SAUR17 and SAUR50 Differentially Regulate PP2C-D1 during Apical Hook Development and Cotyledon Opening in Arabidopsis

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Following germination in the dark, Arabidopsis (Arabidopsis thaliana) seedlings undergo etiolation and develop apical hooks, closed cotyledons, and rapidly elongating hypocotyls. Upon light perception, the seedlings de-etiolate, which includes the opening of apical hooks and cotyledons. Here, we identify Arabidopsis Small Auxin Up RNA17 (SAUR17) as a downstream effector of etiolation, which serves to bring about apical hook formation and closed cotyledons. SAUR17 is highly expressed in apical hooks and cotyledons and is repressed by light. The apical organs also express a group of light-inducing SAURs, as represented by SAUR50, which promote hook and cotyledon opening. The development of etiolated or de-etiolated apical structures requires asymmetric differential cell growth. We present evidence that the opposing actions of SAUR17 and SAUR50 on apical development largely result from their antagonistic regulation of Protein Phosphatase 2C D-clade 1 (PP2C-D1), a phosphatase that suppresses cell expansion and promotes apical hook development in the dark. SAUR50 inhibits PP2C-D1, whereas SAUR17 has a higher affinity for PP2C-D1 without inhibiting its activity. PP2C-D1 predominantly associates with SAUR17 in etiolated seedlings, which shields it from inhibitory SAURs such as SAUR50. Light signals turn off SAUR17 and upregulate a subgroup of SAURs including SAUR50 at the inner side of the hook and cotyledon cells, leading to cell expansion and unfolding of the hook and cotyledons.

INTRODUCTION

Seeds often germinate when buried in the dark in soil. To cope with this condition, dicotyledonous plants such as Arabidopsis (Arabidopsis thaliana) have adopted a developmental strategy known as skotomorphogenesis or etiolation. In this program, the young seedling rapidly elongates its hypocotyl upward, and its cotyledons are tightly closed under an apical hook (Von Arnim and Deng, 1996; Fankhauser and Chory, 1997). The apical hook and folded cotyledons protect the shoot apical meristem from mechanical damage as the seedling penetrates the soil. Once the apical organs emerge and perceive light, de-etiolation occurs as the apical hook unfolds, opens, and separates the two cotyledons to initiate the transition to autotrophic growth (Arsovski et al., 2012; Gommers and Monte, 2018). Etiolated development is essential for plant growth under natural environmental conditions, as it allows the seedling to successfully emerge from the soil and survive the dark-to-light transition (Gallego-Bartolomé et al., 2011; Zhong et al., 2014; Shi et al., 2016).

The formation of an apical hook and its unfolding, as well as cotyledon opening (or cotyledon separation), result from differential cell growth. Extensive studies on apical hook development have revealed a dynamic spatial-temporal process that is elaborately controlled by multiple phytohormones and light signals (Abbas et al., 2013; Mazzella et al., 2014; Wang and Guo, 2019). Based on time-lapsed imaging analysis of Arabidopsis seedlings, the apical hook is established during the initial 1.5 d after germination in the dark (the hook formation phase). This is followed by a hook maintenance phase, and near the end of day 3, the hook starts to slowly open in darkness (the hook opening phase; Raz and Ecker, 1999; Smet et al., 2014; Zhu et al., 2017). Apical hook formation results from asymmetric growth of the cells on the opposite sides of the apical hypocotyl (Silk and Erickson, 1978; Raz and Ecker, 1999). This growth is largely driven by the asymmetric distribution of auxin to the concave side of the hook, causing differential inhibition of cell elongation (Kuhn and Galston, 1992; Žádníková et al., 2016; Cao et al., 2019). Auxin synthesis, distribution, and responses are supported and regulated by other...
phytohormones including ethylene, gibberellins, and brassinosteroids (Li et al., 2004; Vandenbussche et al., 2010; Gallego-Bartolomé et al., 2011; Abbas et al., 2013). In particular, ethylene signals the presence of mechanical pressure from the soil to plants in darkness, which causes an exaggerated apical hook to form and intensifies the etiolation program of apical organs (Guzmán and Ecker, 1990; Zhong et al., 2014). The course of hook development in the dark is promptly disrupted by light exposure, which triggers the hook to fully open within a few hours (Liscum and Hangarter, 1993; Dong et al., 2019).

Less is known about the regulation of cotyledon morphology. In the seed, the two cotyledons are closed together, and they remain closed when the seed germinates in darkness and the seedling becomes etiolated (Wei et al., 1994). The exposure of etiolated seedlings to light triggers the simultaneous opening of cotyledons and hooks, although the two events are not dependent on each other (Liscum and Hangarter, 1993). Apical hook and cotyledon development are regulated by an overlapping yet nonidentical set of regulators, including the phytochrome-interacting factors, ethylene-responsive factors EIN3/EIL1, auxin response factors, brassinosteroid signaling transcription factors, and TCP transcription factors (Li et al., 2004; Leivar et al., 2008; Shin et al., 2009; Zhang et al., 2013; Shi et al., 2018; Zhang et al., 2018; Chen et al., 2019; Dong et al., 2019). These transcription factors regulate a large number of downstream target genes, many of which encode effectors that directly participate in cellular processes such as cell expansion, cell division, and cell wall reconfiguration. Although much is known about these transcription factor networks, our understanding of their downstream effectors that ultimately determine the shape and structure of the hook and cotyledons remains fragmented.

The Small Auxin Up RNA (SAUR) gene family has 79 members in Arabidopsis. This family of plant-specific cellular effectors modulates the growth and development of essentially every plant organ throughout its life cycle (Ren and Gray, 2015; Stortenbeker and Bemer, 2019). SAUR genes were originally identified in soybean (Glycine max) based on their ability to rapidly respond to auxin treatment (McCure and Guilfoyle, 1987). In fact, SAUR genes respond not only to auxin but also to light, brassinosteroids, ethylene, gibberellins, abscisic acid, and high temperature, among other stimuli (Ren and Gray, 2015). Because their mRNAs and proteins tend to be expressed in specific tissues and short lived (Newman et al., 1993; Chae et al., 2012; Spartz et al., 2012; Li et al., 2015); SAUR genes are capable of mounting dynamic spatial-temporal responses according to internal and environmental cues.

The best-known function of SAUR proteins is promoting cell expansion. This function underlies the role of several SAURs in elongation growth of hypocotyls and stamens, as well as tropic growth and leaf size (Chae et al., 2012; Spartz et al., 2012; Kong et al., 2013; Li et al., 2015; Sun et al., 2016; Bemer et al., 2017; van Mourik et al., 2017; Wang et al., 2020). The asymmetric localization of SAURs causes differential cell growth, leading to the bending of stems or roots during tropic growth or changes in plant architecture (Kong et al., 2013; Ren and Gray, 2015; Wang et al., 2020). For example, SAUR10 is strongly expressed in the abaxial epidermal layer of branches where it promotes cell elongation to trigger vertical branch growth (Bemer et al., 2017). In a study of light-regulated genes in separated hypocotyl and cotyledon tissues, Sun et al. (2016) identified 32 SAUR genes as light-induced in cotyledons and/or repressed in hypocotyls SAURs (lirSAURs) that potentially mediate differential growth as plants undergo the dark-to-light transition, or de- etiolation. Among these, SAUR16 and SAUR50 have been demonstrated to facilitate light-induced cotyledon opening (Dong et al., 2019).

A general mechanism by which SAURs promote cell expansion has been elucidated. Spartz et al. (2014) showed that SAUR19 binds to Protein Phosphatase 2C D-clade 1 (PP2C-D1) at the C-terminal conserved motif in its phosphatase domain (Wong et al., 2019) and inhibits the phosphatase activity. This leads to increased phosphorylation and activation of Arabidopsis H^+-ATPases (AHAs; Spartz et al., 2014). This, in turn, results in acidification of the apoplasm, ultimately leading to loosening of the cell wall as well as greater water uptake to increase the turgor pressure, and consequently an increase of cell size (Spartz et al., 2017). In addition to SAUR19, other SAUR proteins including SAURB, SAUR14, SAUR40, SAUR50, SAUR65, and SAUR72 can also bind to and inhibit PP2C-D1 (Spartz et al., 2014; Sun et al., 2016). Nonetheless, not all SAUR proteins promote cell expansion. SAUR32 and SAUR36 have been postulated to negatively regulate cell growth in hypocotyls and leaves, respectively (Park et al., 2007; Hou et al., 2013), although the underlying mechanism remains unknown.

The PP2C-D subfamily in Arabidopsis consists of nine members with diverse expression patterns (Ren et al., 2018). PP2C-D2, PP2C-D5, and PP2C-D6 are expressed throughout dark- and light-grown seedlings and mainly localize to the plasma membrane, whereas PP2C-D1 is expressed in apical regions of dark-grown seedlings, with strong enrichment in the inner side of the apical hook (Ren et al., 2018). The pp2c-d1 and pp2c-d2/d5/d6 triple mutants display reduced apical hook angles and open cotyledons in the dark, indicating that they function in apical organ development in etiolated seedlings (Spartz et al., 2014; Ren et al., 2018).

