Abstract: Among the three primary auxin-induced gene families, Auxin/Indole-3-Acetic Acid (Aux/IAA), Gretchen Hagen3 (GH3) and SMALL AUXIN UP RNA (SAUR), the function of SAUR genes remains unclear. Arabidopsis SAUR genes have been phylogenetically classified into three clades. Recent work has suggested that SAUR19 (clade II) and SAUR63 (clade I) promote cell expansion through the modulation of auxin transport. Herein, we present our work on SAUR41, a clade III SAUR gene with a distinctive expression pattern in root meristems. SAUR41 was normally expressed in the quiescent center and cortex/endodermis initials; upon auxin stimulation, the expression was provoked in the endodermal layer. During lateral root development, SAUR41 was expressed in prospective stem cell niches of lateral root primordia and in expanding endodermal cells surrounding the primordia. SAUR41-EGFP (enhanced green fluorescent protein) fusion proteins localized to the cytoplasm. Overexpression of SAUR41 from the Cauliflower mosaic virus 35S promoter led to pleiotropic auxin-related phenotypes, including long hypocotyls, increased vegetative biomass and lateral root development, expanded petals and twisted inflorescence stems. Ectopic SAUR41 proteins were able to promote auxin transport in hypocotyls. Tissue-specific expression of SAUR41 from the PIN1, WOX5, PLT2 and ACR4 promoters induced the formation of new auxin accumulation/signaling peaks above the quiescent centers, whereas tissue-specific expression of SAUR41 from the PIN2 and PLT2 promoters enhanced root gravitropic growth. Cells in the root stem cell niches of these transgenic seedlings were differentially enlarged. The distinctive expression pattern of the SAUR41 gene and the explicit function of SAUR41 proteins implied that further investigations on the loss-of-function phenotypes of this gene in root development and environmental responses are of great interest.

DOI: https://doi.org/10.1093/pcp/pct028

Accepted Version

Originally published at:
Kong, Yingying; Zhu, Yubin; Gao, Chen; She, Wenjing; Lin, Weiqiang; Chen, Yong; Han, Ning; Bian, Hongwu; Zhu, Muyuan; Wang, Junhui (2013). Tissue-specific expression of SMALL AUXIN UP RNA41 differentially regulates cell expansion and root meristem patterning in Arabidopsis. Plant Cell Physiology, 54(4):609-621.

DOI: https://doi.org/10.1093/pcp/pct028
Tissue-specific Expression of SMALL AUXIN UP RNA41 Differentially Regulates Cell Expansion and Root Meristem Patterning in Arabidopsis

<table>
<thead>
<tr>
<th>Journal:</th>
<th>Plant and Cell Physiology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID:</td>
<td>PCP-2012-E-00558.R1</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Regular Paper</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>n/a</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
<td>Kong, Yingying; Zhejiang University, College of Life Sciences Zhu, Yubin; Zhejiang University, College of Life Sciences Gao, Chen; Zhejiang University, College of Life Sciences She, Wenjing; Zhejiang University, College of Life Sciences Lin, Weiqiang; Zhejiang University, College of Life Sciences Chen, Yong; Zhejiang University, College of Life Sciences ning, han; Zhejiang University, College of life sciences hongwu, bian; Zhejiang University, College of life sciences muyuan, zhu; Zhejiang University, College of life sciences Wang, Junhui; Zhejiang University, College of life sciences</td>
</tr>
<tr>
<td>Keywords:</td>
<td>SAUR, Arabidopsis, Auxin transport, Cell expansion, Root meristem patterning</td>
</tr>
</tbody>
</table>
Running title: SAUR41 in cell expansion and root development

Manuscript type: Regular paper

*Correspondence author:
Junhui Wang
Institute of Genetics, College of Life Sciences, Zhejiang University, 368 Yuhangtang Road, Hangzhou 310058, China
Tel: 0086-571-88206495; Fax: 0086-571-88206535
E-mail: junhuiwang@zju.edu.cn.

Subject area: growth and development

Number of black and white figures: 1
Number of color figures: 5
Number of table: 0
Supplementary data: 1 table.
Tissue-specific Expression of \textit{SMALL AUXIN UP RNA41} Differentially Regulates Cell Expansion and Root Meristem Patterning in Arabidopsis

Yingying Kong	extsuperscript{1,2,3}, Yubin Zhu	extsuperscript{1,2,3}, Chen Gao	extsuperscript{1}, Wenjing She	extsuperscript{1}, Weiqiang Lin	extsuperscript{1}, Yong Chen	extsuperscript{1}, Ning Han	extsuperscript{1}, Hongwu Bian	extsuperscript{1}, Muyuan Zhu	extsuperscript{1} and Junhui Wang	extsuperscript{1,*}

	extsuperscript{1}Institute of Genetics, College of Life Sciences, Zhejiang University, Zijingang Campus, Hangzhou 310058, China

	extsuperscript{2}Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou 310016, China

These authors contributed equally to this work.

Abbreviations: ARF, AUXIN RESPONSE FACTOR; ACR4, ARABIDOPSIS CRINKLY4; Aux/IAA, AUXIN/INDOLE-3-ACETIC ACID; CaMV, Cauliflower Mosaic Virus; DIC, differential-interference-contrast; EGFP, enhanced green fluorescent protein; GA, gibberellins; GH3, Gretchen Hagen3; GUS, beta-glucuronidase; LRC, lateral root cap; NAA, 1-naphthaleneacetic acid; PI, propidium iodide; PIN, PINFORM; PLT2, PLETHORA2; SAUR, SMALL AUXIN UP RNA; TIR1/AFB, TRANSPORT INHIBITOR RESPONSE1/AUXIN-BINDING F-BOX PROTEIN; WOX5, WUSCHEL-RELATED HOMEobox 5.
ABSTRACT

