cells at the post-embryonic stage. Leaves are lateral organs derived from the peripheral zone of the shoot apical meristem. In Arabidopsis, leaf development can be divided into three phases: initiation, primary morphogenesis, and secondary morphogenesis. After the leaf initiates, the primordial cells proliferate robustly for a period of time to form the basic leaf shape during primary morphogenesis (Poethig, 1997; Donnelly *et al.*, 1999; Scarpella *et al.*, 2010). At this phase, several patterning processes are established, such as the axial polarities (the adaxial–abaxial, proximo-distal and medio-lateral axes) and the major venation pattern. At the time of the transition from cell proliferation to cell expansion/differentiation, which coincides with the onset of endo-reduplication, vacuole-associated cell expansion contributes to the increase of organ size during secondary morphogenesis (Poethig, 1997; Donnelly *et al.*, 1999; Beemster *et al.*, 2005; Scarpella *et al.*, 2010).

The balance between cell proliferation and differentiation is precisely controlled during leaf development, and is essential for the final leaf shape and size (Gutierrez, 2005; Tsukaya, 2006). Mutations in several leaf patterning genes, such as *JAGGED* (*JAG*; Dinneny *et al.*, 2004; Ohno *et al.*, 2004), *BLADE-ON-PETIOLE1* (*BOP*; Ha *et al.*, 2003), *ASYMMETRIC LEAVES1* (*AS1*) and *AS2* (Semiarti *et al.*, 2001; Sun

et al., 2002; Zgurski *et al.*, 2005), *TORNADO1* (*TRN1*) and *TRN2* (Cnops *et al.*, 2006), have been reported to cause ectopic cell divisions or an imbalance between cell proliferation and differentiation. Artificial alteration of cell proliferation in developing leaves also affects leaf shape and venation patterning (Wyrzykowska *et al.*, 2002; Kang *et al.*, 2007; Kuwabara *et al.*, 2011; Malinowski *et al.*, 2011). During leaf development, the exit of cells from proliferation progresses from the leaf tip towards the base, and may be modulated by cell division arrest signals (Donnelly *et al.*, 1999; Nath *et al.*, 2003). A mutation in the *CINCINNATA* (*CIN*) gene of *Antirrhinum* enables cells to be less sensitive to the arrest signals, leading to excess cell proliferation in leaf margin and a curved leaf with a wavy edge (Nath *et al.*, 2003). *PEAPOD* (*PPD*) genes negatively regulate the proliferation of dispersed meristematic cells, and excess proliferation in mutant lamina leads to a dome-like leaf blade (White, 2006). In addition, different polarity axes may adopt separate mechanisms to modulate cell proliferation and cell expansion (Tsukaya, 2006), as the *angustifolia* (*an*) and *rotundifolia* (*rot*) mutants exhibit altered cell proliferation or cell expansion in specific medio-lateral or proximo-distal directions (Kim *et al.*, 1998, 2002; Narita *et al.*, 2004).

During leaf development, an important event is the proper differentiation and patterning of specialized cells, a process that largely depends on developmental cues. For instance, vascular precursor cells are selected from homogeneous ground meristem cells of leaf primordia and divide longitudinally to give rise to procambial cells. These cells further divide and differentiate to produce xylem and phloem tissues, generating a continuous, branched and hierarchical venation pattern in Arabidopsis leaves (Foster, 1952; Candela *et al.*, 1999; Scarpella *et al.*, 2004). It has been shown that auxin distribution and polar transport provide positional information for venation patterning (Sachs, 1989; Mattsson *et al.*, 1999; Sieburth, 1999; Mattsson *et al.*, 2003; Scarpella *et al.*, 2006, 2010). The auxin canalization model proposes that auxin itself has a feedback effect on its polar transport mediated by auxin efflux carrier PIN-FORMED (PIN) proteins, and directs the formation of vascular strands (Sachs, 1989; Rolland-Lagan and Prusinkiewicz, 2005; Scarpella *et al.*, 2006, 2010).

Extensive genetic studies have shown that transcriptional networks and auxin-related pathways play important roles in leaf patterning (Barkoulas *et al.*, 2007; Scarpella *et al.*, 2010). However, the upstream components that integrate developmental and environmental cues to control leaf morphogenesis remain largely unknown. In this study, we identified a temperature-sensitive leaf developmental mutant and found it corresponding to the *sub-2* mutant allele of the *SCRAMBLED*/*STRUBBELIG* (*SCM*/*SUB*) gene encoding a receptor-like kinase (RLK). Previous studies showed that SCM/SUB is required for root epidermal cell specification and cell morphogenesis during the development of floral organs and stems, as well as the orientation of cell divisions in the L1 layer of the outer integument and the L2 layer of the floral meristem (Chevalier *et al.*, 2005; Kwak *et al.*, 2005). Here we report previously unknown functions of *SCM*/*SUB* in blade development and venation formation. We found that *SCM*/*SUB* is required to maintain the balance between cell proliferation and cell differentiation in both epidermal and internal tissues during leaf development. Our expression pattern analyses and temperature-shift experiments suggest that SCM/SUB probably mediates a developmental-stage specific signal for early leaf patterning.

RESULTS

sub/*scm* mutants displayed temperature-sensitive leaf developmental defects

In order to understand the mechanisms of leaf patterning and identify the hidden component(s), a recessive temperature-sensitive leaf developmental mutant was isolated from an EMS-mutagenized Arabidopsis mutant pool in the Col-0 background (Yan *et al.*, 2006). Map-based cloning showed that a G → A mutation occurred in the coding region of the *At1g11130* gene encoding the leucine-rich repeat receptor-like kinase (LRR-RLK) SCM/SUB and corresponded to the *sub-2* allele in the L*er* background (Figure S1a,b; Chevalier *et al.*, 2005; Kwak *et al.*, 2005). For confirmation that mutation in the *SCM*/*SUB* gene contributes to mutant phenotypes, a construct containing the full-length *SCM*/*SUB* gene was introduced into mutant plants and successfully complemented the mutant phenotypes (Figure S1c). For consistency, we use SUB as the gene name hereafter. The *sub-2* mutant in the Col-0 background was backcrossed three times to wild-type Col-0, and the resultant mutant line was used for subsequent analyses.

Compared with the wild-type, the *sub-2* leaves displayed varying degrees of defects at 30°C, including reduced leaf outgrowth, bilateral asymmetry, incomplete lamina and twisted petiole (Figure 1a,b). In severe cases, the deformed leaf blade had >180° rotation of petiole (Figure 1c). The shape and size of *sub-2* cotyledons appeared to be similar to Col-0, indicating a role for *SUB* in post-embryonic leaf development. When grown at 22°C, the mutant phenotypes were weakened, except for the reduced leaf size and slightly rough leaf surface (Figure 1d,e). The reduced leaf area in *sub-2* was mainly due to the reduced leaf width and incomplete development of the leaf blade, leading to a significant increase in the leaf index (radio of leaf length to leaf width; Figure 1f–i). In addition, *sub-2* exhibited temperature-sensitive twisting of the stem and reduced plant height (Figure 1j–l). Furthermore, a null T-DNA insertion mutant *scm-2* in the Col-0 background (Kwak *et al.*, 2005), as well as the *sub-2* and *sub-3* mutants in the L*er* background, also showed temperature-sensitive leaf defects (Figures S1d and