In this study, in an attempt to identify cellular effectors of etiolated development among SAUR genes, we focused on SAURs that are expressed in the dark and are downregulated by light. We show that Arabidopsis SAUR17, the most highly expressed SAUR gene in the cotyledons and hook region of etiolated seedlings, plays a key role in maintaining the apical hook and closed cotyledons. By contrast, a group of lirSAURs, as represented by SAUR50, promotes hook and cotyledon opening. Whereas SAUR50 inhibits PP2C-D1, SAUR17 binds to PP2C-D1 with high affinity but without inhibiting its enzymatic activity. We propose that SAUR17 competitively associates with PP2C-D1 and protects it from inhibitory SAURs such as SAUR50. This enables PP2C-D1 to actively suppress cell expansion and maintain apical hook curvature and cotyledon closure in the dark. Our discovery of the antagonistic interplay between SAUR17 and SAUR50 elucidates a possible mechanism used by SAURs to control cell size.

RESULTS

Arabidopsis SAUR17 Is Specifically Expressed in the Hooks and Cotyledons of Dark-Grown Seedlings

In a previous study of organ-specific light-regulated gene expression (Sun et al., 2016), a group of SAUR genes was found to be
expressed specifically in the cotyledons of etiolated seedlings and downregulated by light (Supplemental Figure 1A). Among these SAUR genes, SAUR17 stood out as the most robustly expressed gene in the dark and as highly organ specific. RT-qPCR of dissected tissues confirmed that SAUR17 mRNA was exclusively expressed in the cotyledon and hook areas of dark-grown seedlings (Figure 1). SAUR17 transcript levels dropped drastically within 1 h of light exposure, indicating that SAUR17 expression was strongly inhibited by light (Figure 1A). SAUR17 transcripts were not detected in hypocotyls or roots regardless of light conditions (Figure 1A).

To visualize the cellular localization of SAUR17 expression, we generated transgenic Arabidopsis ProSAUR17:GFP, ProSAUR17:GUS, and ProSAUR17:SAUR17-GFP lines in which the SAUR17 promoter was used to drive the expression of GFP, β-glucuronidase (GUS) reporter, and the SAUR17-GFP fusion

![Figure 1](https://example.com/image1)

**Figure 1.** Arabidopsis SAUR17 Is Specifically Expressed in the Hooks and Cotyledons of Dark-Grown Seedlings.

(A) SAUR17 transcript levels in various organs of the wild-type (Col-0) seedlings. Three-day-old etiolated seedlings (D) were exposed to white light for 0 h (D), 1 h (DL1h), or 6 h (DL6h). Whole seedlings, or dissected tissues of hooks and cotyledons, hypocotyls, and roots were for RNA analysis by RT-qPCR. Error bars represent SD of three biological replicates. PP2A was used as an internal control.

(B) Images showing the apical organ morphology and fluorescence of ProSAUR17:GFP and ProSAUR17:SAUR17-GFP seedlings. Three-day-old dark-grown seedlings (D) were exposed to white light for the indicated number of hours (DL1h to DL12h) or kept in darkness for 12 h (DL24h). Photographs were taken under a Leica stereoscope.

(C) Localization patterns of SAUR17 in hooks and cotyledons. Etiolated 35S:GFP (control), ProSAUR17:GFP, and ProSAUR17:SAUR17-GFP seedlings (3 d) were pressed to separate the cotyledons. The fluorescence was observed under a Zeiss confocal microscope.

(D) SAUR17 transcript levels in the wild-type (Col-0) seedlings during growth in the dark. Dark-grown seedlings at 1 to 7 d of growth (1D to 7D) were collected whole or after cutting below the hook. The upper part (hooks and cotyledons) and lower part (hypocotyls and roots) of the seedling were separately analyzed by RT-PCR. The data represent one of three repeats of independent experiments, with the two other repeats shown in Supplemental Figure 2. Standard deviation (means ± SD, n = 3) is based on three technical repeats. PP2A was used as an internal control.

(E) ProSAUR17:GFP and ProSAUR17:SAUR17-GFP fluorescence signals peak at 2 and 3 d in the dark. D1 to D5 indicate 1- to 5-d dark-grown seedlings, and L3 indicates 3-d light-grown seedlings.
protein, respectively. GFP fluorescence and GUS staining were found mainly in the cotyledon and hook areas of dark-grown seedlings (Figures 1B and 1C; Supplemental Figure 1B). SAUR17-GFP exhibited a distinctive pattern in etiolated cotyledons, sharply contrasting with the uniformly distributed GFP SAUR17-GFP exhibited a distinctive pattern in etiolated cotyledons (Figures 1B and 1C; Supplemental Figure 1B). Altogether, both SAUR17 promoter lines showed expression profiles that resembled endogenous SAUR17 in terms of organ-specific expression as well as light responsiveness. This expression profile hints at a possible role for SAUR17 in hook and cotyledon development in etiolated seedlings.

Expression Dynamics of SAUR17 in the Dark Correlates with Apical Hook Development

The development of the apical hook in darkness has been described in sequential phases: the hook formation phase, maintenance phase, and opening phase (Smet et al., 2014; Zhu et al., 2017). A similar time scale of apical hook development was observed under our experimental conditions (Supplemental Figure 2). Apical hooks of the wild-type Columbia-0 (Col-0) were established during the first 1.5 d after germination in the dark. The hooks were maintained for the next 24 h and gradually unfolded in the following 60 h (Supplemental Figures 2A and 2B).

We monitored SAUR17 expression during growth in the dark by time-course analysis. SAUR17 expression was initially quite low at germination, and it rapidly increased during the first 2 d of growth in the dark, peaking on day 1.5 post-germination, followed by a decrease at 2.5 d post-germination in the dark (Figure 1D; Supplemental Figures 2C and 2D). This expression pattern correlates precisely with the degree of curvature of the apical hooks in etiolated seedlings, as both peaked between 1.5 and 2.5 d post-germination (Supplemental Figures 2A and 2B). ProSAUR17:SAUR17-GFP transgenic plants showed the strongest fluorescence signals in the hooks and cotyledons of 2- to 3-d-old dark-grown seedlings, whereas they were undetectable in light-grown seedlings (Figure 1E). Considering that GFP is relatively stable, this finding is in excellent agreement with the RT-PCR data shown in Figure 1D and Supplemental Figures 2C and 2D.

The dynamic spatial and temporal expression of SAUR17, either in the dark or during the dark-to-light transition, tightly coincided with the developmental progression of apical hooks. In the dark, SAUR17 expression rose during the hook formation phase, peaked around the hook maintenance phase, and declined when the hooks started to relax and slowly open (Figures 1D and 1E; Supplemental Figure 2). Upon irradiation with light, SAUR17 rapidly decreased, correlating with the unfolding of the hook and opening of cotyledons. This expression pattern prompted the hypothesis that SAUR17 functions in the etiolation of apical organs, i.e., apical hooks as well as closed and unexpanded cotyledons.

SAUR17 Promotes Apical Hook Formation and Cotyledon Closure in the Dark

To study the functions of SAUR17, we created clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 mutant lines of saur17 (Supplemental Figure 3). The saur17 single mutant showed the wild type-like apical hooks when grown in normal dark conditions. We then examined the mutant under conditions that would induce the formation of an exaggerated hook angle. The etiolation program in the apical region of a seedling can be reinforced by mechanical pressure of the soil through increased ethylene signaling (Zhong et al., 2014; Shi et al., 2016). Ethylene induces exaggerated apical hook formation in etiolated seedlings as part of the process known as the triple response (Guzmán and Ecker, 1990). When grown on plates containing the ethylene precursor 1-amino-cyclopropanecarboxylic acid (ACC), Col-0 seedlings exhibited twisted and exaggerated apical hooks, but this response was deficient in saur17 mutants, which showed only a slight increase in hook angle (Figures 2A and 2B). This result indicates that SAUR17 plays an important role in modulating apical hook morphology. We also examined the cotyledon opening kinetics of dark-grown seedlings following irradiation with light. The saur17 mutants showed slightly but detectably premature acceleration of cotyledon opening (Figures 2C and 2D), suggesting that the loss of SAUR17 allowed cotyledons to open more readily after light treatment.

The single mutant saur17 showed defects in apical hooks and cotyledons, but the phenotype was rather weak. In fact, the lack of a detectable phenotype in single mutants of SAUR genes is very common, because SAUR genes display strong sequence similarity and genetic redundancy (Ren and Gray, 2015). Phylogenetic analysis indicated that the Arabidopsis SAUR17 subfamily consists of SAUR44, SAUR45, SAUR46, SAUR47, and SAUR57 (Supplemental Figure 4A; Ren and Gray, 2015). Among these genes, SAUR44 and SAUR57 displayed similar expression patterns to SAUR17, whereas SAUR45, SAUR46, and SAUR47 were expressed at very low levels in seedlings (Figure 2E). In addition to the SAUR17 subfamily members, SAUR54 was also expressed in the hook and cotyledons of dark-grown seedlings and was repressed by light, whereas SAUR51, another member of the SAUR54 subfamily, was not light responsive (Figure 2E; Supplemental Figure 1A). We knocked out all genes in the SAUR17 and SAUR54 subfamilies (Supplemental Figure 4A), generating the saur17 saur44 saur45 saur46 saur47 saur51 saur54 saur57 (saur17,44,45,46,47,51,54,57) octuple mutants (Supplemental Figure 3C). To aid with the specific evaluation of SAUR17, we also created the saur44 saur45 saur46 saur47 saur51 saur54 saur57 (saur44,45,46,47,51,54,57) septicum mutant, in which all eight above-mentioned genes except SAUR17 were mutated (Supplemental Figure 3B). For simplicity, we refer to saur44,45,46,47,51,54,57 septicum mutants as saurS lines, and saur17,44,45,46,47,51,54,57 septicum mutants as saurSS saur17 lines.