Among the three primary auxin-induced gene families, Aux/IAA, GH3, and SAUR, the function of SAUR genes remains unclear. Arabidopsis SAUR genes have been phylogenetically classified into three clades. Recent work has suggested that SAUR19 (clade II) and SAUR63 (clade I) promote cell expansion through the modulation of auxin transport. Herein, we present our work on SAUR41, a clade III SAUR gene with a distinctive expression pattern in root meristems. SAUR41 was normally expressed in the quiescent center and cortex/endodermis initials; upon auxin stimulation, the expression was provoked in the endodermis layer. During lateral root development, SAUR41 was expressed in prospective stem cell niches of lateral root primordia and in expanding endodermal cells surrounding the primordia. SAUR41-EGFP fusion proteins localized to the cytoplasm. Overexpression of SAUR41 from the CaMV 35S promoter led to pleiotropic auxin-related phenotypes, including long hypocotyls, increased vegetative biomass and lateral root development, expanded petals and twisted inflorescence stems. Ectopic SAUR41 proteins were able to promote auxin transport in hypocotyls. Tissue-specific expression of SAUR41 from the PIN1, WOX5, PLT2, and ACR4 promoters induced the formation of new auxin accumulation/signaling peaks above the quiescent centers, whereas tissue-specific expression of SAUR41 from the PIN2 and PLT2 promoters enhanced root gravitropic growth. Cells in the root stem cell niches of these transgenic seedlings were differentially enlarged. The distinctive expression pattern of SAUR41 gene and the explicit function of SAUR41 proteins implied that further investigations on the loss-of-function phenotypes of this gene in root development and environmental responses are of great interest.

Key words: SAUR; Arabidopsis; Auxin transport; Cell expansion; Root meristem patterning
Introduction

The plant hormone auxin modulates many aspects of plant growth and development, and recent decades have seen rapid progress in our understanding of the molecular mechanism of auxin biology (Woodward and Bartel, 2005; Santner and Estelle, 2009; Vanneste and Friml, 2009). For instance, in the field of auxin control of root development, numerous fundamental insights have been achieved on the contribution of shoot-derived auxin and root-generated auxin in root development (Overvoorde et al., 2010), establishment and maintenance of root stem cell niches (Aichinger et al., 2012; Perilli et al., 2012; Petricka et al., 2012), hormone interactions in coordinated root growth (Petricka et al., 2012; Ubeda-Tomáš et al., 2012), and root growth in response to the environment (Jones and Ljung, 2012; Petricaka et al., 2012).

Auxin regulates cell division, differentiation and elongation in part by changing gene expression (Woodward and Bartel, 2005; Paponov et al., 2008; Santner and Estelle, 2009; Vanneste and Friml, 2009; Hayashi, 2012). Primary auxin response genes consist of members of three gene families (Hagen and Guilfoyle, 2002): Aux/IAA (Auxin/Indole-3-Acetic Acid), GH3 (Gretchen Hagen), and SAUR (SMALL AUXIN UP RNA). Aux/IAA proteins are transcription repressors (Ulmasov et al., 1997; Worley et al., 2000; Gray et al., 2001; Ouelllet, et al., 2001; Ramos et al., 2001; Reed, 2001; Rogg et al., 2001; Tiwari et al., 2001). Their domain II interacts with the TIR1/AFB family of auxin receptors, while their domains III and IV dimerize with the ARF family transcription factors (Kim et al., 1997; Gray et al., 2001). Upon an auxin stimulus, Aux/IAA proteins are ubiquitinated by the TIR1/AFB proteins and then degraded, which release the activity of ARF proteins (Dharmasiri et al., 2005; Kepinski and Leyser, 2005; Tan et al., 2007). The GH3 family of acyl acid amido synthetases contributes to the amino acid conjugation of indole acetic acid, jasmonic acid, or salicylic acid (Ljung et al., 2002; Staswick et al., 2005; Park et al., 2007). The crystal structure and putative mechanisms of two GH3 proteins had been reported recently (Westfall et al., 2012). In contrast to Aux/IAA and GH3 proteins, the function of SAUR proteins remains unclear, most likely due to genetic redundancy within the
large SAUR gene family (Hagen and Guilfoyle, 2002; Jain et al., 2006).

Recent studies have begun to address the function of SAUR proteins in rice (*Oryza sativa*) and Arabidopsis. *OsSAUR39* has been identified as a negative regulator of auxin synthesis and transport, since rice plants overexpressing *OsSAUR39* exhibit reduced lateral root development and shoot and root length (Kant et al., 2009). The SAUR19 subfamily proteins (SAUR19-24) of Arabidopsis have been reported to be highly unstable; however, the addition of an N-terminal tag increases the stability of these proteins (Spartz et al., 2012). Arabidopsis plants overexpressing these stabilized SAUR fusion proteins exhibited increased hypocotyl elongation and larger leaf size, while seedlings expressing an artificial microRNA targeting the *SAUR19* subfamily genes exhibit opposite phenotypes (Franklin et al., 2011; Spartz et al., 2012). In another report on Arabidopsis, transgenic plant lines overexpressing *SAUR63:GFP* or *SAUR63:GUS* displayed long hypocotyls, petals and stamen filaments; in contrast, transgenic plants expressing artificial microRNA constructs targeting to the *SAUR63* subfamily genes (*SAUR61-68* and *SAUR75*) had slightly reduced hypocotyl and stamen filament elongation (Chae et al., 2012). The *SAUR19* subfamily genes were expressed in elongating tissues, including the root elongation zone (Spartz et al., 2012) while the *SAUR63* subfamily genes were not expressed in roots (Chae et al., 2012). As far as the mechanism is concerned, Arabidopsis SAUR proteins have been proposed to promote cell expansion through the modulation of auxin transport (Spartz et al., 2012; Chae et al., 2012).

The *SAUR* gene family could be phylogenetically classified into three clades (Kodira et al., 2011). The genes in clades I and II had a tendency to display higher expression levels in leaves and lower expressions in roots, while the genes in clade III demonstrated opposite expression patterns (Kodira et al., 2011). The *SAUR63* subfamily and the *SAUR19* subfamily were clade I and clade II *SAUR* genes, respectively (Kodira et al., 2011). Herein, we presented our work on *SAUR41*, a clade III *SAUR* gene displayed a distinctive expression pattern in Arabidopsis root meristems.