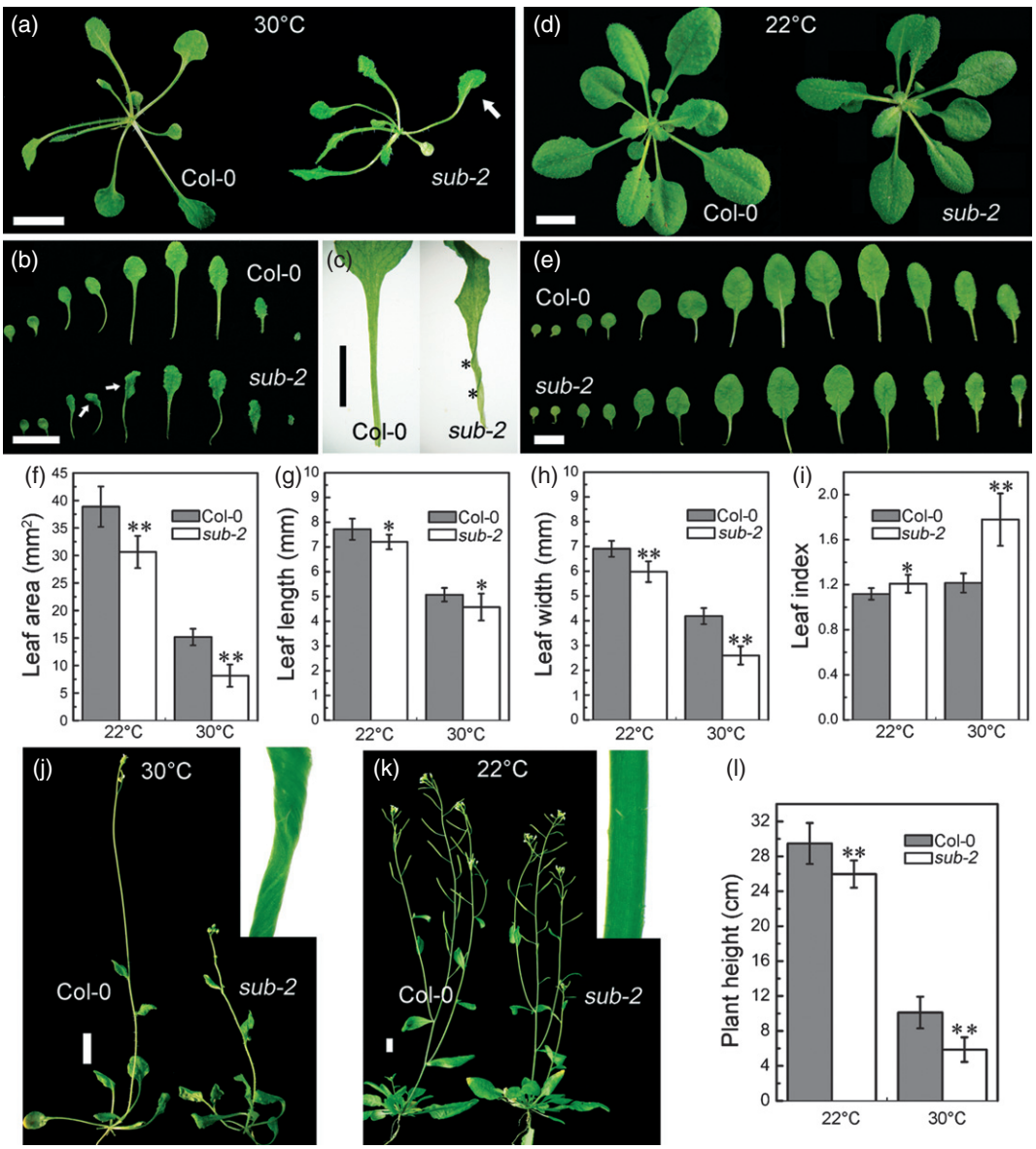

Figure 1. Morphological phenotypes of the temperature-sensitive mutant *sub-2*.
(a,b) Twenty-day-old wild-type Col-0 and *sub-2* plants grown at 30°C. Leaves including cotyledons are shown from left to right in (b). Arrows indicate the narrow and incomplete *sub-2* leaves.
(c) Close-up views of a Col-0 leaf and a deformed mutant leaf with twisted petiole (asterisks) grown at 30°C.
(d,e) Twenty-eight-day-old Col-0 and *sub-2* plants grown at 22°C. Cotyledons and rosette leaves are shown from left to right in (e).
(f–i) Analyses of leaf area (f), leaf length (g), leaf width (h) and leaf index (i) for the first-pair fully expanded leaves. Values are means ± SD ($n \geq 20$).
(j,k) Thirty-five-day-old Col-0 and *sub-2* plants grown at 30°C (j) and 50-day-old plants grown at 22°C (k). Twisted stems were present in *sub-2* plants at 30°C but not at 22°C (insets).
(l) Comparison of plant heights of Col-0 and *sub-2* plants in (j) and (k). Values are means ± SD ($n = 12$).
Scale bars = 1 cm. Asterisks indicate statistically significant differences compared to wild-type by Student's *t* test (*$P \leq 0.01$; **$P \leq 0.001$).

S2). Therefore, we conclude that the mutation in the *SUB* gene is responsible for the mutant phenotypes.

Altered cell proliferation and expansion during early leaf patterning in *sub-2*

To monitor the timing of SUB action during leaf development, we first examined the early leaf morphology at 30°C using scanning electron microscopy (SEM). At leaf stage 1 (Carland and McHale, 1996), the leaf shape and size of *sub-2* appeared similar to that of Col-0 (Figure 2a,b). However, an irregular leaf margin appeared at leaf stage 2 in *sub-2* plants (Figure 2c,d), and morphological abnormalities progressed at leaf stage 3, including reduced leaf outgrowth, bilateral asymmetry, and an irregular leaf margin with ectopic hyd-