When grown in the dark, the apical hooks of the saurSS and saurSS saur17 mutants were not tightly formed during the hook formation phase, and they unfolded prematurely (Figures 2F and 2H). The hook angles of the saurSS saur17 mutants were...
consistently more relaxed, that is, reduced, than those of saur<sup>56</sup> mutants up to day 3 in the dark (Figures 2F and 2H). These results suggest that SAUR17 plays an important role in promoting hook curvature during the hook formation and maintenance phases. These results also indicate that some of the seven other SAUR genes are also involved in this process (Figures 2F and 2H).

In the dark, the saur<sub>17</sub> single and saur<sup>56</sup> mutants largely had closed cotyledons similar to those of the wild type, whereas 30 to 40% of the saur<sup>56</sup> saur<sub>17</sub> mutants exhibited open cotyledons on day 3 in the dark (Figures 2F and 2G), indicating that SAUR17 plays a critical role in cotyledon closure in the dark. The open-cotyledon phenotype was also evident in another saur<sub>17</sub>-containing multiplex mutant: the saur<sub>17</sub>51,54,57 quadruple mutant population contained significantly more seedlings with open-cotyledons in the dark compared to saur51,54,57 triple mutants and the Col-0 wild-type seedlings (Supplemental Figures 4B and 4C). Analyses of both higher order mutants suggested that the cotyledon-opening phenotype was more specifically associated with the saur<sub>17</sub> mutation, albeit in the background of other saur mutations.

Surprisingly, SAUR17-overexpressing lines showed somewhat similar phenotypes to saur<sub>17</sub> higher order mutants, as they had weaker apical hooks and increased cases of open-cotyledons in the dark (Supplemental Figure 5). As explained in the Discussion, we suspected that these irregular and inconsistent phenotypes were most likely artifacts of overexpression. Together with the results of loss-of-function analyses, we suggest that SAUR17 is involved in maintaining closed cotyledons in the dark and that it plays an important role in apical hook development, particularly under increased ethylene levels. This observation suggests that SAUR17 activity becomes more important when young seedlings encounter strong mechanical pressure from the soil during etiolation growth.
A Group of SAURs Promotes Hook Unfolding and Cotyledon Opening

In contrast to the dark-expressed SAURs represented by SAUR17, another group of SAURs including SAUR6, SAUR12, SAUR14, SAUR16, and SAUR50, is induced by light in cotyledon and hook regions (Sun et al., 2016), as confirmed by RT-qPCR (Supplemental Figure 6A). This expression profile would be in line with a role in facilitating de-etiolation in apical organs, which has been confirmed for SAUR16 and SAUR50 during light-induced cotyledon opening (Sun et al., 2016; Dong et al., 2019). Here, we tested the idea that not only SAUR16 and SAUR50 but also all five of the SAURs promote the de-etiolation of apical organs. We generated the saur6 saur12 saur14 saur16 saur50 (saur6,12,14,16,50) quintuple mutant lines via CRISPR/Cas9 gene editing (Supplemental Figure 7). As shown in Figure 3,

**Figure 3.** A Group of lirSAURs Promotes Hook and Cotyledon Opening.

(A) to (C) saur6,12,14,16,50 quintuple mutants exhibit stronger apical hooks in the dark and slower hook and cotyledon opening than the wild type. Two independent mutant lines were examined. (A) Apical morphology of the seedlings grown in the dark for 3 d and transferred to white light (80 μmol/m²/s) at the indicated time points. Bar = 1 mm. (B) Hook angles of the wild type and saur6,12,14,16,50 during the dark-to-light transition. Data are shown as means ± se. n > 50 for each sample point. One-way ANOVA was used to calculate significant differences. Bonferroni’s post test, P < 0.01. (C) Percentage of seedlings exhibiting open cotyledons at the indicated time points after light exposure. Cotyledon separation angles greater than 20° were scored as open cotyledons. Three biological replicates were performed. n > 50 for each replicate. Data are shown as means ± se (one-way ANOVA, Bonferroni’s post test, P < 0.01). (D) Localization pattern at the apical region in 2.5-d dark-grown ProSAUR6:GFP, ProSAUR12:GFP, ProSAUR14:GFP, ProSAUR16:GFP, and Pro-SAUR50:GFP seedlings. Insets inside the panels show enlarged images of the hook region. The fluorescence was observed under a Leica stereoscope. Bar = 0.5 mm.
saur6, 12, 14, 16, 50 quintuple mutants showed significantly delayed cotyledon opening compared to the wild type after the light treatment (Figures 3A and 3C), a phenotype more severe than that of the saur6 saur50 double mutant (Sun et al., 2016; Dong et al., 2019). In addition, saur6, 12, 14, 16, 50 exhibited a striking defect in the rate of light-induced apical hook unfolding (Figures 3A and 3B). It is important to point out that the mutants developed significantly greater hook angles even in the dark (dark-to-light time 0 [DL0]; Figures 3A and 3B), prior to the light exposure that enhanced the expression of this group of SAUR genes. These results indicate that these SAUR genes are also expressed in the dark, albeit at low levels, and that they function in unfolding of the hook.

The promoter-reporter lines of ProSAUR6:GFP and ProSAUR14:GFP showed strong light-induced GFP expression, as indicated by increased fluorescence after the dark-grown plants were exposed to light (Supplemental Figure 6B), which is consistent with the expression profiles of the endogenous genes (Supplemental Figure 6A). The ProSAUR12:GFP, ProSAUR16:GFP, and ProSAUR50:GFP lines showed weaker light induction of the GFP reporter compared to their respective endogenous genes in cotyledon-hook tissue (Supplemental Figure 6C). This is probably due to the lack of the mRNA-destabilizing element that is often found at the 3’ untranslated regions of SAUR transcripts (Newman et al., 1993). Thus, the GFP reporter transcripts were more stable, and GFP signals were easily visualized in dark-grown seedlings (Figure 3D). The expression of these genes was observed in cotyledons (SAUR6, SAUR14) or both the cotyledon and hook areas (SAUR12, SAUR16, SAUR50; Figure 3D). Notably, SAUR12, SAUR16, and SAUR50 displayed stronger expression on the inner side of the apical hook (Figure 3D). SAUR50 has been shown to inhibit PP2C-D1 and induce cell expansion (Sun et al., 2016). The expression of SAUR50 on the inner side of the hook and evenly in cotyledons could explain the cellular basis for SAUR50’s function in apical hook unfolding and cotyledon enlargement.

SAUR17 Binds to PP2C-D1 in Vitro, but Does Not Inhibit Its Phosphatase Activity

Thus far, we have identified two groups of SAUR genes that modulate the apical morphology of the seedlings in opposite manners: those functioning to maintain etiolated morphology, as represented by SAUR17, and those promoting de-etiolated morphology, as represented by SAUR50. All of the SAUR proteins that have been tested, including SAUR9, SAUR19, and SAUR50, inhibit PP2C-D1 activity and promote cell expansion (Spartz et al., 2014; Sun et al., 2016). We performed the same in vitro PP2C-D1 enzymatic test on SAUR17 (Figure 4), but SAUR17 showed no effect on PP2C-D1 activity, whereas the positive controls SAUR14 and SAUR50 inhibited its phosphatase activity in the same experiment (Figure 4A). We repeated the assay using increasing amounts of SAUR17, but PP2C-D1 activity levels remained unchanged even at the highest concentration, whereas SAUR50 inhibited PP2C-D1 activity in a concentration-dependent manner (Figure 4B).

We investigated whether SAUR17 could bind to PP2C-D1 as strongly as SAUR50. By measuring the equilibrium dissociation constants ($K_d$) of the respective pairs in vitro, we found that SAUR17 actually exhibited a higher affinity for PP2C-D1 than did SAUR50 (Figure 4C). The PP2C-D1 and SAUR50 interaction had a $K_d$ of $4.643 \times 8 \text{ M}$, whereas the PP2C-D1 and SAUR17 interaction had a $K_d$ of $2.733 \times 8 \text{ M}$. Thus, SAUR17 was capable of binding to PP2C-D1 more tightly than SAUR50, but did not inhibit its activity. These results indicate that the binding of PP2C-D by the SAUR proteins does not in itself inhibit its phosphatase activity.

PP2C-D1 Preferentially Associates with SAUR17 over Other SAURs in Etiolated Seedlings

PP2C-D1 is capable of interacting with many SAUR proteins in vitro; conversely, a SAUR protein is often capable of interacting with several different PP2C-D phosphatases in vitro or in yeast two-hybrid systems (Spartz et al., 2014; Sun et al., 2016; Ren et al., 2018). However, little is known about whether any PP2C-D member(s) actually associates with any specific SAUR protein in planta. We set out to identify proteins that specifically bind to SAUR17 or SAUR50 in dark-grown seedlings by immunoprecipitation followed by mass spectrometry (IP-MS). GFP-tagged transgenic lines SAUR17-GFP, SAUR50-GFP, or 35S-GFP (negative control) were used in this analysis (Supplemental Data Set 1). Remarkably, after filtering out GFP binding noises, PP2C-D1 was identified as the top second protein in the list from SAUR17-GFP IP-MS, and PP2C-D2 was identified as the top protein from SAUR50-GFP IP-MS out of more than 2600 individual proteins identified in this experiment (Supplemental Data Set 1). Altogether, SAUR17-GFP IP-MS identified six of nine PP2C-D clade members, with PP2C-D1 listed as the predominant SAUR17 binding phosphatase (Figure 4D). SAUR50-GFP IP identified seven PP2C-D clade members including PP2C-D1, whereas PP2C-D2 was the major binding partner of SAUR50 (Figure 4D). 35S-GFP, which was used as the negative control, did not pull out any PP2C-D phosphatases (Figure 4D; Supplemental Data Set 1), indicating that the binding of PP2C-Ds with the SAURs was specific. These results also demonstrate that PP2C-D phosphatases are key binding partners of SAUR proteins in vivo.