The *SAUR41* subfamily contained four members: *SAUR41* (At1g16510), *SAUR40*...
SAUR71 (At1g56150) and SAUR72 (At3g12830). Their amino acid sequences differed from other SAUR families in the N-terminus. In prior microarray experiments, the expression of SAUR41 has been reported to be regulated by circadian rhythm (Mazzella et al., 2005; Darrah et al., 2006), biotic stress (Zhang et al., 2007; Peltier et al., 2011), and mitochondrial dysfunction and reactive oxygen species (Carrie et al., 2010; Gleason et al., 2011). The expression of SAUR40 and SAUR71 was responsive to abscisic acid signaling (Leondardt et al., 2004; Zeng et al., 2012) and the functional status of chloroplasts (Bosco et al., 2004; Estavillo et al., 2011). SAUR71 and SAUR72 were expressed in the vascular development (Nagawa et al., 2006; Shirakawa et al., 2009).

We found that SAUR41 was normally expressed in the quiescent center and cortex/endodermis initials, but it was provoked in the endodermis layer upon an auxin or gravitropic stimulation. During lateral root development, SAUR41 was distinctively expressed in prospective stem cell niches of lateral root primordia and in expanding endodermal cells surrounding the primordia. SAUR41-EGFP fusion proteins localized to the cytoplasm, unlike EGFP-SAUR19 and SAUR63-EGFP which localized predominantly to the plasma membrane. Interestingly, although the gene expression pattern and the protein localization pattern of SAUR41 were different from SAUR19 and SAUR63, the phenotypes resulting from overexpression of SAUR41 driven by the CaMV 35S promoter shared many similarities with those of SAUR19 and SAUR63 subfamily genes. Tissue-specific expression of SAUR41 from promoters of auxin transporter genes and root meristem patterning genes differentially modulated root meristem development, root cell expansion, and root gravitropic growth. The distinctive expression pattern of SAUR41 gene and the explicit function of ectopic SAUR41 proteins implied that further investigations on the loss-of-function phenotypes of this gene family in root development are of great interest.

Results
SAUR41 had a distinctive expression pattern in Arabidopsis root meristems

Previously, to counteract possible position effects in plant promoter analysis, we used the gypsy-Su(Hw) system of Drosophila in a novel approach that facilitated high and precise expression of reporter genes (She et al., 2010). Using this system, together with the GATEWAY recombination approach, we generated sets of promoter reporter lines for the PIN gene family encoding auxin carriers and the TIR1/AFB gene family encoding auxin receptors (She et al., 2010). We extended this system to promoter analysis of certain SMALL AUXIN UP RNA (SAUR) genes, and found that the SAUR41 (At1g16510) gene showed a distinctive expression pattern in root meristems.

In generating promoter reporter lines for SAUR41, an ~1800 bp DNA fragment upstream of the ATG start codon of SAUR41, as predicted by AtcisDB (Davuluri et al., 2003), was fused with the EGFP-GUS reporter gene. Viewed by confocal microscopy, SAUR41 was found to be specifically expressed in the quiescent center and cortex/endodermis initials of root stem niches (Fig. 1A). Considering that SAUR genes had been proposed to function in auxin-mediated growth events, we examined the expression of SAUR41 under gravitropic stimulation and auxin treatment. Gravitropism allows plant roots to grow directionally, whereas auxin is an essential regulator in this process (Harrison and Masson, 2008). During the gravitropic response, the expression of SAUR41 was provoked on both sides of the endodermis at the proximal meristem region and on the upper side of the endodermis at distal elongation zone, indicating that this gene might act to coordinate root elongation rather than simply respond to auxin redistribution (Fig. 1B). SAUR41 was specifically induced in the endodermis with 1 h of auxin (10 µM 1-naphthaleneacetic acid, NAA) treatment (Fig. 1C). Since the endodermis was recently reported as the primary responsive tissue for gibberellins (GA) to coordinate root growth (Ubeda-Tomás et al., 2008, 2009, 2012), we then tested whether or not SAUR41 was a GA-responsive gene. As shown in Fig. 1D, SAUR41 was unresponsive to GA treatment. This result was in agreement with the results of a microarray experiment (Josse et al., 2011).

Extending the duration of auxin treatment to 12 h, and detected by overnight
histochemical staining, *SAUR41* was found to be induced in multiple cell layers at the root meristem and transition zone (Fig. 1E). However, in the elongation zone, it was specifically induced in the endodermal cells (Fig. 1F).

During lateral root development, *SAUR41* was expressed in the prospective quiescent center of lateral root primordia (Fig. 1G, H). Interestingly, *SAUR41* was also specifically expressed in the endodermal cells surrounding the lateral root primordia during the process of the lateral root primordia breaking through the endodermis (Fig. 1G). In the newly formed lateral roots, *SAUR41* was strongly expressed in the quiescent center and initial cells, and weakly expressed in the endodermis (Fig. 1I). In hypocotyls, petioles and cotyledons, *SAUR41* was predominantly expressed in the vascular tissues (Fig. 1J, K and L).

**SAUR41-EGFP fusion protein accumulated in the cytoplasm**

To check the subcellular localization of the SAUR41 protein, we generated a transgenic construct in which the CaMV (Cauliflower Mosaic Virus) 35S promoter drove a C-terminal translational fusion between the full-length SAUR41 and the EGFP protein. Location of the fusion protein in hypocotyls and root tips of stably-transformed Arabidopsis plants was examined by confocal microscopy. Results showed that the EGFP fluorescence was identified at the cytoplasm of epidermal and cortical cells in hypocotyls (Fig. 1M, N); and at the cytoplasm of all types of cells in root tips, including quiescent center cells, cortex/endodermis initial cells and epidermal cells (Fig. 1O, P).

**Overexpression of *SAUR41* conferred pleiotropic auxin-related phenotypes**

To explore potential roles for *SAUR41* in Arabidopsis growth and development, we first screened T-DNA and transposon insertion lines from the Arabidopsis Biological Resource Center (ABRC) and the Rikagaku Kenkyusho Bioresource Center (RIKEN-BRC), respectively. The line SALK_056968 from the ABRC stocks
contained a T-DNA at the promoter region of \textit{SAUR41}. However, RT-PCR analysis revealed that this insertion did not impair the expression of \textit{SAUR41} (data not shown). In three additional lines (SALK\_121397, PST\_11030 and PST\_17947), we failed to identify DNA insertions inside the \textit{SAUR41} gene.