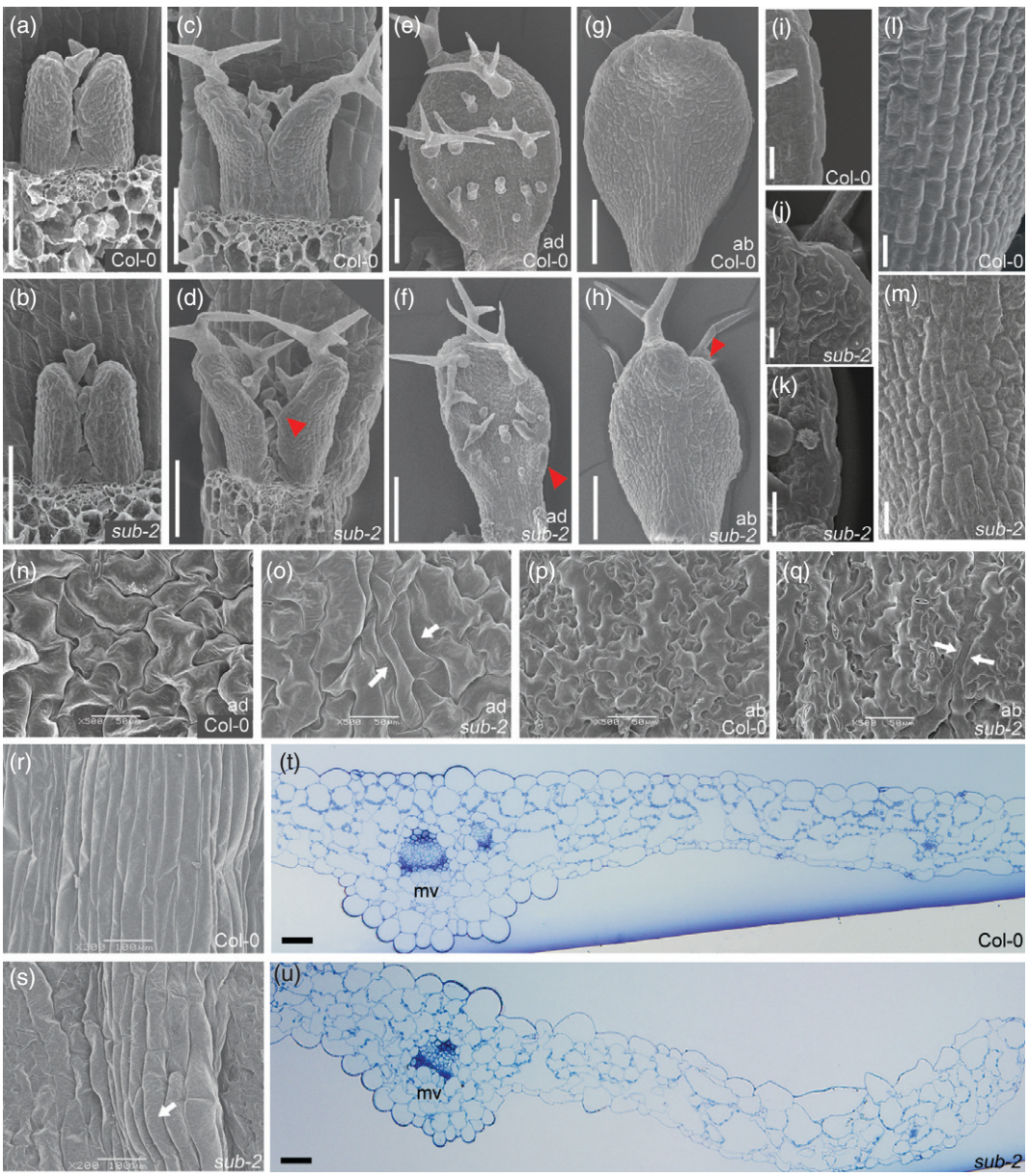

Figure 2. Developmental defects in epidermal and internal tissues of *sub-2* plants at 30°C.
(a,b) Leaf development of Col-0 and *sub-2* plants at stage 1.
(c,d) Leaf development of Col-0 and *sub-2* plants at stage 2. Note the slightly irregular leaf margin in *sub-2* (arrowhead).
(e–h) Adaxial (ad) and abaxial (ab) views of Col-0 and *sub-2* leaves at stage 3, showing impaired leaf development and ectopic hydathodes (arrowheads) in *sub-2* plants.
(i–k) Higher magnifications of (e), (h) and (f), respectively, showing the irregular leaf margin in *sub-2* plants.
(l,m) Higher magnifications of (g) and (h) showing the distorted epidermal cells along the *sub-2* midrib compared with the elongated cells in Col-0.
(n–q) Adaxial and abaxial surfaces of mature first-pair leaves from Col-0 and *sub-2* plants, showing non-fully expanded cells (arrows) in both sides of the *sub-2* leaves.
(r,s) Twisted abaxial epidermal cells (arrow) underlying the midrib in a mature *sub-2* leaf (s), compared with elongated cells in the Col-0 leaf (r).
(t,u) Transverse sections through the midvein (mv) and adjacent regions of mature fifth leaves. Note the irregular cells in both the epidermal and internal tissues of *sub-2* leaves.
Scale bars = 100 μm (a–h), 20 μm (i–m) and 50 μm (n–u).

athodes (Figure 2e–h). The elongated epidermal cells along the leaf margin and midrib appeared to be distorted in *sub-2* plants (Figure 2i–m). Study of the growth dynamics of leaf primordia also confirmed that early leaf outgrowth was largely inhibited in the medio-lateral axis of *sub-2*, leading to the reduced leaf size (Figure S3). At the late developmental stage, non-fully expanded epidermal cells were visible in both the adaxial and abaxial leaf surfaces of the *sub-2* blade

(Figure 2n–q). The uneven expansion of these cells may lead to the rough and crinkled leaf surfaces as shown in Figure 2(u). In addition, the abaxial epidermal cells along the midvein were obviously twisted (Figure 2r,s), and the shape and arrangement of mesophyll cells were irregular in the *sub-2* leaves (Figure 2t,u). These results indicate that the *SUB* gene is required for cell shape/expansion in both epidermal and internal tissues.

Transverse sections through the apex of 5-day-old seedlings (approximately leaf stage 3) grown at 30°C showed that the number of proliferating cells was reduced, but more vacuolated cells and intercellular gaps appeared in young leaves of *sub-2*, suggesting an early transition from cell proliferation to expansion (Figure 3a,b). Statistical analyses showed that the number of exterior epidermal cells at this stage and the number of palisade cells in the 25-day-old first

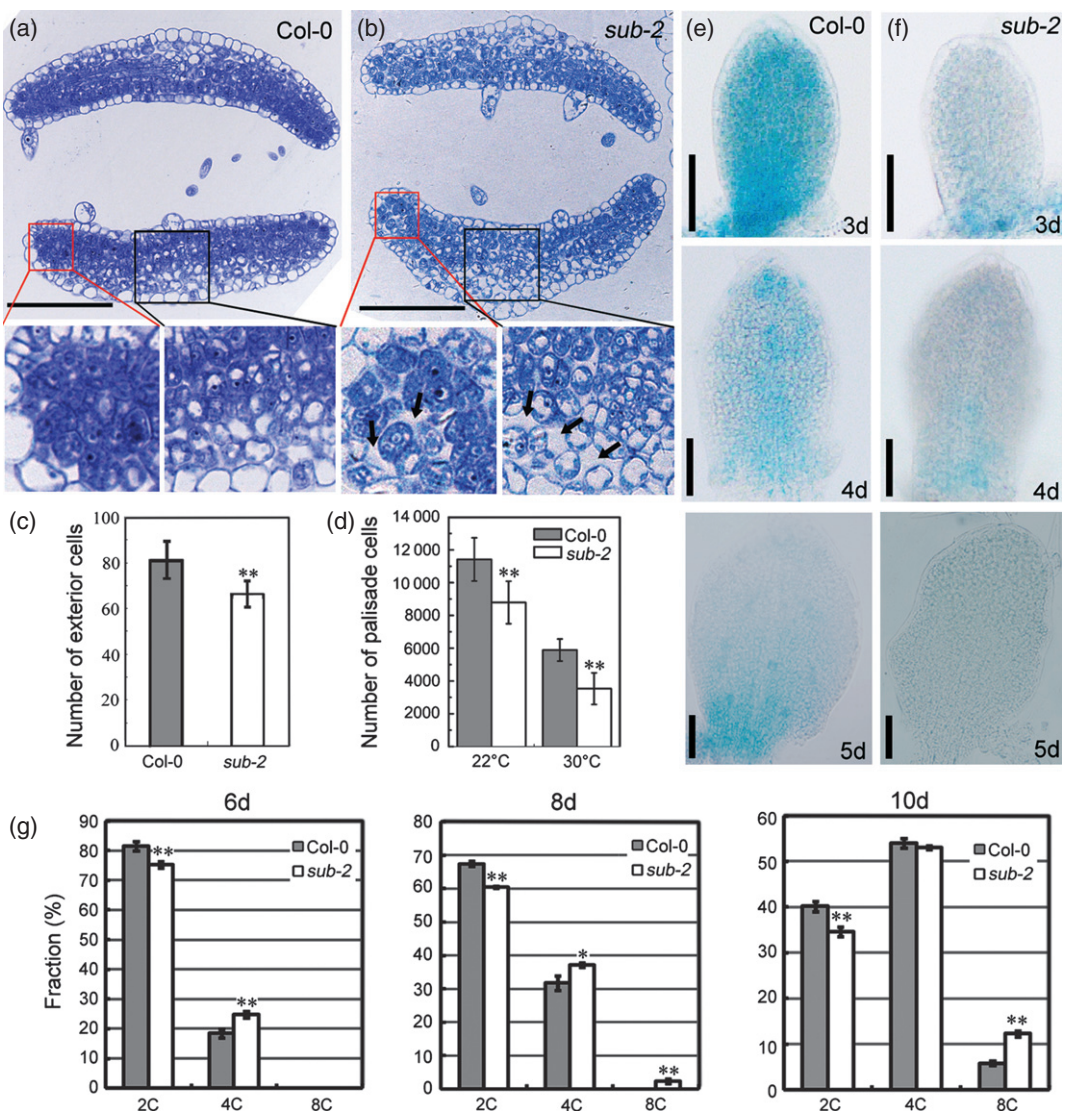

Figure 3. Mutation in *SUB* reduces cell proliferation.
(a,b) Transverse sections through leaves of 5-day-old Col-0 and *sub-2* seedlings grown at 30°C, showing the precociously expanding cells in lateral meristem regions (red boxes) and midrib regions (black boxes) of *sub-2*. Arrows indicate intercellular gaps.
(c) Number of exterior cells per leaf section in (a) and (b). Asterisks indicate statistically significant differences compared to wild-type by Student's *t* test (**$P \leq 0.001$; $n = 6$ for Col-0 and $n = 10$ for *sub-2*).
(d) Number of palisade cells in the first pair of leaves from 25-day-old Col-0 and *sub-2* plants. Asterisks indicate statistically significant differences compared to wild-type by Student's *t* test (**$P \leq 0.001$; $n \geq 20$).
(e,f) CYCB1;1–GUS activity in 3–5-day-old Col-0 and *sub-2* first leaves grown at 30°C. Note the weaker CYCB1;1–GUS expression in *sub-2* than Col-0.
(g) Distribution of nuclear ploidy in the first pair of leaves of 6-, 8- and 10-day-old seedlings grown at 30°C. Asterisks indicate statistically significant differences compared to wild-type by Student's *t* test (*$P \leq 0.01$;**$P \leq 0.001$; $n = 3$).
Scale bars = 100 μm (a,b) and 50 μm (e,f).

leaf of *sub-2* plants were reduced, indicating that cell proliferation was impaired during *sub-2* leaf development (Figure 3c,d).