We reversed the bait and used PP2C-D1-GFP for IP-MS in dark-grown seedlings. PP2C-D1-GFP IP-MS identified four endogenous SAUR proteins in etiolated seedlings, none of which were found in the 35S-GFP control (Supplemental Data Set 2). Strikingly, SAUR17 stood out as the most abundantly bound protein among all of the SAURs, whereas SAUR50 was not detected (Figure 4E; Supplemental Data Set 2). The same experiment also identified AHA1 and AHA2, the known substrates of PP2C-D phosphatases, at a comparable scale to SAUR17. Thus, SAUR17 is a major binding partner of PP2C-D1 in the dark. Together, these IP-MS data sets demonstrate that, among the many members of the PP2C-D clade and SAUR proteins, the SAUR17–PP2C-D1 pair not only exists but also it might represent a prevalent protein complex that is fairly abundant in etiolated Arabidopsis seedlings.

SAUR17 Protects PP2C-D1 Activity against SAUR50 via a Competitive Association

Although SAUR17 is the most highly expressed SAUR gene in etiolated hook and cotyledons, SAUR50-subgroup genes are also
expressed at low levels in these tissues, where they work to promote hook opening (DL0; Figures 3A and 3B). Given their overlapping spatial-temporal expression patterns, we studied the interplay of SAUR17 and SAUR50 on their common target, PP2C-D1. In an in vitro assay in which PP2C-D1 activity was inhibited by SAUR50, the phosphatase activity of PP2C-D1 was gradually restored by adding increasing amounts of SAUR17 protein to the reactions; this restoration was dependent on SAUR17 protein concentration (Figure 5A). These results indicate that SAUR17 interferes with the inhibitory function of SAUR50 on PP2C-D1.

Because SAUR17 has a higher binding affinity for PP2C-D1 compared to SAUR50, we examined whether SAUR17 would impede the binding of SAUR50 to PP2C-D1. In a yeast three-hybrid system, coexpression of SAUR17 clearly weakened the interaction of SAUR50 with PP2C-D1, but coexpression of SAUR50 had very little effect on the interaction of SAUR17 with PP2C-D1 (Figure 5B). For quantitative competition analysis, we performed an in vitro pull-down assay. GST-SAUR17 (or GST-SAUR50) was pre-bound to His-PP2C-D1 proteins, increasing amounts of His-SAUR50 (or His-SAUR17) competitor proteins were added to the reactions, and His-PP2C-D1 pulled down by glutathione agarose was examined (Figure 5C). SAUR17 effectively dislodged SAUR50 from PP2C-D1, whereas SAUR50 prevented SAUR17 from binding to PP2C-D1, but only at sufficiently high concentrations. Specifically, 0.1 μg of His-SAUR50 competitors had little effect on SAUR17-PP2C-D1 binding, but the

Figure 4. In Vitro and in Vivo Binding of SAUR17 to PP2C-D1 and the Lack of Inhibition of PP2C-D1 Phosphatase Activity.

(A) SAUR50, but not SAUR17, inhibits the phosphatase activity of PP2C-D1. The pNPP phosphatase assays contained GST-PP2C-D1 (0.3 μM) with 1 μM GST, GST-SAUR17, GST-SAUR14, or GST-SAUR50. Error bars indicate ± sd (n = 4).

(B) Increasing concentrations of SAUR17 have no effects on PP2C-D1 phosphatase activity, in contrast to SAUR50. The pNPP phosphatase assays contained GST-PP2C-D1 (0.3 μM) with the indicated concentrations of GST-SAUR17 or GST-SAUR50. Data are shown as means ± sd (n = 3).

(C) PP2C-D1 exhibits higher binding affinity to SAUR17 than to SAUR50. Equilibrium Kd's between PP2C-D1 and SAUR50 (left) or between PP2C-D1 and SAUR17 (right) were determined using Biacore T200 software. His-PP2C-D1 was bound to CM5 chip, and the indicated concentrations of His-SAUR17 or His-SAUR50 were used.

(D) SAUR17 predominantly associates with endogenous PP2C-D1 in etiolated seedlings. Three-day-old dark-grown seedlings of the indicated transgenic lines were used for IP-MS. The PSM scores of PP2C-D members from 35S:GFP, 35S:SAUR17-GFP, and 35S:SAUR50-GFP parallel IP-MS are shown. The nomenclature of PP2C-D members is according to Spartz et al. (2014). Also see Supplemental Data Set 1.

(E) PP2C-D1 primarily binds to endogenous SAUR17 in etiolated seedlings. 35S:GFP and 35S:PP2C-D1-GFP seedlings were grown in the dark for 3 d and collected for IP-MS. Four SAURs were identified from the PP2C-D1 IP, along with AHA1 and AHA2. The PSM scores for the respective proteins are shown. Also see Supplemental Data Set 2.
same amount of His-SAUR17 competitor protein substantially destabilized SAUR50-PP2C-D1 binding (Figure 5C, bottom). These results suggest that SAUR17 and SAUR50 likely bind to the same site on PP2C-D1 and are physically competitive against each other. However, under similar protein concentrations, SAUR17 is a preferred PP2C-D1 binding partner over SAUR50, likely because SAUR17 intrinsically has a higher affinity for PP2C-D1 (Figure 4C). This in vitro result is in agreement with the finding from yeast three-hybrid analysis (Figure 5B), and it explains at the molecular level the finding from IP-MS that SAUR17, not SAUR50, was predominantly bound to PP2C-D1 in etiolated seedlings (Figure 4E).

Analysis of SAUR17 and SAUR50 for the PP2C-D1 Binding and Inhibition Domain

To identify the structural features distinctive to SAUR17 that may contribute to its functional difference from other SAURs, we compared its protein sequence with those of the SAURs that have been experimentally shown to inhibit PP2C-D1 (Figure 6A). We also performed secondary structure modeling of representative SAURs (Supplemental Figure 8). SAUR17 has the lowest hydropathy score and the highest fraction of charged residues compared to the other SAUR proteins examined (Holehouse et al., 2017). Nevertheless, SAUR17 contains a conserved SAUR domain with a similar predicted secondary structure to other SAURs (Buchan and Jones, 2019). Within the SAUR domain, SAUR17 contains a triple Lys cluster (K-40, -41, -42) and another Lys (K-78) that appear to be unique. Interestingly, the triple-K cluster is located between the two beta strands (Supplemental Figure 8). The SAUR17 N-terminal extension (NTD) contains a unique stretch of Glu residues (E-14 to E-18), which results in a highly acidic SAUR17 NTD compared to other SAUR NTDs. In addition, the SAUR17 NTD lacks the long N-terminal a-helical structure present in all other SAUR NTDs analyzed (Supplemental Figure 8). The small helical structure in SAUR17 NTD was predicted at low confidence (Buchan and Jones, 2019).

Because SAUR17-specific structural features can be found in both the NTD and SAUR domain, we first attempted to separate these two domains and define their respective roles in the binding and inhibition of PP2C-D phosphatases. The yeast two-hybrid data show that the SAUR domain in SAUR17 or SAUR50 is necessary and sufficient for binding to PP2C-D1 (Figure 6B). However, the SAUR domain fragment of SAUR17 (SAUR17 29-95) showed a weaker interaction, suggesting that the N- and C-terminal extensions of SAUR17 could enhance the interaction of its SAUR domain with PP2C-D1. By contrast, the SAUR domain fragment of SAUR50 (SAUR50 44-107) showed a stronger interaction than full-length SAUR50 (Figure 6B), suggesting that SAUR50 NTD might hinder the binding to PP2C-D1. The complete results of the yeast two-hybrid interaction experiments are shown in Supplemental Figure 9A, which include the interaction data on the deletion mutants of PP2C-D1 and full-length PP2C-D2 and PP2C-D5. We found that the phosphatase domain on PP2C-D1 mediated its binding with the SAURs (Supplemental Figure 9A); this observation is consistent with a recent finding by Wong et al. (2019).
We swapped the SAUR domains and NTDs of SAUR17 and SAUR50 and produced two chimeric SAUR proteins. SAUR171-28 SAUR5044-107 exhibited a stronger interaction with PP2C-D1 compared to SAUR50, whereas SAUR501-43SAUR1729-95 did not detectably interact with PP2C-D1 (Figure 6B; Supplemental Figure 9B). This result is consistent with the idea that SAUR17 NTD enhances, whereas SAUR50 NTD suppresses, the interaction of the downstream SAUR domain with PP2C-D1. PP2C-D2 and PP2C-D5 behaved similarly to PP2C-D1 in how they interacted with the SAUR proteins tested (Supplemental Figure 9).