We then used a gain-of-function approach and generated transgenic Arabidopsis plants overexpressing untagged (35S::\textit{SAUR41}) or MYC-tagged (35S::\textit{SAUR41-MYC}) \textit{SAUR41} gene, in addition to the 35S::\textit{SAUR41-EGFP} plants described above. More than 20 independent lines were obtained for each transgenic construct. Plants overexpressing untagged \textit{SAUR41} had strong phenotypes, similar to that of MYC-tagged \textit{SAUR41}. In contrast, plants overexpressing EGFP-tagged \textit{SAUR41} had the weakest phenotypes. We chose 35S::\textit{SAUR41} plants for detailed study.

The 35S::\textit{SAUR41} transgenic lines displayed pleiotropic auxin-related phenotypes. Light-grown seedlings exhibited 1.7-2.0-fold longer hypocotyls than wild-type controls (Fig. 2A, B, E; $P<0.01$, \textit{t}-test). Surprisingly, these seedlings also displayed 1.3-1.5-fold longer primary roots compared to wild-type controls (Fig. 2C, D, F; $P<0.05$, \textit{t}-test). In addition, overexpression of \textit{SAUR41} increased root waving on vertically-oriented agar plates (Fig. 2D). After 10 d of growth, 35S::\textit{SAUR41} seedlings had 1.5-1.9-fold increase in lateral root numbers ($P<0.05$, \textit{t}-test) and 20-30\% increase in vegetative biomass as measured by the fresh weight of shoots (Fig. 2G, H; $P<0.05$, \textit{t}-test).

Adult 35S::\textit{SAUR41} plants had twisted inflorescence stems (Fig. 2I). Furthermore, the petals of transgenic flower organs were over-expanded and defective in opening completely (Fig. 2J, K and L), resulting in reduced seed setting in many siliques (Fig. 2I).

To determine if the elongated hypocotyl phenotype induced by ectopic \textit{SAUR41} was due to increased cell expansion, we measured hypocotyl epidermal cell length in 6-d-old seedlings. The change in hypocotyl epidermal cell length was parallel to the change in hypocotyl length (Fig. 3A, Fig. 2E). We then directly measured $^{3}$H IAA transport in hypocotyls and detected a 40-70\% increase in basipetal IAA transport in hypocotyls of 35S::\textit{SAUR41-EGFP}, 35S::\textit{SAUR41-MYC}, and 35S::\textit{SAUR41} seedlings.
Tissue-specific expression of SAUR41 from the PIN1 promoter induced alterations in root meristem patterning

As SAUR41 had a distinctive expression pattern in Arabidopsis root meristems, and rice and Arabidopsis SAUR proteins have been proposed to modulate auxin transport (Kant et al., 2009; Spartz et al., 2012; Chae et al., 2012), we next implemented tissue-specific expression of SAUR41 from promoters of auxin transporter genes and root meristem patterning genes. To facilitate the examination of auxin signaling and distribution, all transgenic plants were generated in a DR5rev::GFP background (Friml et al., 2003; Fig. 4B).

We first expressed SAUR41 from the PIN1 and PIN2 promoters. In Arabidopsis roots, PIN1 promoter activity was strong in stele cells and weak in endodermis and the quiescent center (She et al., 2010; Fig. 4C). Ectopic expression of SAUR41 driven by the PIN1 promoter led to auxin retention in stele initials transporting auxin, resulting in a large auxin accumulation/signaling peak (Fig. 4D-G). In addition, PIN1::SAUR41 roots had additional tiers of distal stem cells (Fig. 4E, G), while wild-type roots typically had one tier of distal stem cells (Ding and Friml, 2010; Fig. 4A, B). Non-differentiated distal stem cells below the quiescent center were characterized by the absence of starch grains (Ding and Friml, 2010; Fig. 4H, L). Finally, PIN1::SAUR41 roots had supernumerary cell layers (Fig. 4E, G), while wild-type roots exhibited distinctive root radial patterns (Fig. 4A, B). Statistically, 80-90% PIN1::SAUR41 roots displayed abnormalities in root meristem patterning (n=40, P<0.05, t-test).

PIN2 promoter activity was detected in the epidermis, cortex and lateral root cap (She et al., 2010; Fig. 4I). Ectopic expression of SAUR41 from the PIN2 promoter induced expansion of epidermal and cortical cells, but the auxin accumulation/signaling pattern appeared normal (Fig. 4J). PIN2::SAUR41 roots exhibited a root-waving phenotype on vertically-oriented agar plates, and the
epidermal and cortical cells in the two sides of root transition zones were irregularly
and asymmetrically elongated (Fig. 4K).

Comparison of tissue-specific expression of SAUR41 and IAA2<sup>P65S</sup> from
promoters of root meristem patterning genes

We also expressed SAUR41 from the promoters of root meristem patterning genes.
Three promoters were chosen: WOX5, ACR4, and PLT2. WOX5 encoded a
homeodomain transcription factor (Sarkar et al., 2007), PLT2 encoded an AP-2 type
transcription factor (Aida et al., 2004; Galinha et al., 2007), and ACR4 encoded a
receptor-like kinase (De Smet et al., 2008). They were three of the major regulators of
root stem cell activity (Aichinger et al., 2012; Perilli et al., 2012; Petricka et al., 2012).
To compare the effects between tissue-specific expression of a SAUR protein and a
stabilized AUX/IAA protein, we introduced IAA2<sup>P65S</sup> that harbored a site-directed
mutation in the proline residue of conserved domain II. As one of 29 Aux/IAA genes
of Arabidopsis, IAA2 was highly auxin inducible and expressed in vascular tissues and
auxin accumulation/signaling peaks (Swarup et al., 2001).

WOX5 was expressed in the quiescent center of root stem cell niches (Sarkar et al.,
2007; Fig. 5A). Tissue-specific expression of SAUR41 from the WOX5 promoter
induced the formation of additional auxin accumulation/signaling peaks in stele
initials above the quiescent center (Fig. 5B-F). In addition, WOX5::SAUR41 roots had
supernumerary cell layers (Fig. 5C, D, F), like those observed in PIN1::SAUR41 roots
(Fig. 4E, G). As expected, tissue-specific expression of IAA2<sup>P65S</sup> driven by the WOX5
promoter inhibited auxin accumulation/signaling in the quiescent center (Fig. 5G, H).
Interestingly, it also inhibited auxin accumulation/signaling in the stele initials (Fig.
5G, H), opposite to the effects observed in tissue-specific expression of SAUR41 from
the WOX5 promoter (Fig. 5B-F).