During leaf development, basipetal exit of cell division can be visualized by accumulation of the cell-cycle marker CYCB1;1–GUS (Colon-Carmona *et al.*, 1999; Donnelly *et al.*, 1999). The CYCB1;1–GUS activity decreased earlier in *sub-2* plants than in Col-0 at both temperatures (Figure 3e,f and S4), consistent with the reduced cell number as shown in Figure 3(d). As transition from cell proliferation to cell expansion is coupled with the onset of endo-reduplication (Beemster *et al.*, 2005), an alternative cycle in which cells undergo rounds of DNA replication without division, we measured the DNA content using flow cytometry to examine cell growth in the developing first leaves. As shown in Figure 3(g), the proportions of 4C and 8C cells in *sub-2* leaves were prematurely increased, while that of 2C cells was reduced. These results indicate that the mutation in *SUB* results in reduced cell proliferation and earlier onset of endo-reduplication during leaf development.

Altered venation and auxin patterning in *sub-2*

During leaf development, the formation of leaf shape and venation pattern are temporally coordinated (Dengler and Kang, 2001; Scarpella *et al.*, 2010). We found that the complexity and continuity of *sub-2* venation was significantly reduced at 30°C, with fewer branching points and areoles but visible vascular islands (Figure 4a and Table 1). Similar to leaf shape, these defects were also alleviated at 22°C, although the venation complexity was still reduced because of the decreased number of higher-order veins in *sub-2* leaves (Figure 4b and Table 1). To examine the early vascular development, we introduced *ATHB8–GUS*, a marker for procambial cells (Baima *et al.*, 1995), into *sub-2* plants. Although expression of *ATHB8–GUS* showed no obvious difference between *sub-2* and Col-0 at leaf stage 1 (Figure 4c), the subsequent formation of continuous loops was disturbed in the *sub-2* leaves (Figure 4d,e). It is known that polar auxin transport plays important roles in venation patterning (Mattsson *et al.*, 1999; Sieburth, 1999; Mattsson *et al.*, 2003; Scarpella *et al.*, 2006). Expression of the auxin efflux carrier PIN-FORMED1 (PIN1) precedes expression of *ATHB8–GUS* in leaf primordia, and thus represents an earlier marker for procambium formation (Scarpella *et al.*, 2006). We used a functional PIN1–GFP reporter (Benkova *et al.*, 2003) to examine PIN1 expression. During formation of continuous veins in wild-type leaf primordia, the PIN1–GFP expression domain was broad at first but was gradually restricted to a narrow site and became connected to the midvein PIN1–GFP expression domain or other pre-existing PIN1–GFP expression domains (Scarpella *et al.*, 2006). In contrast, some PIN1–GFP expression domains were ectopic or even missing, or failed to extend in *sub-2* plants (Figure 4f), suggesting that the auxin flow may be interrupted. However, we found that induction of vascular continuity along leaf margin by naphthylphthalamic acid, an inhibitor of polar auxin transport, was decreased in the *sub-2* leaves at 30°C (Figure S5). These results indicate that both *SUB* and polar auxin transport are required for venation patterning.

We further examined the auxin response using a synthetic auxin reporter *DR5–GUS* (Ulmasov *et al.*, 1997). Expression of *DR5–GUS* in *sub-2* leaves was slightly weaker in both tip and vascular zones but increased in the ectopic hydathodes (Figure S6a,b). Exogenous application of indole-3-acetic acid (IAA) induced *DR5–GUS* expression in both Col-0 and *sub-2* seedlings, showing that auxin perception and response were normal in the *sub-2* mutant (Figure S6c,d). In addition, *AXR1* encodes an ubiquitin-activating enzyme E1 that is required for auxin signaling (Leyser *et al.*, 1993). Compared with the *sub-2* or *axr1-3* single mutant, the *sub-2 axr1-3* double mutant showed reduced complexity of venation patterning at both temperatures (Figure 4g,h and Table 1). Discontinuous veins were significantly increased and more vascular islands appeared in *sub-2 axr1-3* leaves, particularly at 30°C (Figure 4h and Table 1), suggesting that *SUB* acts synergistically with *AXR1* in venation patterning.

SUB-mediated early leaf patterning is critical for the final leaf shape

Leaf shape may be affected at various stages during leaf development. To determine the stage(s) that is severely affected by the *sub-2* mutation, we performed temperature-shift experiments (Figure 5). Both Col-0 and *sub-2* seedlings grown at 22°C were transferred to 30°C between 1 and 14 days after germination (DAG), and vice versa (experiments 22/30 and 30/22, respectively). The length and width of the first rosette leaf was measured at 16 DAG, and the leaf index was used as a parameter to reflect the degree of leaf defects. In both experiments, the leaf index of Col-0 plants was relatively steady and similar to that of seedlings continuously grown at 22 or 30°C. In contrast, the leaf index of *sub-2* seedlings shifted at between 1 and 7 DAG in the 22/30 shift experiment was significantly reduced, especially between 4 and 7 DAG (from 1.84 to 1.37; Figure 5a,b). In the 30/22 shift experiment, the *sub-2* mutant shifted at between 1 and 7 DAG displayed more severe defects the longer they were held at 30°C (Figure 5c,d). However, the leaf index of mutant seedlings shifted after 7 DAG changed little in either experiment. Based on an analysis of leaf developmental stages at the time of shifting (Figure S7), we conclude that *SUB* predominantly functions in the early stage of primary morphogenesis during leaf development (leaf stages 1–4).

The expression pattern of *SUB* during leaf development

To examine the expression pattern of *SUB* during leaf development, promoter activity was analyzed using the *proSUB:GUS* reporter line (Figure 6a–o). The GUS signal was weak in the shoot apical meristem but increased