To study the role of the NTD and the SAUR domain in inhibiting PP2C-D1 activity, we purified the SAUR17 and SAUR50...
truncation mutants and the chimeric fusion proteins and then tested their effects on PP2C-D1 activity in an in vitro phosphatase assay (Figure 6C). The SAUR50 SAUR domain, either alone (GST-SAUR5014-107) or in the context of SAUR17 NTD (GST-SAUR1718-28SAUR5044-107), exhibited inhibitory activity similar to GST-SAUR50. By contrast, the SAUR17 SAUR domain alone or with SAUR50 NTD (GST-SAUR5014-28SAUR1718-28) did not significantly inhibit PP2C-D1 (Figure 6C). Because GST-SAUR5014-28 SAUR1718-28 may not bind to PP2C-D1, this lack of inhibition could be due to either the lack of binding, the lack of inhibitory activity, or both. Apart from this protein, all other proteins that inhibited PP2C-D1 contained the SAUR50 SAUR domain, supporting the idea that the phosphatase inhibitory function of this protein is primarily associated with its SAUR domain.

SAUR17 and SAUR50 Modulate PP2C-D1 Activity in Opposite Manners to Influence Apical Hook and Cotyledon Development

PP2C-D1 is expressed most strongly in the inner side of the apical hook in the dark (Ren et al., 2018), and the pp2c-d1 single mutant shows reduced apical hook angles (Spartz et al., 2014). These findings suggest that PP2C-D1 plays an important role in apical hook development. We therefore generated PP2C-D1 overexpression lines. These 35S:PP2C-D1-GFP (PP2C-D1OE) seedlings exhibited tighter apical hooks (greater hook angles) and delayed hook unfolding after light exposure compared to the wild type (Figures 7A and 7B). Light-induced cotyledon opening was also slower in 35S:PP2C-D1-GFP than in the wild-type control (Figures 7A and 7C). These data support that PP2C-D1-mediated suppression of cell expansion strongly contributes to the development of etiolated apical organs.

To investigate how SAUR17 and SAUR50 modulate the PP2C-D1 gain-of-function phenotype in vivo, we generated the saur17 saur44 saur57 (saur17,44,57) mutant by CRISPR/Cas9-mediated gene editing (Supplemental Figure 10) and SAUR50 overexpression lines in the PP2C-D1OE background. In both cases, PP2C-D1 expression levels were not affected by saur17,44,57 mutations or SAUR50 overexpression (Supplemental Figure 11). Mutations in saur17,44,57 resulted in premature cotyledon opening in the dark both the wild-type and PP2C-D1OE backgrounds, and they negated the sharp apical hooks associated with PP2C-D1 overexpression (Figures 7D to 7F). These results indicate that saur17,44,57 strongly suppressed most of the altered apical phenotypes associated with PP2C-D1 overexpression. These results suggest that the role of PP2C-D1 in maintaining a tight apical hook is strongly supported by SAUR17, SAUR44, and SAUR57. Given that SAUR17 was identified as the main interacting SAUR of PP2C-D1, it is reasonable to conclude that SAUR17 likely plays a key role in maintaining PP2C-D1 activity.

The phenotype of PP2C-D1OE was also compromised by overexpression of SAUR50. Overexpressing SAUR50 resulted in open cotyledons in the dark (Figures 7G to 7I) and completely suppressed the altered hook curvature and cotyledon-opening phenotypes of PP2C-D1OE (Figures 7G to 7I). This result suggests that the function of PP2C-D1 is blocked by overproducing SAUR50, which inhibits PP2C-D1 activity. These results are consistent with the hypothesis that SAUR17 and SAUR50 antagonistically regulate PP2C-D1 to modulate apical morphological development in seedlings.

SAUR57 Enhances Apical Hook Formation by Inhibiting PP2C-D1 Activity in Convex Cells of the Hook

We studied another dark-expressed SAUR gene, SAUR57 (Figure 8), even though its expression level in the dark is not as high as SAUR17 (Figure 2E). In ProSAUR57:GFP transgenic lines, SAUR57 was mainly expressed on the outer side of apical hook and uniformly in etiolated cotyledons (Figures 8A and 8B). After 12 h of light irradiation, the GFP fluorescent intensity in cotyledons decreased, and its overall localization pattern was altered (Figure 8C). Therefore, the dynamic spatial-temporal changes in the expression of SAUR57 differed from that of SAUR17.

We purified SAUR57 protein and tested its effect on PP2C-D1 activity. SAUR57 inhibited PP2C-D1 activity to the same extent as SAUR50 (Figure 8D). Based on this information, we propose that by blocking PP2C-D1 activity, SAUR57 specifically enlarges the convex cells of the hook in the dark, which facilitates hook bending and formation. Thus, not all dark-expressed SAURs behave like SAUR17 in terms of how they regulate PP2C-D1.

DISCUSSION

Upon germination in the dark, Arabidopsis seedlings undergo etiolation. This developmental program is required for seedlings to successfully emerge from the darkness in soil (Von Arnim and Deng, 1996; Fankhauser and Chory, 1997). A great deal is known about the general signaling determinants of etiolation, from photomorphogenesis repressors such as CONSTITUTIVE PHOTOMORPHOGENIC1 and DE-ETIOLATED1 to transcription factors such as phytochrome-interacting factors, EIN3/EIL1, and so on (Chory et al., 1989; Deng et al., 1992; Leivar et al., 2008; Shin et al., 2009; Zhang et al., 2013, 2018; Shi et al., 2018). However, the downstream effectors regulated by these transcription factors, which carry out specific cellular activities to modulate structures such as apical hooks or cotyledon opening and closure, have not been clearly defined. In this study, we obtained several lines of evidence that SAUR17 serves as an effector of etiolation development specific for the apical structures. SAUR17 is strongly and specifically expressed in apical organs of dark-grown young seedlings and is repressed by light (Figure 1). SAUR17 expression also precisely correlates with apical hook development during extended growth in the dark (Supplemental Figure 2). Genetic and molecular studies indicated that SAUR17 functions in the formation of etiolated apical structures by binding to and protecting the activity of the cell expansion inhibitor PP2C-D1.

SAUR17-PP2C-D1 Complex Maintains Etiolated Apical Structures

Many SAURs induce cell expansion. Spartz et al. (2014) initially proposed that SAURs function by binding to and inhibiting D clade PP2C phosphatases. The IP-MS data presented in this study provide in vivo evidence to support this conclusion, as SAURs and PP2C-D phosphatases are intimate binding partners in plant cells.
Figure 7. Function of PP2C-D1 in Apical Hook Development Is Antagonistically Regulated by SAUR17 and SAUR50.

(A) to (C) PP2C-D1 overexpression promotes apical hook development in the dark and delays light-induced apical hook and cotyledon opening. (A) Apical phenotype of 35S:PP2C-D1-GFP seedlings grown in the dark for 3.5 d and transferred to white light (70 μmol/m²/s) at the indicated time points. (B) Hook
Among all of the SAUR17 and SAUR50 interacting proteins identified in dark-grown seedlings, PP2C-D phosphatases were the most abundant (Supplemental Data Set 1). Conversely, among PP2C-D1 interacting proteins, SAUR17 was identified at a level on par with PP2C-D1’s known substrates AHA1 and AHA2 (Figure 4E; Supplemental Data Set 2). These data convincingly validate the in vivo relevance of SAUR proteins as regulators of PP2C-D phosphatases.

Arabidopsis contains 79 SAUR genes and 9 PP2C-D clade genes. Each of the SAUR proteins is capable of interacting with a number of different PP2C-Ds in vitro (Ren and Gray, 2015; Stortenbeker and Bemer, 2019). Indeed, promiscuous interactions have often hampered the identification of specific SAUR-PP2C-D pairs in the cells of interest. Here, we demonstrated that SAUR17 was predominantly associated with PP2C-D1 among the nine members of the Arabidopsis PP2C-D clade and that PP2C-D1 was primarily bound by endogenous SAUR17 of all of the SAUR proteins in dark-grown seedlings (Figures 4D and 4E). The two proteins shared overlapping, but not identical, localization patterns in the hook and cotyledons, whereas the SAUR17-PP2C-D1 pair combination stood out unambiguously. In addition, SAUR32, which has a similar localization to PP2C-D1 at the inner side of the hook (Park et al., 2007; Ren et al., 2018), was also identified by IP-MS, but at a much lower level than SAUR17 (Figure 4E).

Overexpression of PP2C-D1 results in shorter hypocotyls (Spartz et al., 2014) and greater hook angles (tighter hooks) than the wild type (Figure 7). We suggest that most of these functions of PP2C-D1 are performed in the form of the SAUR71-PP2C-D1 complex, given the predominance of this pair association. This idea is also supported by the observation that sau17,44,57 strongly suppressed the apical phenotype of plants overexpressing PP2C-D1 (Figures 7D to 7F). PP2C-D1 is also expressed in light-grown seedlings and in other plant organs (Ren et al., 2018), where it would be regulated by other SAURs. However, we propose that in apical organs of dark-grown seedlings, PP2C-D1 is associated with SAUR17 to maintain etiolated apical morphology.

SAUR17 Protects PP2C-D1 against Inhibitory SAURs Such as SAUR50

PP2C-D subfamily members are Mg²⁺/Mn²⁺-dependent phosphatases that dephosphorylate and thereby deactivate H⁺-ATPases (AHA1 and AHA2), thus impairing the cell expansion capacity (Shi, 2009; Fuchs et al., 2013; Spartz et al., 2014). The binding of SAUR proteins to PP2C-D2 usually leads to the inhibition of its phosphatase activity that, in turn, activates cell expansion (Ren and Gray, 2015). SAUR17 distinguishes itself from inhibitory SAURs such as SAUR50 in that it binds to PP2C-D1 without inhibiting its phosphatase activity (Figure 4). In addition, due to its higher affinity for PP2C-D1 than SAUR50, SAUR17 is able to compete against SAUR50, thereby shielding PP2C-D1 from SAUR50 and likely other inhibitory SAURs.