ACR4 was expressed in the root stem cell niche and its surrounding cells, including
young epidermal cells, cortical cells and columella cells (De Smet et al., 2008; Fig.
5I). Ectopic expression of SAUR41 from the ACR4 promoter led to the expansion of
cells expressing ACR4 (Fig. 5J, K). Formation of auxin accumulation/signaling peaks in stele initials was visible (Fig. 5J, K), but not as dramatically as that in the WOX5::SAUR41 roots (Fig. 5B-F), indicating that the balanced expression of SAUR41 in the entire root stem cell niche attenuated the effects of ectopic SAUR proteins on auxin transport, compared to the selected expression of SAUR41 in the quiescent center by the WOX5 promoter. On the other hand, tissue-specific expression of IAA2p65S from the ACR4 promoter had a more remarkable impact on root meristem development compared to that seen in WOX5::IAA2p65S roots. As shown in Fig. 5, ACR4::IAA2p65S roots had two separate auxin accumulation/signaling maxima: one in the quiescent center, the other in the young columella cells (Fig. 5L). 

PLT2 was gradiently expressed in root meristems and lateral cap cells (Aida et al., 2004; Galinha et al., 2007; Fig. 5M). Tissue-specific expression of SAUR41 from the PLT2 promoter exhibited triple effects as addressed above: additional auxin accumulation/signaling peaks in stele initials, and supernumerary cell layers in the proximal meristem zone (Fig. 5N, O). On the other hand, tissue-specific expression of IAA2p65S from the PLT2 promoter inhibited auxin accumulation/signaling in the quiescent center, promoted auxin accumulation/signaling in stele initials, and led to establishment of new auxin accumulation/signaling peaks in the adjacent provascular cells (Fig. 5P), similar to that seen for relocalization of auxin accumulation/signaling peaks upon polar auxin transport inhibitor treatment (Sabatini et al., 1999).

Ectopic SAUR41 proteins differentially regulated root cell expansion and root gravitropic growth

We measured cell areas of stele initial cells, quiescent center cells, and distal stem cells in root meristems of transgenic seedlings expressing SAUR41 from promoters of auxin transporter genes and root meristem patterning genes. Results showed that PIN1::SAUR41, WOX5::SAUR41, and PLT2::SAUR41 roots had a 30-60% increase in cell areas of stele initial cells and quiescent center cells, while ACR4::SAUR41 roots had enlarged quiescent center cells and distal stem cells (Fig. 6A, P<0.05, t-test).
The root gravitropic responses in these transgenic seedlings were also analyzed. 

ACR4::SAUR41 roots showed delayed gravitropic growth (Fig. 6B), coinciding with their higher auxin accumulation/signaling in young columella cells but lower auxin accumulation/signaling in root lateral cap cells (Fig. 5I). PIN2::SAUR41 roots had advanced gravitropic growth 2 h after the gravitropic stimulation, while PLT2::SAUR41 roots displayed enhanced gravitropic growth 3 h after the gravitropic stimulation (Fig. 6B). It has been suggested that auxin transport in lower side of lateral cap cells and auxin accumulation in lower side of epidermal and cortical cells were essential for root gravitropic response (Ottenschläger, et al., 2003). Our results were consistent with the expression pattern of PIN2 and PLT2 promoters. PIN2 was expressed in both lateral cap cells and epidermal and cortical cells (Fig. 4I), while PLT2 was expressed in lateral cap cells (Fig. 5M).

Discussion

The expression pattern of SAUR41 in root meristems was distinctive. SAUR19 subfamily genes were expressed in growing hypocotyls in response to shade avoidance, and in root elongation zones in response to auxin treatment (Spartz et al., 2012). SAUR63 and members of its clade were expressed in growing regions of hypocotyls, cotyledons, petiole, young rosette leaves and inflorescence stems, but not in roots (Chae et al., 2012). Herein, we found that the expression of SAUR41 was normally restricted to the quiescent center and cortex/endodermis initials of root meristems; upon an auxin or gravitropic stimulation, it was provoked in the endodermis at proximal meristem region and transition zone of Arabidopsis roots (Fig. 1A-F). Furthermore, SAUR41 was differentially expressed during lateral root development, as manifested by GUS activity in prospective stem cell niches of lateral root primordia, and in expanding endodermal cells surrounding the lateral root primordia (Fig. 1G-I). In prior microarray experiments, the expression of SAUR41 has been reported to be regulated by multiple environmental signals (Mazzella et al., 2005; Darrah et al., 2006; Zhang et al., 2007; Peltier et al., 2011). Taken together, it seemed
that \textit{SAUR41} was actively expressed in response to both developmental and environmental cues.

\textit{SAUR41}-EGFP fusion proteins localize to the cytoplasm (Fig. 1M-P), while EGFP-\textit{SAUR19} and \textit{SAUR63}-EGFP localize predominantly to the plasma membrane (Spartz et al., 2012; Chae et al., 2012). Although the protein localization pattern and the gene expression pattern of \textit{SAUR41} were different from \textit{SAUR19} and \textit{SAUR63}, the phenotypes resulting from overexpression of \textit{SAUR41} driven by the CaMV \textit{35S} promoter shared many similarities with those of \textit{SAUR19} and \textit{SAUR63} subfamily genes. In all three cases, transgenic seedlings had elongated hypocotyls, expanded hypocotyl epidermal cells, and enhanced hypocotyl basipetal IAA transport, compared to wild type (Fig. 2A, B, E; Fig. 3; Spartz et al., 2012; Chae et al., 2012). In the cases of \textit{SAUR41} and \textit{SAUR19}, transgenic seedlings displayed waving roots and increased vegetative biomass (Fig. 2C, D, H; Spartz et al., 2012); while in the cases of \textit{SAUR41} and \textit{SAUR63}, transgenic plants had expanded petals and twisted inflorescence stems (Fig. 2I-L; Chae et al., 2012). Interestingly, only in the case of \textit{SAUR41} did the transgenic seedlings have increased primary root length and lateral root development compared to wild-type controls (Fig. 2F, G). Thus, it seems likely that Arabidopsis \textit{SAUR} proteins have some similarities but also specificity in terms of molecular functions. Alternatively, \textit{SAUR} proteins may share a similar molecular function, but different \textit{SAUR} proteins require different tissue-specific partners.