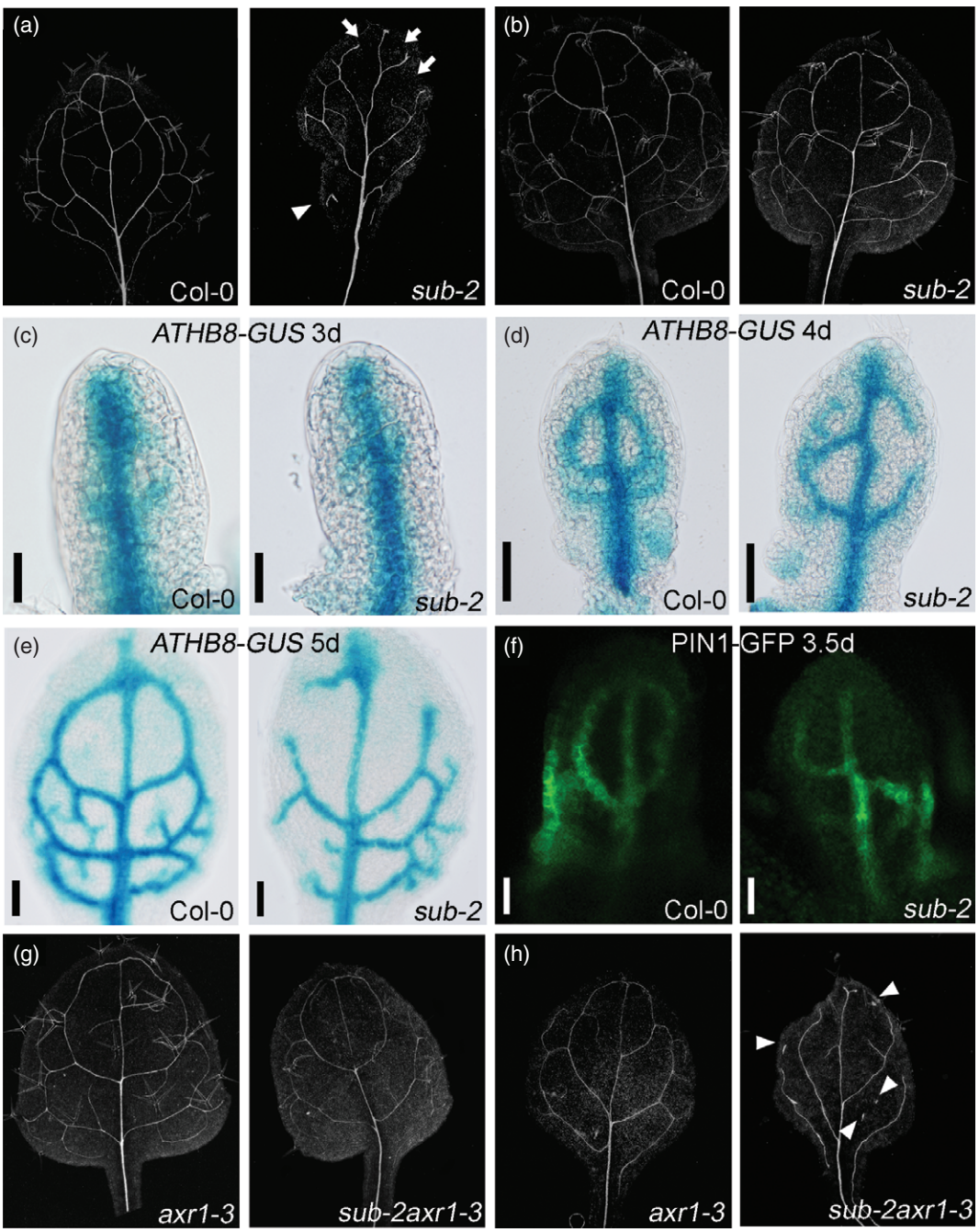

Figure 4. Altered venation patterning in *sub-2* leaves.
(a,b) Venation patterning of the first leaves from 12-day-old seedlings grown at 30°C (a) and 22°C (b). Note the open loops (arrows) and vascular island (arrowhead) in *sub-2* plants grown at 30°C.
(c–e) *ATHB8–GUS* expression in 3–5-day-old first leaves at 30°C, indicating disrupted formation of venation loops in *sub-2* leaves.
(f) PIN1–GFP signals in 3–5-day-old first leaves at 30°C, showing ectopic formation of PIN1–GFP expression domains in *sub-2* leaves.
(g,h) Cleared first leaves of *axr1-3* and *sub-2 axr1-3* seedlings grown at 22°C (g) and 30°C (h). Vascular islands increased in *sub-2 axr1-3* leaves at 30°C (arrowheads).
Scale bars = 20 μm (c,f) and 50 μm (d,e).

throughout young primordia, with higher activity in provascular tissues and the adaxial–abaxial juxtaposition region. During leaf expansion, GUS expression was basipetally reduced and finally restricted to vascular cells that were still dividing and differentiating.

To further examine the accumulation of SUB protein during leaf development, we generated transgenic plants expressing *SUB–YFP* under the control of a native *SUB* promoter (*proSUB:SUB-YFP*) in the *sub-2* background. The SUB–YFP fluorescence signal was very weak in the leaf

Table 1 Analysis of venation pattern in the first leaves of 12-day-old *sub-2*, *axr1-3* and *sub-2 axr1-3* plants

	Genotype	Number of areoles	Number of branching points	Number of island veins	*n*
22°C	Col-0	10.65 ± 1.99	26.30 ± 3.70	0	15
	sub-2	**7.17 ± 1.95**	**19.50 ± 3.09**	0	12
	axr1-3	**6.47 ± 1.68**	**16.07 ± 3.43**	0	15
	sub-2 axr1-3	***4.15 ± 1.04***	***12.50 ± 2.59***	0	20
30°C	Col-0	9.90 ± 1.66	25.60 ± 5.40	0	10
	sub-2	**2.50 ± 1.08**	**11.80 ± 2.49**	**0.50 ± 0.84**	10
	axr1-3	**4.88 ± 1.25**	**13.88 ± 2.75**	0	8
	sub-2 axr1-3	***1.90 ± 1.66***	***9.00 ± 2.16***	***1.10 ± 1.29***	10

Values represent means ± SD. Values that are significantly different from Col-0 are indicated in bold; values for double mutants that are significantly different from the single mutant are indicated in italics (Student's *t* test, $P \leq 0.01$).

primordia of most transgenic plants, although the leaf phenotypes of *sub-2* were rescued. We thus selected complemented plants with strong YFP signal but reduced leaf size, which resemble the phenotypes of weak *SUB*-OE plants (see below), for analysis. Consistent with the GUS activity of *proSUB:GUS*, SUB–YFP accumulation in young primordia was basipetally diminished during leaf development and became restricted to developing vascular cells (Figure 6p–r). In addition, SUB–YFP accumulated in the cell periphery of epidermal tissues (Figure 6s), similar to previous reports in root and sepal epidermal cells (Kwak and Schiefelbein, 2008; Yadav *et al.*, 2008). Interestingly, accumulation of SUB–YFP was predominant at the junctions of two neighboring procambial cells (arrows in Figure 6t), suggesting a potential polar localization of SUB during vascular differentiation. These results indicate that *SUB* expression is temporally and spatially regulated during leaf development, and the expression pattern coincides with its roles in early leaf development and vascular formation.

Constitutive expression of SUB inhibits leaf development

To learn more about SUB function, we generated transgenic plants expressing *SUB* under the control of the CaMV 35S promoter in the Col-0 background. Among 86 independent *SUB* over-expression (*SUB*-OE) T_1 plants, 54 lines showed varying degrees of dwarfing and reduced organ size (Figure 7a,b). Real-time RT-PCR analysis showed that SUB acts in a dose-dependent manner, and the individuals with strong phenotypes accumulated more *SUB* transcripts (Figure 7c). In severe cases, growth of almost all the organs was inhibited from seedling stage to adult stage (Figure 7a,b and S8).

We then examined the cellular basis of impaired leaf development in the *SUB*-OE plants with strong phenotypes. Compared with the fully expanded cells in Col-0 (Figure 7d,g,i), a large number of small cells were clustered together in the epidermal and internal tissues of *SUB*-OE leaves (Figure 7e,h,j). Both cell size and the number of single-layer palisade cells were decreased (Figure 7m–o), and the number of cell layers between adaxial and abaxial epidermis was increased in the *SUB*-OE leaves (Figure 7h). Some smaller cells were observed in both epidermis and internal tissues, suggesting that progression of cell growth was inhibited and was less synchronous in *SUB*-OE plants than in Col-0 (arrows in Figure 7f,h,j). In addition, development of vascular tissues was also inhibited in *SUB*-OE leaves

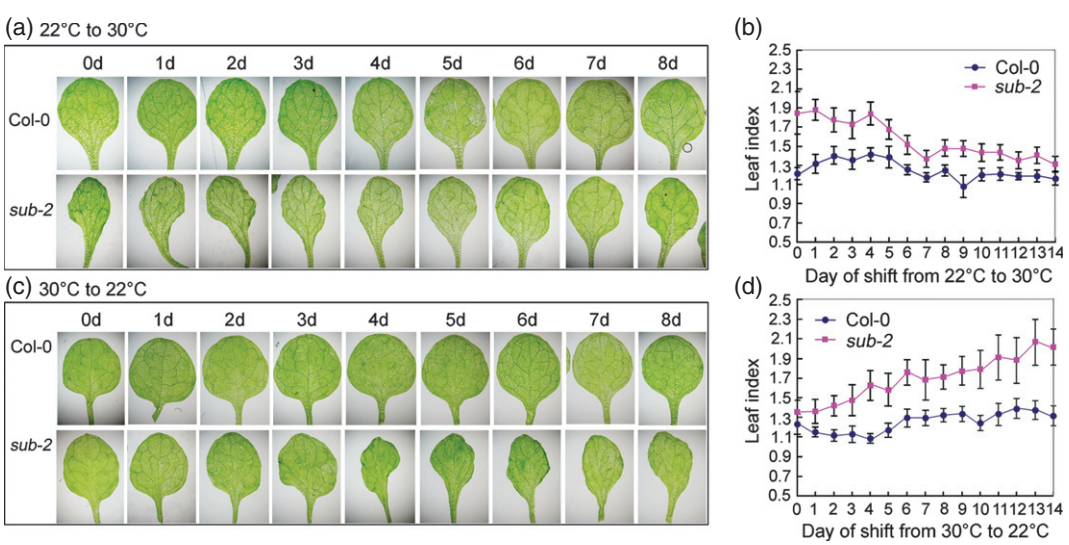

Figure 5. Temperature-shift experiments show the role of *SUB* in early leaf patterning.
(a) Representative first leaves of 16-day-old plants shifted at between 1 and 8 DAG in the 22/30 experiment.
(b) Analysis of leaf index in the 22/30 experiment. Values are means ± SD ($n \geq 12$).
(c) Representative first leaves of 16-day-old plants shifted at between 1 and 8 DAG in the 30/22 experiment.
(d) Analysis of leaf index in the 30/22 experiment. Values are means ± SD ($n \geq 12$).