As illustrated in Figure 9, we hypothesize that the antagonistic interplay of SAUR proteins contributes to the development of the seedling’s apical structure during etiolation and the release of this structure upon de- etiolation. In the dark, SAUR17 binds to PP2C-D1, thereby protecting this phosphatase from inhibitor SAURs such as SAUR50. The SAUR17-associated PP2C-D1 phosphatase actively suppresses cell expansion in certain cells, including the concave side of the hook, which must be kept small in order for apical hooks to form and for cotyledons to remain closed. Upon light irradiation, SAUR17 levels drastically decline while SAUR50 levels increase. As a result, SAUR50, and probably other SAURs, bind to and inhibit PP2C-D1 activity, which induce general cell expansion, ultimately leading to hook and cotyledon opening and cotyledon enlargement (Figure 9A).

It should be pointed out that SAUR17 also interacted with PP2C-D2 and PP2C-D5 in etiolated seedlings (Supplemental Figure 9). PP2C-D2 and PP2C-D5 are most strongly expressed in hypocotyls, but they are also expressed in the hook and cotyledons (Ren et al., 2018), where endogenous SAUR17 is located. Whether SAUR17 regulates PP2C-D2 and PP2C-D5 in a similar fashion to PP2C-D1 remains to be studied. Even though SAUR proteins can interact with multiple members of the PP2C-D family (Spartz et al., 2014; Sun et al., 2016; Wen et al., 2019; Wong et al., 2019), their regulatory effects vary. For example, Spartz et al. (2014) showed that SAUR19 inhibited PP2C-D1, but not PP2C-D2 or PP2C-D4, activity in vitro, whereas SAUR5 inhibited all of these D-clade phosphatases in the same assay. This raises the question of whether SAUR19 may function as an inhibitor of PP2C-D1 but a protector of PP2C-D2 and PP2C-D4. This speculation requires extensive experimentation. Altogether, the mechanism of SAUR17 and its antagonistic relationship with SAUR50 reveals a layer of complexity and versatility in the SAUR-PP2C-D system.

Specificities of the SAUR Overexpression Lines

Our data show that functions of SAUR50, SAUR6, SAUR12, SAUR14, and SAUR50 are associated with de- etiolation in apical...
organs (notably SAUR50 promotes etiolation growth in hypocotyl cells; Sun et al., 2016; Dong et al., 2019; this study). This conclusion is in agreement with genetic phenotypes of both the loss-of-function mutants and SAUR50 overexpression lines (Figures 3 and 7G to 7I). By contrast, SAUR17 facilitates etiolation development, which is supported by the phenotypes of loss-of-function mutants of saur17 and its higher order mutants (Figures 2 and 7D and 7F; Supplemental Figure 4).

Surprisingly, overexpressing SAUR17 as in 35S:SAUR17 or ProSAUR17:SAUR17-GFP lines did not result in a stronger etiolation phenotype, as anticipated. Instead, these SAUR17 overexpression lines displayed a partially de-etiolated apical phenotype

Figure 8. SAUR57 Displays an Asymmetric Localization to the Outer Side of the Hook and Inhibits PP2C-D1.
(A) SAUR57 displays stronger expression on the outer side of the apical hook. ProSAUR57:GFP was grown in the dark for 2 d, and the images were acquired under a fluorescent stereoscope.
(B) SAUR57 is evenly distributed in etiolated cotyledons. ProSAUR57:GFP was grown in the dark for 3 d. Cotyledons were pressed to separate for imaging observation using a laser scanning confocal microscope.
(C) Tissue-specific expression pattern of SAUR57 is altered after light irradiation. ProSAUR57:GFP seedlings were grown in the dark for 2 d (D) and transferred to the light for 12 h (DL12h).
(D) SAUR57 inhibits the phosphatase activity of PP2C-D1. GST-PP2C-D1 (0.25 μM) was incubated with 1 μM GST, 1 μM GST-SAUR50, or 1 μM GST-SAUR57, and pNPP phosphatase assays were performed. Data are shown as means ± sd (n = 3).

Figure 9. Schematic Diagrams of the Roles of SAUR17/SAUR50 in Apical Development and the Localization Patterns of the SAURs in the Apical Hook.
(A) A simplified schematic representation of the differential regulation of PP2C-D1 by SAUR17 and SAUR50 that contributes to apical morphological development during etiolation and de-etiolation. In etiolated seedlings, the SAUR17-PP2C-D1 complex abundantly accumulates in the hook and cotyledon region, where PP2C-D1 is active in suppressing cell expansion. This is critical for apical hook formation and maintenance as well as cotyledon closure. Upon illumination, light turns off SAUR17 and upregulates SAUR50 in cotyledons and hooks. This allows SAUR50 to bind to PP2C-D1 and inhibits its activity, resulting in cell expansion and ultimately hook and cotyledon opening.
(B) Localization patterns of relevant SAURs and PP2C-D1 in apical hooks. SAUR57 is enriched at the convex cells of the hook, whereas SAUR12, SAUR16, and SAUR50, as well as SAUR32 (Park et al., 2007) and PP2C-D1 (Ren et al., 2018), are enriched in the concave cells of the hook. SAUR17 is uniformly localized throughout the hook.
in the dark (Supplemental Figure 5), which resembled that of lines overexpressing SAUR50, even though these two SAURs show opposite light responsiveness, contrasting loss-of-function phenotypes, and antagonistic roles in regulating PP2C-D1. Even more perplexing, all of the SAUR overexpression lines we examined so far displayed a similar partially de-etiolated apical phenotype. This phenotype is also very common among reported SAUR overexpression lines, including plants overexpressing SAUR19 (Spartz et al., 2012), SAUR36 (Stamm and Kumar, 2013), SAUR53 (Kathare et al., 2018), and SAUR50 and SAUR85 (Sun et al., 2016). In particular, SAUR32, which inhibits cell expansion and plays a role in hook maintenance (Park et al., 2007), also showed the same overexpression phenotype. Considering these observations, we suggest that extra caution should be taken in interpreting the de-etiolated apical phenotype of SAUR overexpression lines and that this phenotype may not necessarily indicate a specific function of the SAURs in apical organ development. SAURs are known for their functional redundancy and sequence similarity. Over-accumulation and/or ectopic expression might alter or reduce the specificity of a SAUR protein in terms of the cellular locations of its action, protein interaction patterns, or even how it regulates PP2C-D phosphatases. This problem might be better tolerated in simpler structures such as hypocotyls, but it is likely aggravated in complex structures such as folding and unfolding apical hooks and cotyledons, where cell expansion and the suppression of cell expansion occur simultaneously in different parts of a structure. More studies are needed to understand the specificities versus redundancies of SAUR proteins.

Localization Patterns of the SAURs in Apical Hook

Specific cellular localization is of key importance to the SAUR-PP2C-D system (Ren and Gray, 2015; Stortenbeker and Bemer, 2019). In this study, we revealed localization patterns of several SAUR genes in the apical organs of dark-grown seedlings, as summarized in Figure 9B. SAUR17 exhibits a uniform expression pattern in the hook area. SAUR12, SAUR16, and SAUR50, whose expression is enhanced by light, are preferentially distributed on the inner (concave) side of the apical hook. The same area is also preferentially enriched with SAUR32 (Park et al., 2007) and PP2C-D1 (Ren et al., 2018). It should be mentioned that the main site of SAUR32 expression is in hypocotyls, where it is not regulated by light (Supplemental Figure 1A). On the other side of the hook, SAUR57 asymmetrically accumulates at the outer (convex) side of the apical hook, and its localization changes after light irradiation (Figure 8C). Both SAUR50 and SAUR57 inhibit the phosphatase activity of PP2C-D1, suggesting they modulate hook curvature by promoting cell expansion at the inner and outer side of the apical hook, respectively. Interestingly, a SAUR50-like gene from sunflower (Helianthus annuus) localizes to the east side of the stem, and its expression correlates with the diurnal bending of the apex toward the sun (Atamian et al., 2016). Thus, the functionality and localization of SAUR50 appear to be conserved. It is unclear how the asymmetric localization patterns of these genes are established. Given that auxin is asymmetrically distributed across the apical hook and that the auxin gradient is critical for hook morphology (Abbas et al., 2013), it is tempting to speculate that auxin plays a role in the asymmetric expression and accumulation of certain SAUR genes at the hook.

METHODS

Plant Materials and Growth Conditions

All mutants and transgenic plants were in the Arabidopsis (Arabidopsis thaliana) Col-0 ecotype background. 35S:SAUR50-GFP seeds were described previously (Sun et al., 2016).

The seeds were treated with 15% (w/v) NaClO for 5 to 10 min and washed four or five times with sterile double-distilled water. The surface-sterilized seeds were sown on Murashige and Skoog medium containing 4.4 g/L Murashige-Skoog powder, 1% (w/v) Suc, and 0.6 to 0.8% (w/v) agar, pH 5.8. The seeds were cold stratified in the dark for 3 to 4 d at 4°C and transferred to white light (70 μmol·m−2·s−1) for 6 to 12 h to promote germination. For light-grown seedlings, seedlings were kept in a Percival light chamber with continuous white light (70 μmol·m−2·s−1) at 22°C. Dark-grown seedlings were incubated in darkness at 20°C. For chemical treatment, ACo (A3903-100MG; Sigma-Aldrich) was supplied to the Murashige and Skoog medium for phenotypic analysis.