Tissue-specific expression of \textit{SAUR41} from \textit{PIN1}, \textit{WOX5}, \textit{ACR4} or \textit{PLT2} promoters caused new auxin accumulation/signaling peaks in stele initial cells transporting auxin (Fig. 4D-G; Fig. 5B-F, N, O). Roots of \textit{PIN1::SAUR41} seedlings had additional tiers of distal stem cells below the quiescent center and supernumerary cell layers in root meristems (Fig. 4D-G, H, L, Q). It has been reported that auxin regulates distal stem cell differentiation in Arabidopsis roots, and defects in auxin transport would lead to additional tiers of distal stem cells (Ding and Friml, 2010). Thus, it seemed likely that \textit{SAUR41} induced perturbation of auxin transport in root meristems as it was expressed above the stem cell niches (from the \textit{PIN1} promoter).

In contrast, tissue-specific expression of \textit{SAUR41} from the \textit{PIN2} promoter did not
induce alterations in root meristem patterning, but caused alterations in cell expansion in the corresponding cell lineages (Fig. 4J, K). In addition, PIN2::SAUR41 and PLT2::SAUR41 roots had enhanced gravitropic growth (Fig. 6B), indicating that ectopic SAUR41 proteins promoted root basipetal auxin transport for root gravitropic responses. Taken together, it seems likely that ectopic SAUR41 proteins retard auxin transport in root stem cell niches, but promote auxin transport in root lateral cap cells and epidermal and cortical cells.

Previously, it has been proposed that rice SAUR39 acted as a negative regulator of organ growth and auxin transport (Kant et al. 2009), while Arabidopsis SAUR19 and SAUR63 acted as positive regulators of cell expansion and auxin transport (Spartz, et al., 2012; Chae et a., 2012). Herein, in terms of cell expansion, we found that SAUR41 promoted cell expansion, as it was constitutively expressed from the CaMV 35S promoter (Fig. 2, 3). In addition, stele initial cells, quiescent center cells, and distal stem cells in root meristems of transgenic seedlings expressing SAUR41 from promoters of auxin transporter genes and root meristem patterning genes were differentially enlarged (Fig. 6A). Thus, similar to SAUR19 with N-terminal tags and SAUR63 with C-terminal tags, untagged SAUR41 promoted cell expansion, as it was ectopically expressed. However, in terms of auxin transport, the functions of SAUR proteins appeared to be more complicated. The observed higher flow rate of labeled IAA in hypocotyls could be an indirect effect of SAUR protein overexpression. Two questions, why ectopic SAUR41 proteins retarded auxin transport in root stem cell niches but promoted basipetal auxin transport, and why rice OsSAUR39 (analogous to Arabidopsis SAUR63, clade I) inhibited auxin transport but Arabidopsis SAUR63 promoted auxin transport, remained unanswered. It should be noticed that the DR5::GFP marker basically indicated the status of auxin signaling but not the auxin transport. Currently, direct measurement of auxin transport in root stem cell niches is unavailable. It was tempting to speculate that SAUR41 proteins used different mechanism to regulate auxin transport for cell elongation and for root meristem patterning.

Tissue-specific expression of IAA2P65S from WOX5, ACR4 and PLT2 promoters
displayed fundamentally different effects on root meristem patterning compared to that observed for *SAUR41* (Fig. 5). The mechanism of stabilized Aux/IAA proteins is clear. They impaired the SCF\textsuperscript{TIR1} pathway of auxin signaling to regulate cell division, differentiation and elongation. They also disturbed auxin transport by transcriptional modification of the auxin export machinery (Hayashi, 2012; Scherer et al., 2012). On the contrary, the precise mechanism by which SAUR proteins regulate cell expansion and auxin transport remains unclear. It will be interesting to learn whether there exist epistatic interactions between the *IAA2* and the *SAUR41* gain of function phenotypes. We are currently crossing the *IAA2*\textsuperscript{P65S} lines with the corresponding *SAUR41* lines to answer this question.

The *SAUR41* function reported here was solely depends on the ectopic expression data, while its endogenous role in stem cell maintenance remained unclear. The gene could be involved in the regulation of cell sizes of quiescent center and cortex/endodermis initials, and/or in the modulation of auxin transport in these cells. In addition, the *SAUR41* subfamily contains four members: *SAUR40, SAUR41, SAUR71* and *SAUR72*. Further investigations on promoter activity and protein localization patterns of other *SAUR41* subfamily members, as well as on loss-of-function phenotypes of the *SAUR41* gene family, are required and of great interest.
Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia-0 and the DR5rev::GFP background (Friml et al., 2003) were used as sources of wild-type plant materials. Promoter reporter lines for the PIN gene family and the TIR1/AFB gene family (She et al., 2010) have been donated to ABRC. Seeds were surface-sterilized and cultured aseptically on 9 cm Petri dishes containing Gamborg’s B5 medium with 1% (w/v) sucrose and 1% (w/v) agar. The plates were maintained at 4˚C for 2 d, and then transferred to a culture room (23˚C, 80 µM m⁻² s⁻¹ irradiance with a 16-h photoperiod, 30-40% RH).

Vector construction and plant transformation

We used the GATEWAY™ system for vector construction. Entry vectors were created using the pENTR™/D-TOPO kits (Invitrogen). The PCR primers for construction of entry vectors for the coding region of SAUR41 and for promoter regions of SAUR41, WOX5, ACR4 and PLT2 were listed in Table S1. IAA2P65S was generated by overlapping PCR using primers in Table S1. Each entry clone was confirmed by DNA sequencing. GATEWAY™ compatible destination vectors for protein subcellular localization, overexpression, MYC tagging and promoter analysis were used (Karimi et al., 2002; Earley et al., 2006; She et al., 2010). The LR Reaction was conducted to generate different expression vectors. To facilitate tissue-specific gene expression from various promoters, the CaMV 35S promoter in the overexpression constructs (35S::SAUR41 and the 35S::IAA2P65S) was replaced with a ccdB fragment by a method described previously (Yang et al., 2012). Briefly, the ccdB fragment was PCR-amplified using pH7FWG2,0 as a template, with the primers ccdB-Up and ccdB-Dn, containing an introduced HindIII and SpeI site, respectively. The ccdB fragment was then digested to replace the 35S promoter sequence, thus forming new destination vectors for tissue-specific expression.