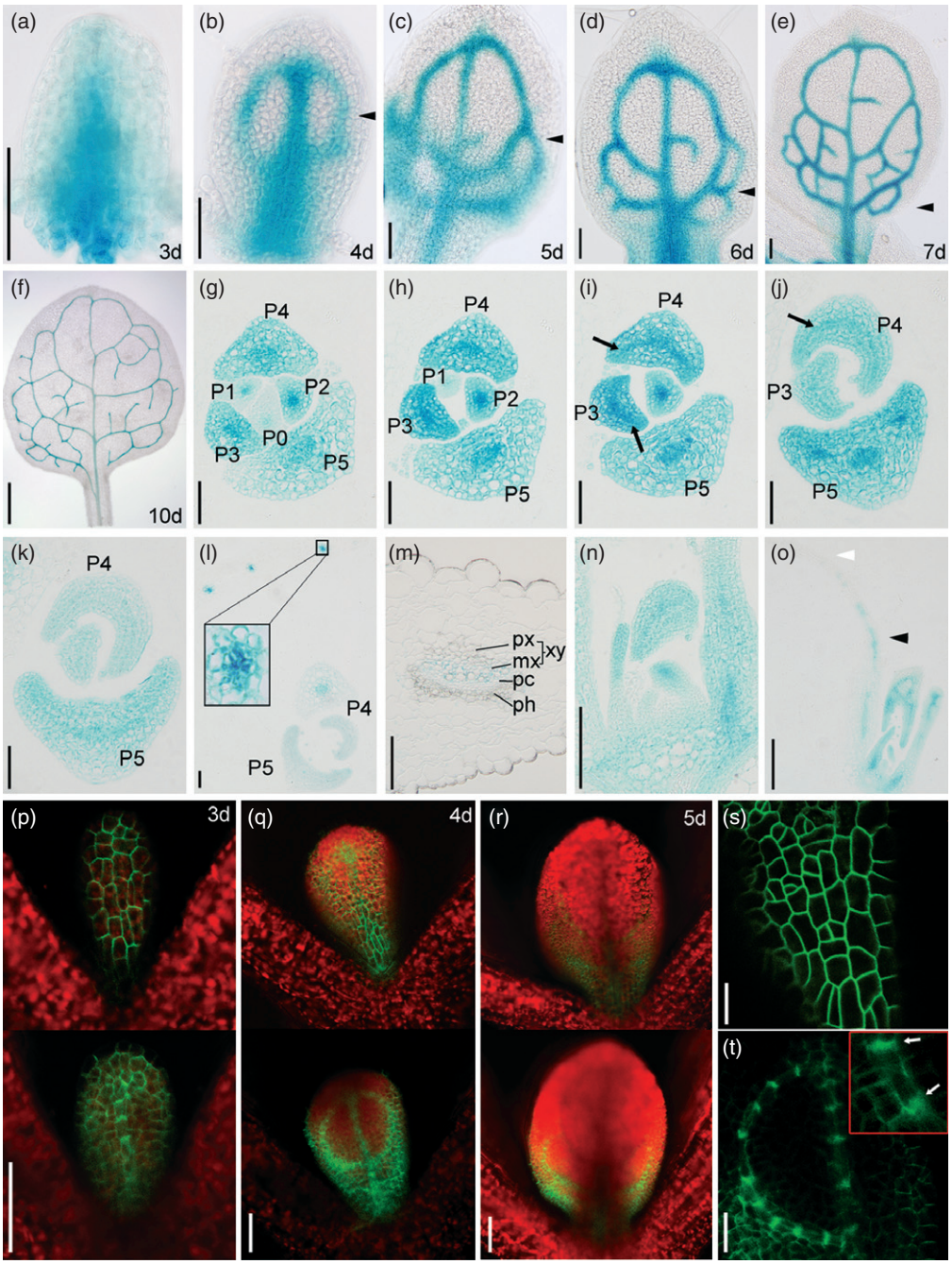

Figure 6. Spatio-temporal expression of *proSUB:GUS* and *proSUB:SUB-YFP* during leaf development.
(a–f) GUS staining of the first leaves at 3-7 DAG (a–e) and 10 DAG (f). Arrowheads in (b–e) indicate the transition regions of *SUB* expression in lamina.
(g–l) Representative images of transverse serial sections through the shoot apex of 12-day-old transgenic seedlings from the base (g) to the tip (l). The GUS signals were strong throughout young primordia (P0–P3) and gradually reduced in the distal part of older primordia [P4 and P5 in (k) and (l)]. Higher levels were detected at the adaxial–abaxial boundary [black arrows in (i) and (j)] and the developing vasculature [inset in (l)].
(m) Transverse section through the midrib of the fifth leaf from 28-day-old plants. GUS activity was evident in metaxylem (mx) and procambial (pc) cells, but weak in other vascular cells. xy, xylem; px, protoxylem; ph, phloem.
(n,o) Longitudinal sections through the shoot apex. GUS activity faded from the leaf tip (white arrowhead) to the basal part (black arrowhead) in late-stage leaf primordia (o).
(p–r) SUB–YFP expression in 3–5-day-old primordia. Signals (green) were detected in the abaxial epidermal view (upper) or median view (lower).
(s) SUB–YFP expression in abaxial epidermal cells.
(t) SUB–YFP expression in procambial cells showing predominant accumulation at the junctions of neighboring cells (arrows in inset).
Scale bars = 50 μm (a–e,g–l,p–r), 200 μm (m–o), 0.5 mm (f) and 20 μm (s,t).