Constructs Used this Study

All primers are listed in Supplemental Data Set 3. To generate ProSAUR17:GFP, the SAUR17 promoter was cloned from genomic DNA using primers SAUR17 promoter F_SbfI and SAUR17 Promoter R_XbaI and inserted into pJim19-GFP (Basta) digested with SbfI and XbaI to delete the cauliflower mosaic virus (CaMV) 35S promoter.

For ProSAUR17:SAUR50-GFP, the genomic fragment of SAUR17 without the stop codon was amplified using primers SAUR17 Promoter_SbfI F2 and SAUR17 CDS without SC_XhoI R and cloned into the SbfI and XhoI restriction sites of pJim19-GFP (Basta) to yield pJim19-SAUR50-GFP (Basta).

For ProSAUR7:GUS, the SAUR17 promoter was cloned from genomic DNA using primers SAUR17 Promoter_BstXI F and SAUR17 Promoter_SaiI R and inserted into pCambia1381 digested with BstXI and SaiI.

To generate ProSAUR14:GFP and ProSAUR50:GFP, the SAUR14 and SAUR50 promoters were cloned from genomic DNA using primer pair SAUR14 promoter F_SbfI/SAUR14 Promoter R_XbaI and SAUR50 promoter F_SbfI/SAUR50 Promoter R_XbaI, respectively, and inserted into pJim19-GFP (Basta) digested with SbfI and XbaI to delete the CaMV 35S promoter.

For ProSAUR6:GFP and ProSAUR16:GFP, the pJim19-GFP (Basta) vector was digested with SbfI and XhoI to delete the CaMV 35S promoter. The SAUR6 and SAUR16 promoters were amplified from the wild-type genomic DNA with primer pair SAUR6 Promoter F_SbfI/SAUR6 Promoter R_XhoI and SAUR16 Promoter F_SbfI/SAUR16 Promoter R_SaiI, respectively. Finally, the amplified SAUR6 and SAUR16 promoters were digested with SbfI/Xhol and SbfI/SaiI, respectively, and ligated into the digested pJim19-GFP vector (Basta).

To generate ProSAUR12:GFP, the SAUR12 promoter was amplified from genomic DNA with primer pair SAUR12 Promoter F_Stul and SAUR12 Promoter F_Agel and cloned into the Stul and AgeI restriction sites of pEGAD.

For 35S:PP2C-D1-GFP, the PP2C-D1 coding sequence was amplified from the wild-type cDNA with primers PP2C-D1 CDS_XbaI F and PP2C-D1 CDS without SC_XhoI R, and inserted into pJim19-GFP (Basta), which was digested with XbaI and Xhol.

For SAUR50Ce (in the 35S:PP2C-D1-GFP background), the 35S:SAUR50-GFP sequences were amplified from the pJim19-SAUR-GFP vector (Sun et al., 2016) and inserted into the EcoRI and HindIII restriction sites of pCambia1300.
To generate 3SS:SAUR17, 3SS:SAUR17-GFP, and 3SS:GFP, the SAUR17 coding sequence was amplified from the wild-type cDNA using primer pair SAUR17 CDS_F/SAUR17 CDS with out SC_Stul. The PCR product was cloned into the Xhol and StuI restriction sites of pGEM19 or pGEM19-GFP (Sun et al., 2016), respectively, to yield vectors over-expressing SAUR17 or SAUR17-GFP. pGEM19-GFP was used as the 3SS:GFP control.

To generate the GST-SAUR17, GST-SAUR57, GST-PP2C-D2, and GST-PP2C-D5 expression vectors, gene fragments were amplified from the wild-type cDNA using primer pairs SAUR17_Sall F/SAUR17_NotI R, SAUR57_Sall F/SAUR57_NotI R, PP2C-D2-Xhol F/PP2C-D2_NotI R, and PP2C-D2_Sall F/PP2C-D2_NotI R, respectively, and inserted into the pGEX-4T-1 vector following digestion with Sall and NotI to generate the GST-SAUR17, GST-SAUR57, GST-PP2C-D2, and GST-PP2C-D5 constructs.

For GST-SAUR1729-95 and GST-SAUR5744-107, the gene fragments were synthesized and cloned into the Sall and NotI restriction sites of pGEX-4T-1.

For GST-SAUR171-28 and GST-SAUR501-43, the gene fragments were synthesized and cloned into the Sall and NotI sites of pGEX-4T-1 using primer pairs S17-N-S50-C_Sall F/S17-N-S50-C_NotI R and S50-N-S17-C_Sall F/S50-N-S17-C_NotI R, respectively.

To produce the His-PP2C-D1, His-SAUR17, and His-SAURO5 Escherichia coli expression vectors, the coding sequences of PP2C-D1, SAUR17, and SAUR50 were cloned into the pET-28a vector using primer pairs PP2C-D1_EcoRI F/PP2C-D1_Xhol R, SAUR17_CDS_BamHI F/SAUR17_CDS_Sall R, and SAUR50_CDS_BamHI F/SAUR50_CDS_Sall R, respectively.

The yeast two-hybrid constructs BD-PP2C-D1, BD-PP2C-D2, BD-PP2C-D5, and BD-SAUR50 were previously described by Sun et al. (2016). Different domains of PP2C-D1 were cloned into the EcoRI and Xhol restriction sites of pBlueScript using the primers PP2C-D1_EcoRI F/PP2C-D1_Xhol R and digested with EcoRI and Xhol to remove the GAL4 activation domain. The SAUR17, SAUR50, and SAUR51 were digested using SaI and NotI.

For GST-PP2C-D2 and GST-PP2C-D5 expression vectors, the coding sequences of PP2C-D2, SAUR50, and SAUR57 were amplified from the wild-type cDNA using primer pairs SAUR50_SalI F/SAUR50_NotI R, SAUR57_SalI F/SAUR57_NotI R, and SAUR51_SalI F/SAUR51_NotI R and digested with SalI and NotI.

Total RNA was extracted from the samples using RNeasy Plant Mini kits (REF 74,904; Qiagen) following the manufacturer’s protocol.

To generate the CRISPR/Cas9 mutants saur17, saur51 saur54 saur57 (saur51,54,57), saur17 saur54 saur57 (saur17,54,57), saur17 saur51 saur54 (saur17,51,54,57), and saur57 (saur57), the CRISPR/Cas9 gene editing system was used (Wang and Chen, 2020). The forward and reverse sgRNA primers were annealed and cloned into the BbsI site of the pAiU6-26-M vector (Wang and Chen, 2020). All sgRNA primers are listed in Supplemental Data Set 3. Next, the tandem sgRNA cassette was digested with KpnI andSalI and ligated into p3SS-Cas9-P2A-GFP (Hyg) or pUBQ10-Cas9-P2A-GFP (Gent; Wang and Chen, 2020). All constructs were transferred into Agrobacterium strain GV3101 and transformed into Arabidopsis by the floral dip method. All CRISPR/Cas9-edited lines have been confirmed to be stable germine mutants.

RNA Extraction and RT-qPCR Analysis

The respective constructs were transferred into Agrobacterium tumefaciens strain GV3101 and transformed into Arabidopsis using the floral dip method (Clough and Bent, 1998).

To generate the CRISPR mutants saur17, saur51 saur54 saur57 (saur51,54,57), saur17 saur51 saur54 saur57 (saur17,51,54,57), saur17 saur44 saur57 (saur17,44,57), saur17 saur14 saur57 (saur14,57), saur17 saur14 saur57 (saur17,57), and saur8 saur12 saur14 saur16 saur50 (saur16,12,14,16,50), the CRISPR/Cas9 gene editing system was used (Wang and Chen, 2020). For the saur51,54,57, saur17,51,54,57, saur14,54,57, saur17,44,57, saur17,44,57,3SS:PP2C-D1-GFP, saur56, and saur56 saur17 mutants, two single-stranded guided RNAs (sgRNAs) were designed to target SAUR17, SAUR51, SAUR54, and SAUR57 and one sgRNA was designed to target SAUR44, SAUR45, SAUR46, and SAUR47. For the saur17 mutants, only SAUR17 sgRNA1 was used to edit SAUR17. To generate the saur6,12,14,16,50 quintuple mutants, the plasmid for editing SAUR6 (one sgRNA), SAUR12 (two sgRNAs), and SAUR14 (one sgRNA) was transformed into plants in the saur16 saur50 #17 background (Sun et al., 2016). The forward and reverse sgRNA primers were annealed and cloned into the BbsI site of the pAiU6-26-M vector (Wang and Chen, 2020). All sgRNA primers are listed in Supplemental Data Set 3. Next, the tandem sgRNA cassette was digested with KpnI and SalI and ligated into p3SS-Cas9-P2A-GFP (Hyg) or pUBQ10-Cas9-P2A-GFP (Gent; Wang and Chen, 2020). All constructs were transferred into Agrobacterium strain GV3101 and transformed into Arabidopsis by the floral dip method. All CRISPR/Cas9-edited lines have been confirmed to be stable germine mutants.