All of the expression vectors were electroporated into Agrobacterium tumefaciens strain GV3101. Plants were transformed using the vacuum infiltration method.
(Bechtold et al., 1993). Transgenic plants were selected on B5 plates with 12.5 µg/mL hygromycin or 25 µg/mL kanamycin depending on the selection markers. Single-locus and homozygous transgenic lines were characterized as we described previously (She et al., 2010).

**Microscopic analysis and Histochemical detection**

For histochemical detection of GUS activities, young seedlings at different developmental stages and different parts from transgenic plants were collected. They were stained at 37°C overnight in 1 mM 5-bromo-4-chloro-3-indolyl-b-D-glucuronic acid (X-Gluc), 1 mM potassium ferricyanide, 0.1% Triton X-100, and 0.1 M sodium phosphate buffer, pH 7.0 with 10 mM EDTA. Samples were washed in 70% ethanol to remove chlorophyll. DIC (differential-interference-contrast) images were visualized using a microscope (Nikon Eclipse 80i, Japan) with DXM1200 CCD camera and EclipseNet software. For the localization of fluorescence fusion proteins, a confocal microscope system (Zeiss LSM510, Germany) was used. Without specification, 5-d-old seedlings were mounted in water. Starch grains in columella cells were stained with I$_2$-KI as described previously (Ding and Friml, 2010).

**Hypocotyl IAA transport assay**

IAA transport in hypocotyls was measured as previously described (Chae, 2012). [$^3$H]IAA was a product of American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). The radioisotope counts of [$^3$H] IAA was detected using a low-noise scintillation counter (MicroBeta 2, Perkin Elmer).

**Growth and cell measurement, statistical analysis, and image processing**

After incubations for the durations indicated in the text, the plates were digitally photographed. Root and hypocotyl length was measured using magnified images. Lateral root (>1 mm) numbers were counted using each seedling as an individual sample. For hormone treatment, seedlings were transferred onto a medium containing 10 µM NAA or GA$_3$ for 1 or 12 h. For gravitropism assays, the protocol of Weijers et
al. (2005) was adopted. The mean hypocotyl epidermal cell length and the cell area of
root stem cell niches were measured as described by Spartz et al. (2012). Each
treatment contained 30-50 seedlings and was replicated 3 times. Statistical analysis of
the data was performed using Microsoft Excel and Student’s *t*-test. Images were
processed using Adobe Photoshop CS2.
Supplementary data

Supplementary data are available at PCP online.

Funding

This work was supported by funding from the National Natural Science Foundation of China (grant no. 31170211 to J.W.).

Acknowledgements

We are grateful to ABRC and RIKEN-BRC for the distribution of Arabidopsis materials. No conflict of interest is declared.

References


Kodaira K.-S., Qin, F., Tran, L.-S.P., Maruyama, K., Kidokoro, S., Fujita, Y. et al.


She, W., Lin, W., Zhu, Y., Chen, Y., Jin, W., Yang, Y., et al. (2010) The gypsy insulator of *Drosophila melanogaster* together with its binding protein *Su(Hw)* (Suppressor of Hairy-wing) facilitate high and precise expression of transgenes in *Arabidopsis thaliana*. *Genetics* 185: 1141-1150

Vacuolar SNAREs function in the formation of the leaf vascular network by regulating auxin distribution. *Plant Cell Physiol.* 50: 1319-1328


*Science* 336: 1708-1711.


*Plant J.* 21: 553–562


Doi:10.1104/pp.112.206946


Figure legends

Fig. 1. The expression pattern of the *SAUR41* gene, and the subcellular localization of *SAUR41* fusion protein. An ~1800 bp promoter region of *SAUR41* was fused with the *EGFP-GUS* reporter gene. Green EGFP (enhanced green fluorescent protein) signals and red PI (propidium iodide, staining cell wall here) signals were viewed by confocal microscopy. GUS (beta-glucuronidase) activity stained in blue was viewed by DIC (differential-interference-contrast) microscopy. *SAUR41* was normally restricted to the quiescent center and cortex/endodermis initials at the root meristems (A). Upon a gravitropic (B) or auxin (C) stimulation, *SAUR41* was provoked in the endodermis layer. (D) *SAUR41* was unresponsive to GA treatment. After extended auxin treatment, *SAUR41* was induced in multiple cell layers at the root meristem (E); however, in the elongation zone, it was specifically induced in the endodermal cells (F). During lateral root development, *SAUR41* was expressed in the prospective quiescent center and initial cells of lateral root primordia (G, H) and in the endodermal cells surrounding the lateral root primordia (G). Insets are enlargements of lateral root primordia. In a new lateral root, *SAUR41* was strongly expressed in quiescent center and initial cells (I). *SAUR41* was predominantly expressed in the vascular tissues of hypocotyls (J), petioles (K) and cotyledons (L). *SAUR41*-EGFP fusion proteins localized to the cytoplasm of epidermal cells and cortical cells in hypocotyls (1M, N) and to the cytoplasm of quiescent center cells, cortex/endodermis initial cells and epidermal cells in root tips (O, P). LRC, lateral root cap; Ep, epidermis; C, Cortex; En, endodermis. Bars: 25 µm.

Fig. 2. Overexpression of *SAUR41* from the CaMV 35S promoter confered pleiotropic auxin- or cell expansion-related phenotypes. (A-E) Light-grown 6-d-old seedlings. (F-H) Light-grown 10-d-old seedlings. (I-L) Adult plants. Hypocotyls of wild-type controls (A) and 35S::*SAUR41* seedlings (B). Roots of wild-type controls (C) and 35S::*SAUR41* seedlings (D). (E-H) Statistical comparison of hypocotyl length (E), primary root length (F), lateral root number (G) and vegetative biomass (H) of wild...
type controls with 35S::SAUR41 seedlings. (I-L) Adult 35S::SAUR41 plants had twisted inflorescence stems (I) and over-expanded petals (J-L). Roots or flowers marked by arrows were enlarged in the subsequent images. **, P<0.01; *, P<0.05, t-test.

Fig. 3. Ectopic SAUR41 promoted hypocotyl epidermal cell expansion and IAA transport. (A) Mean hypocotyl epidermal cell length in 6-d-old seedlings. (B) Relative 3H IAA basipetal transport in hypocotyls of 7-d-old seedlings. **, P<0.01; *, P<0.05, t-test.