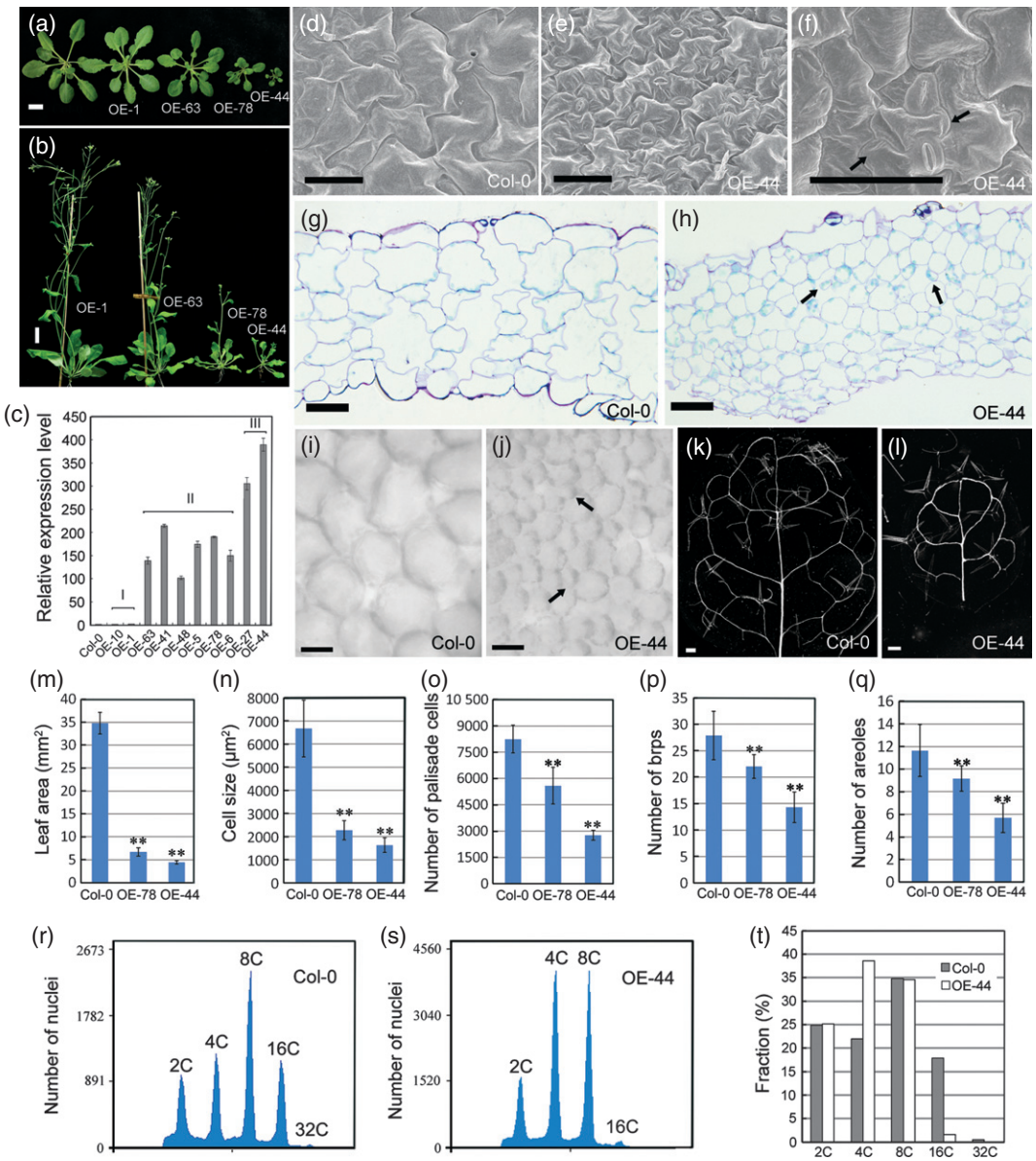

Figure 7. Constitutive expression of *SUB* inhibits leaf development.
(a,b) Twenty-eight-day-old rosettes (a) and 7-week-old plants (b) of representative *SUB*-OE lines (OE-1, OE-44, OE-63 and OE-78).
(c) Quantitative RT-PCR analysis of *SUB* expression in Col-0 and *SUB*-OE lines with various severity levels (I–III).
(d–f) Abaxial epidermis of first leaves from 28-day-old Col-0 and OE-44 plants. Arrows indicate stomata precursor cells.
(g,h) Transverse sections showing the reduced cell size and additional cell layers in OE-44 leaves. Arrows indicate the intermingled small cells in OE-44.
(i,j) Smaller and uneven palisade cells (arrows) in the OE-44 first leaf (j), compared with a Col-0 leaf (i).
(k,l) Venation pattern of the first leaves showing the reduced venation complexity in OE-44 (l) compared with Col-0 (k).
(m–q) The leaf area (m), cell size of palisade cells (n), number of palisade cells (o), number of vascular branching points (p) and number of areoles (q) were measured on the first leaves of Col-0 and *SUB*-OE lines. Values are means ± SD. Asterisks indicate statistically significant differences compared to wild-type by Student's *t* test (**$P \leq 0.001$; $n \geq 12$).
(r–t) Ploidy distributions of the first leaves from 16-day-old Col-0 (r) and OE-44 (s) plants. The quantification result is shown in (t).
Scale bars = 1 cm (a,b), 50 μm (d–j) and 100 μm (k,l).

with reduced complexity (Figure 7k,l,p,q). Nuclear ploidy analysis showed that the population of 4C cells was increased, but the populations of 16C and 32C cells were greatly reduced in the leaves of *SUB*-OE plants compared with Col-0 (Figure 7r–t), indicating that endo-reduplication was inhibited in the leaves of *SUB*-OE plants. Together with the cellular observations, these results show that constitutive expression of *SUB* restricts organ growth by inhibiting progression from cell proliferation to cell expansion, in contrast to the loss-of-function mutant.

DISCUSSION

The hidden roles of *SUB* in leaf patterning

Although *SUB* was previously shown to be required for pattern formation in root epidermis and floral organ development (Chevalier *et al.*, 2005; Kwak *et al.*, 2005), its roles in leaf development have not been reported. We observed that several leaf patterning processes were affected in the *sub* mutant at high ambient temperature, suggesting that either SUB has specialized functions in distinct developmental processes or SUB is involved in a general aspect of leaf development such as cell patterning. As a developmental trigger, the phytohormone auxin has been recognized to modulate organ growth and patterning formation (Benkova *et al.*, 2003; Zgurski *et al.*, 2005; Scarpella *et al.*, 2006, 2010; Bilsborough *et al.*, 2011). The altered expression pattern of DR5–GUS and PIN1–GFP in *sub-2* plants suggests that SUB may coordinate multiple processes together with the auxin-mediated pathway.

An intriguing question is why loss-of-function mutations in the *SUB* gene cause severe leaf developmental defects under high temperature. One possibility is that SUB-mediated early leaf patterning processes are sensitive to high temperature. The temperature sensitivities of several leaf developmental mutants such as *as1* and *as2* (Qi *et al.*, 2004), *phantastic* (Waites and Hudson, 1995; Waites *et al.*, 1998) and *varicose* (Deyholos *et al.*, 2003) support this possibility. Alternatively, other genes that are functionally redundant with *SUB* may complement the *sub-2* phenotypes at 22°C but not at 30°C. SUB belongs to an LRR-V RLK sub-family with nine members whose expression in rosettes has been detected to varying extents (Eyuboglu *et al.*, 2007). The functions and temperature responses of other members remain to be investigated.

SUB coordinates cell proliferation, differentiation and leaf development

During leaf development, expression of *SUB* is initially ubiquitous in the young leaf primordia, then basipetally reduced along the lamina and finally restricted to the leaf veins (Figure 6). Our results demonstrated that precise spatio-temporal expression of *SUB* is important for cell proliferation and differentiation/expansion during leaf development. First, high expression of *SUB* in young leaf primordia is required for cell proliferation, as the cell number and CYCB1;1–GUS activity were largely reduced in *sub-2* leaves. Reduced cell proliferation in *sub-2* plants may contribute to incomplete lateral organs and reduced leaf outgrowth, similar to that in *jag*, *trn1* and *trn2* mutants (Dinneny *et al.*, 2004; Ohno *et al.*, 2004; Cnops *et al.*, 2006). Second, the early expanded cells in the *sub-2* leaves appear not to differentiate well later, given the mis-shapen epidermal and mesophyll cells in mature leaves. This suggests that SUB mediates a differentiation signal for cell expansion in both epidermal and internal tissues. Third, timely arrest of *SUB* expression in differentiating cells is also important for subsequent organ growth. Constitutive expression of *SUB* inhibited cell expansion and endo-reduplication. Fourth, in contrast to the *AN* and *ROT* genes that modulate cell proliferation or expansion along specific polarity axes (Kim *et al.*, 1998, 2002; Narita *et al.*, 2004), cell number was reduced in the horizontal direction and increased along the adaxial–abaxial axis of *SUB*-OE leaves, indicating intricate spatiotemporal regulation of cell proliferation during leaf development. Finally, expression of *SUB* also maintains the synchronous progression of cell proliferation or expansion in bilateral leaf primordia. Mutation of *SUB* results in asymmetric cell growth (Figure 3b), which may partially contribute to the asymmetric leaf shape (Semiarti *et al.*, 2001; Zgurski *et al.*, 2005; Cnops *et al.*, 2006).

Our temperature-shift experiments indicated that SUB predominantly functions in the early stage of leaf development. As *SUB* encodes an RLK, we propose that SUB may mediate a developmental stage-specific signal to coordinate cell proliferation and differentiation for leaf patterning. It has been recognized that RLKs modulate cell proliferation and differentiation in a wide range of growth and developmental processes (De Smet *et al.*, 2009). For example, the ERECTA RLK family members control aerial organ shape and size primarily by promoting cell proliferation, and specify stomatal patterning by repressing asymmetric cell divisions during guard-cell differentiation (Shpak *et al.*, 2003, 2004, 2005; Pillitteri *et al.*, 2007). Our results also show that SUB promotes cell proliferation and participates in vascular differentiation during leaf development. These findings suggest a dual function of these RLKs in cell proliferation and patterning of specialized cells during organ growth. The BARELY ANY MERISTEM 1 (BAM1), BAM2 and BAM3 RLKs are required for leaf development (DeYoung *et al.*, 2006). Similar to *sub-2*, the *bam1 bam2* and *bam1 bam2 bam3* mutants exhibit reduced leaf size, asymmetric leaf shape and venation patterning (DeYoung *et al.*, 2006), indicating functional redundancy of these RLKs for leaf patterning. Recently, direct interaction of peptide ligands and ERECTA family receptors was demonstrated for specification of stomatal patterning (Lee *et al.*, 2012). In addition, overexpression of a small peptide ROT4 also decreases cell proliferation and alters leaf shape (Narita *et al.*, 2004). These findings indicate the existence of peptide-mediated signaling pathways for leaf development. Further determination of SUB-interacting proteins or ligands will shed light on the molecular mechanism of RLK-mediated leaf development.

Roles of SUB in vascular development

Our results showed that venation patterning was altered in *sub-2* leaves, consistent with strong expression of *SUB* in leaf veins. The simultaneous alteration of venation patterning and leaf shape in *sub-2* indicates a strong connection

between the two developmental processes. Auxin has been proposed as an important modulator for both leaf blade development and venation patterning (Scarpella *et al.*, 2010). It has also been shown that auxin maxima at the leaf margin and the PIN1 protein-mediated polar auxin transport in marginal epidermal tissues play important roles in positioning vascular strands (Scarpella *et al.*, 2006). The overlapping functions of auxin and SUB and the irregular leaf margin in *sub-2* suggest that SUB may coordinate venation patterning and leaf shape by directly or indirectly modulating auxin patterning at the leaf margin. However, the impaired venation in *sub-2* may not be the consequence of altered leaf shape, because the complexity of venation was still reduced in *sub-2* leaves with the nearly normal leaf shape at 22°C. SUB strongly accumulates in developing procambial cells (Figure 6t), and the number of vascular cells was reduced in both leaves (Figure 2u) and stems (Chevalier *et al.*, 2005) of *sub* mutants. These findings suggest a direct role for SUB in vascular differentiation. Thus, SUB may be required for vascular patterning throughout the lamina and within vascular bundles.