RNA Extraction and RT-qPCR Analysis

Total RNA was extracted from the samples using RNeasy Plant Mini kits (REF 74,904; Qiagen) following the manufacturer’s protocol. The first-strand cDNA was synthesized from 2 μg of RNA using 5x All-In-One RT Master Mix (catalog no. G490; Applied Biological Materials). The cDNA was diluted to 50 ng/μL. RT-qPCR was performed using SYBR Premix Ex Taq (RR420A; Takara). Two microliters of cDNA was added as a qPCR template, and the total reaction volume was 20 μL. The samples were amplified using the 7500 real-time PCR system (Applied Biosystems). The program was as follows: melting at 95°C for 30 s and amplification with 40 cycles of 95°C for 5 s and 60°C for 34 s. All data analyses used SERINE/THREONINE PROTEIN PHOSPHATASE2A (PP2A) as an internal control. The primers used for RT-qPCR are listed in Supplemental Data Set 3.

With the exception of Figure 1D, all RT-qPCR data presented were obtained from experiments performed with three biological repeats (separate biological materials and extraction processes). The three independent repeat experiments shown in Figure 1D and Supplemental Figures 2C and 2D were each performed with three technical repeats (same sample and RNA extraction with three RT-qPCRs) due to the limited amounts of the samples.

Histochemical GUS Staining

Three-day-old dark-grown seedlings were fixed in 90% (v/v) cold acetone on ice for 30 min and washed three times with ice-cold PBS buffer. The seedlings were stained with GUS staining buffer (50 mM sodium phosphate buffer, pH 7.2, 10 mM EDTA, 1 mM K3[Fe(CN)6], 0.5 mM K4[Fe(CN)6], 3 H2O, 0.1% (v/v) Triton X-100, 1 mg/ml X-Gluc) at 37°C until the color was visible. Chlorophyll was removed from the samples with 70% (v/v) ethanol. Images were captured under a Leica stereoscope.

Hook Angle and Cotyledon Opening Angle Measurements

Seeds were placed on agar plates, and images were taken under a Leica stereoscope. The hook angles and/or cotyledon separation angles of individual seedlings were digitally measured using Leida Application Suite 4.8 software. Only seedlings that displayed clear angle information in an image were scored. The hook bending angles were measured as described by Vandenbussche et al. (2010). Cotyledon separation angles were measured according to Fankhauser and Casal (2004). In graphs where yes-no binary scores were presented to describe cotyledon opening/separation (Figures 2G and 3C), cotyledon angles greater than 20° were scored as open cotyledons.
Plant Protein Extraction and Immunoblot Analysis

Seedlings were frozen in liquid nitrogen and ground into a powder with a mortar and pestle. The sample was transferred to a 1.5-mL tube containing denaturing extraction buffer (8 M urea, 100 mM NaH2PO4, 1 mM phenylmethylsulfonyl fluoride, 1× protease inhibitor [Roche], 100 mM Tris-HCl, pH 8.0), followed by centrifugation (13,000 rpm for 10 min) at 4°C. Protein concentration was determined using the Bradford protein assay (Bio-Rad). SDS loading buffer was added to the protein extraction buffer, and the samples were heated to 100°C for 10 min.

For immunoblot analysis, protein samples were separated by 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% milk for 1 h and incubated with primary antibody overnight at 4°C. After washing three times for 15 min with 1× PBST, the membrane was incubated with secondary antibody for 1 h at room temperature, followed by three 15-min washes. Signals on the membrane were developed with ECL primer (GE Healthcare) and detected on X-ray film (GE Healthcare).

Antibodies used in this study include the following: anti-His (catalog no. H1029-0.2mL; Sigma-Aldrich) at 1:1000 (v/v) dilution; anti-GST (catalog no. G1160-0.2mL, Sigma-Aldrich) at 1:5000 (v/v) dilution; anti-GFP (catalog no. M20004; Abmart) at 1:1000 (v/v) dilution; anti-Actin (BE0027-100; EASYBIO) at 1:5000 (v/v) dilution; and anti-RPN6 polyclonal antibody (laboratory storage).

In Vitro Phosphatase Assays

The constructs used to express GST-PP2C-D1, GST-SAUR14, and GST-SAUR50 were described previously (Sun et al., 2016), and other constructs are described above. All constructs were expressed in E. coli strain BL21 (DE3) pLysS or Rosetta (DE3) under the induction of 1 mM isopropyl-β-D-thiogalactopyranoside for 16 to 20 h at 16°C. Proteins were purified with glutathione agarose beads (GE Healthcare).

For the in vitro phosphatase assays, GST-PP2C-D of the indicated molarity was pre-incubated with GST or GST-SAUR proteins of the indicated molarity for 10 min. Assay buffer (75 mM Tris, pH 7.6, 10 mM MnCl2, 100 mM NaCl, 0.5 mM EDTA, and 5 mM p-nitrophenyl phosphate [pNPP]) was then added to the pre-incubated protein mixture to obtain a final volume of 200 µL. SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The gel was stained with Coomassie Brilliant Blue R250, and protein-containing gel slices were excised and analyzed by MS. Peptide spectrum matches (PSMs), that is, the total number of identified peptide spectra matched for the proteins, are presented in the supplemental data sets and figures.

Equilibrium Kd Measurements

Equilibrium Kd measurements were carried out using a Biacore T200 system (GE Healthcare). A CM5 sensor chip (GE Healthcare) was used to couple His-PP2C-D1, and the indicated concentrations of His-SAUR17 or His-SAUR50 were flowed across the surface of the chip. The Kd was used to evaluate the binding affinity, which was determined using Biacore T200 Evaluation software.

Statistical Analysis

For all multiple comparisons, the significance of the difference between different groups was analyzed by one-way analysis of variance (ANOVA) along with Bonferroni’s multiple comparison test at a significance level of 0.01 using Prism software (GraphPad; https://www.graphpad.com/scientific-software/prism/). Different lowercase letters above the bars indicate significantly different groups: "P < 0.05, "P < 0.01, and ""P < 0.001.

Detailed statistical data with ANOVA data for all of the relevant figures are provided in the Supplemental File.

Accession Numbers

Sequence data from this article can be found in the EMBL/GenBank data libraries under the following accession numbers: SAUR1 (AT4G34770);
SAUR6 (AT2G21210); SAUR12 (AT2G21220); SAUR14 (AT4G38840); SAUR16 (AT4G38860); SAUR17 (AT4G09530); SAUR32 (AT2G46690); SAUR37 (AT4G31320); SAUR44 (AT5G30310); SAUR45 (AT2G36210); SAUR46 (AT2G37030); SAUR47 (AT3G20220); SAUR50 (AT4G34760); SAUR51 (AT1G75580); SAUR54 (SAUR54); SAUR57 (AT3G53250); PP2C-D1 (AT5G02760); PP2C-D2 (AT3G17090); PP2C-D3 (AT3G12620); PP2C-D4 (AT3G55050); PP2C-D5 (AT4G38520); PP2C-D6 (AT3G1370); PP2C-D7 (AT5G66800); PP2C-D8 (AT4G33920); PP2C-D9 (AT5G06750); AHA1 (AT2G18960); AHA2 (AT4G30190); and PP2A (AT1G69960).

Supplemental Data

Supplemental Figure 1. SAUR17 is specifically expressed in the apical organs of dark-grown seedlings.

Supplemental Figure 2. Development of apical hooks and expression of SAUR17 in Col-0 seedlings during extended growth in the dark.

Supplemental Figure 3. Mutation sites in saur17 and higher-order mutants.

Supplemental Figure 4. SAUR17 and SAUR54 subfamily genes function redundantly in promoting hook curvature and cotyledon closure in the dark.

Supplemental Figure 5. Apical phenotypes of SAUR17 overexpression lines in the dark.

Supplemental Figure 6. The transcript levels of the five lirSAURs in apical organs increase after light treatment.

Supplemental Figure 7. Mutation sites of the saur6,12,14,16,50 mutants.

Supplemental Figure 8. Prediction of the secondary structure of SAUR17 compared to representative SAURs that inhibit PP2C-D1 activity.

Supplemental Figure 9. Yeast-two-hybrid assay to identify interaction domains between PP2C-Ds and SAUR17 or SAUR50.

Supplemental Figure 10. Mutation sites of the saur17,44,57 and saur17,44,57/35S:PP2C-D1-GFP mutants.

Supplemental Figure 11. Protein analyses of PP2C-D1 and SAUR50 in transgenic lines related to PP2C-D1 overexpression.

Supplemental Data Set 1. IP-MS identification of proteins associated with SAUR17 or SAUR50 in dark-grown seedlings.

Supplemental Data Set 2. IP-MS identification of proteins associated with PP2C-D1 in dark-grown seedlings.

Supplemental Data Set 3. Primers used in this study.

Supplemental File. ANOVA tables.

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AUTHOR CONTRIBUTIONS

H.C., X.W.D., and N.W. conceived and supervised the research. J.W., N.W., and H.C. designed the experiments. N.S. and J.W. generated the SAUR17 constructs for transgenic plant production and analyzed RNA-sequencing data; F.Z. performed PP2C-D1 IP-MS; R.Y. generated the SAUR17 constructs for the yeast experiments; and J.W. performed all other experiments. J.W., H.C., and N.W. analyzed the data. J.W. and N.W. wrote the article. J.W., N.W., X.W.D., and H.C. edited the article.

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SAUR17 and SAUR50 Differentially Regulate PP2C-D1 during Apical Hook Development and Cotyledon Opening in Arabidopsis

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