During leaf development, expansion/differentiation of mesophyll cells may interfere with extension of procambial domains (Scarpella *et al.*, 2004). Decreased cell proliferation in leaves also reduced the complexity of the venation pattern (Kang *et al.*, 2007). Consistent with these findings, *sub-2* exhibited impaired vascular development as well as reduced cell proliferation and premature cell expansion. However, the venation complexity was also reduced in *SUB*-OE leaves in which cell expansion was inhibited. Similar phenotypes were also observed in our previous analysis with gibberellin-deficient plants (Zhang *et al.*, 2011), suggesting the importance of the balance between cell proliferation and expansion/differentiation for vascular formation. It was shown that a high auxin level in developing leaves is strongly correlated with high cell-division activity, whereas a low auxin level triggers the mitosis to endo-reduplication transition (Ljung *et al.*, 2001; Ishida *et al.*, 2010). As the genetic analysis showed that *SUB* acts synergistically with *AXR1* in venation patterning (Figure 4g,h), auxin and SUB RLK-mediated signaling pathways probably together control the balance between cell proliferation and differentiation for vascular development.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

The Arabidopsis *sub-2* mutant in the Col-0 background was isolated from an EMS-mutagenized mutant pool (Yan *et al.*, 2006). Seeds of *scm-2* (SALK_086357C) were obtained from the Arabidopsis Biological Resource Center. The marker lines *ATHB8–GUS* (Col-0) and *PIN1–GFP* (Col-0) were obtained from the Nottingham Arabidopsis Stock Centre (identification numbers are N296 and N9362, respectively). Seeds of *sub-2* and *sub-3* in the Landsberg *erecta* (L*er*) background (Chevalier *et al.*, 2005), and the reporter lines *DR5–GUS* (Col-0) and *CYCB1;1–GUS* (Col-0) were kindly provided by Kay Schneitz (Plant Developmental Biology, Science Center Weihenstephan, Technical University of Munich), Hai Huang and Jirong Huang (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences), respectively. The reporters were introduced into *sub-2* (Col-0) by crossing. Seeds were surface-sterilized and sown on half-strength Murashige and Skoog medium with 0.7% agar. After 3–4 days at 4°C in the dark, plates were transferred to a growth chamber at 20–22°C or 28–30°C under light (120 μmol m^{-2} sec^{-1}) with a 16 h light/8 h dark cycle. At 6 DAG, seedlings were transferred to the soil.

Plasmid constructions and Arabidopsis transformation

The primers used for cloning are listed in Table S1. For complementation, the 8.4 kb genomic fragment containing the full-length *SUB* gene plus 3430 bp upstream and 406 bp downstream sequences was released and cloned into the vector pCAMBIA1301 (GenBank accession number AF234297), resulting in p1301-gSUB. The 3056 bp promoter of *SUB* was amplified using primers pSUB(-*Sal*I)-F and pSUB(*Sma*I)-R, and cloned into vector pBI101.1 (GenBank accession number U12639.1) to generate the *proSUB:GUS* reporter. For the *proSUB:SUB-YFP* construct, two fragments including the coding region and introns without the stop codon were amplified from genomic DNA using primers gSUBma(*Kpn*I) F and gSUBa(*Bam*HI)-R, and gSUBb-F and gSUBmb(*Bam*HI)-R, and then fused with YFP. 3′ UTR sequence of *SUB* amplified using UTR(SacI)-F and UTR(EcoRI) primers, fusion fragment of *gSUB-YFP*, and the 3430 bp *SUB* promoter were sequentially cloned into a pCAMBIA1301 vector, generating *proSUB:SUB-YFP*. For the over-expression construct, two genomic fragments were amplified using primers gSUBa(*Kpn*I)-F and gSUBa(*Bam*HI)-R, and gSUBb-F and gSUBb(*Bam*HI)-R, and then sequentially placed downstream of the 35S promoter in the overexpression vector pCAMBIA1300S (provided by Yinong Yang, Department of Plant Pathology, the Pennsylvania State University). All the constructs were introduced into Arabidopsis plants by the *Agrobacterium tumefaciens*-mediated transformation procedure (Clough and Bent, 1998).

Microscopy and histology

Leaf morphology and cell number were analyzed as described by Horiguchi *et al.* (2006). The images were photographed using an Olympus SZX7 stereomicroscope or BX51 compound microscope (http://www.olympus.com/). Leaf length, width and size were measured using ImageJ software (http://www.rsb.info.nih.gov/ij). Scanning electron microscopy, confocal laser-scanning microscopy and preparation of half-thin resin sections were performed as described by Zhang *et al.* (2011). To examine venation patterning, leaves were fixed in FAA solution (3.7% formaldehyde, 5% acetic acid, and 50% ethanol), and then incubated with clearing solution (80 g chloral hydrate, 30 ml water, 10 ml glycerol) overnight. The cleared leaves were photographed under dark-field illumination. Histochemical detection of GUS activity was performed as described by Scarpella *et al.* (2004). The samples were incubated in staining solution containing ferricyanide (3 mM for *ATHB8–GUS*; 1 mM for *proSUB:GUS*; 0.5 mM for *DR5–GUS* and *CYCB1;1–GUS*) at 37°C for 2 h (*ATHB8–GUS* and *proSUB:GUS*) or overnight (*DR5–GUS* and *CYCB1;1–GUS*) before clearing.

For serial sections, 12-day-old seedlings expressing *proSUB:GUS* were fixed in 100 mM sodium phosphate buffer (pH 7.0) containing 0.5% formaldehyde and 0.1% Triton X-100 for 45 min, rinsed three times for 5 min each in 100 mM sodium phosphate, and then incubated in staining solution containing 1 mM ferricyanide for 6 h at 37°C. After washing for 5 min with the phosphate buffer, the

seedlings were fixed in FAA solution overnight, dehydrated via a series of ethanol gradients followed by substitution with xylene, and then embedded in Paraplast (Sigma-Aldrich, http://www.sigmaaldrich.com/). Serial 7 μm thick sections were cut using a rotary microtome.

Flow cytometric analysis

Ploidy levels were measured using a MoFlo XDP flow cytometer (Beckman Coulter, http://www.beckmancoulter.com/). The nuclei were released using 4′,6-diamidino-2-phenylindole (DAPI)-containing nuclear isolation medium (NIM-DAPI 10, Beckman Coulter) by chopping fresh leaves with a razor blade followed by filtration through a 40 μm cell strainer (BD Falcon, http://www.bdbiosciences.com/). For each sample, at least 10 000 nuclei from approximately 30 leaves were counted. Each assay was repeated at least twice with similar results.

RNA isolation and real-time RT-PCR

Total RNAs were extracted from the shoots of 4-week-old *SUB*-OE and Col-0 plants using TRIzol reagent (Invitrogen, http://www.invitrogen.com/). cDNAs were synthesized from 3 μg total RNAs using oligo(dT) primer and SuperScript III reverse transcriptase according to the manufacturer's instructions (Invitrogen). Real-time RT-PCR analysis was performed using SYBR Premix Ex Taq (TaKaRa, http://www.takara.com.cn/) and gene-specific primers (Table S1).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the on-line version of this article:

Figure S1. Map-based cloning of the *SCM*/*SUB* gene.

Figure S2. Temperature-sensitive phenotypes of the *sub* mutants in the L*er* background.

Figure S3. Time course of early leaf growth.

Figure S4. CYCB1;1–GUS activity in Col-0 and *sub-2* leaves at 22°C.

Figure S5. Venation pattern in naphthylphthalamic acid-treated *sub-2* leaves.

Figure S6. *DR5–GUS* expression in Col-0 and *sub-2* seedling with or without exogenous indole-3 acetic acid treatment.

Figure S7. Analysis of leaf developmental stage at the time of temperature shift.

Figure S8. Comparison of wild-type Col-0 and *SUB*-OE seedlings.

Table S1. Primers used in this study.

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