Fig. 4. Tissue-specific expression of SAUR41 from the PIN1 and PIN2 promoters. Green EGFP (enhanced green fluorescent protein) signals and red PI (propidium iodide, staining cell wall here) signals were viewed by confocal microscopy. Purple starch grains stained by Lugol’s solution were viewed by DIC (differential-interference-contrast) microscopy. (A) The root stem cell niches. (B) Auxin accumulation/signaling pattern in root meristems of the DR5rev::GFP background line. White arrow indicated the quiescent center, while blue arrow indicates the tier of distal stem cells. Boxed areas were magnified in the subsequent images. Insects were enlargements of root stem cell niches. (C) Promoter activity of PIN1. (D-F) Tissue-specific expression of SAUR41 from the PIN1 promoter led to auxin retention in stele initials transporting auxin (pink arrow), additional tiers of distal stem cells (blue arrow), and supernumerary cell layers (white bracket). (H, L) Differentiation status of columella cells in control roots (H) and PIN1::SAUR41 roots (L) determined by Lugol’s solution staining. (I) Promoter activity of PIN2. (J-K) Tissue-specific expression of SAUR41 from the PIN2 promoter did not induce auxin retention but caused irregular cell expansion in the corresponding cells. LRC, lateral root cap; Ep, epidermis; C, Cortex; En, endodermis. Bars: 25 µm.

Fig. 5. Tissue-specific expression of SAUR41 and IAA2P65S from promoters of root meristem patterning genes. Green EGFP (enhanced green fluorescent protein) signals
and red PI (propidium iodide, staining cell wall here) signals were viewed by confocal microscopy. Boxed areas were magnified in the subsequent images. Insects were enlargements of root stem cell niches. (A) Auxin accumulation/signaling pattern in root meristems of the DR5rev::GFP background line (left) and the promoter activity of WOX5 (right). (B-F) Tissue-specific expression of SAUR41 from the WOX5 promoter. (G, H) Tissue-specific expression of IAA2P65S from the WOX5 promoter. (I) Promoter activity of ACR4. (J, K) Tissue-specific expression of SAUR41 from the ACR4 promoter. (L) Tissue-specific expression of IAA2P65S from the ACR4 promoter. (M) Promoter activity of PLT2. (N, O) Tissue-specific expression of SAUR41 from the PLT2 promoter. (P) Tissue-specific expression of IAA2P65S from the PLT2 promoter. Arrow indicated the division of endodermal cells. < indicated additional auxin accumulation/signaling peaks; * indicated the quiescent center; + indicated tiers of distal stem cells; and [ indicated the supernumerary cell layers. Bars: 25 µm.

Fig. 6. Ectopic SAUR41 proteins differentially regulated root cell expansion and root gravitropic growth. (A) Cell areas of stele initial cells, quiescent center cells and distal stem cells in roots of 5-d-old seedlings. (B) Root gravitropic responses in 6-d-old seedlings. *, P<0.05, t-test.
Fig. 1. The expression pattern of the SAUR41 gene, and the subcellular localization of SAUR41 fusion protein. An ~1800 bp promoter region of SAUR41 was fused with the EGFP-GUS reporter gene. Green EGFP (enhanced green fluorescent protein) signals and red PI (propidium iodide, staining cell wall here) signals were viewed by confocal microscopy. GUS (beta-glucuronidase) activity stained in blue was viewed by DIC (differential-interference-contrast) microscopy. SAUR41 was normally restricted to the quiescent center and cortex/endodermis initials at the root meristems (A). Upon a gravitropic (B) or auxin (C) stimulation, SAUR41 was provoked in the endodermis layer. (D) SAUR41 was unresponsive to GA treatment. After extended auxin treatment, SAUR41 was induced in multiple cell layers at the root meristem (E); however, in the elongation zone, it was specifically induced in the endodermal cells (F). During lateral root development, SAUR41 was expressed in the prospective quiescent center and initial cells of lateral root primordia (G, H) and in the endodermal cells surrounding the lateral root primordia (G). Insets are enlargements of lateral root primordia. In a new lateral root, SAUR41 was strongly expressed in quiescent center and initial cells (I). SAUR41 was predominantly expressed in the vascular tissues of hypocotyls (J), petioles (K) and cotyledons (L). SAUR41-EGFP fusion proteins localized to the cytoplasm of epidermal cells and cortical cells in hypocotyls (1M, N) and to the cytoplasm of quiescent center cells, cortex/endodermis initial cells and epidermal cells in root tips (O, P). LRC, lateral root cap; Ep, epidermis; C, Cortex; En, endodermis. Bars: 25 µm.
Fig. 2. Overexpression of SAUR41 from the CaMV 35S promoter conferred pleiotropic auxin- or cell expansion-related phenotypes. (A-E) Light-grown 6-d-old seedlings. (F-H) Light-grown 10-d-old seedlings. (I-L) Adult plants. Hypocotyls of wild-type controls (A) and 35S::SAUR41 seedlings (B). Roots of wild-type controls (C) and 35S::SAUR41 seedlings (D). (E-H) Statistical comparison of hypocotyl length (E), primary root length (F), lateral root number (G) and vegetative biomass (H) of wild type controls with 35S::SAUR41 seedlings. (I-L) Adult 35S::SAUR41 plants had twisted inflorescence stems (I) and over-expanded petals (J-L). Roots or flowers marked by arrows were enlarged in the subsequent images. **, P<0.01; *, P<0.05, t-test.
Fig. 3. Ectopic SAUR41 promoted hypocotyl epidermal cell expansion and IAA transport. (A) Mean hypocotyl epidermal cell length in 6-d-old seedlings. (B) Relative 3H IAA basipetal transport in hypocotyls of 7-d-old seedlings. **, P<0.01; *, P<0.05, t-test.

99x137mm (600 x 600 DPI)
**Fig. 4.** Tissue-specific expression of SAUR41 from the PIN1 and PIN2 promoters. Green EGFP (enhanced green fluorescent protein) signals and red PI (propidium iodide, staining cell wall here) signals were viewed by confocal microscopy. Purple starch grains stained by Lugol’s solution were viewed by DIC (differential-interference-contrast) microscopy. (A) The root stem cell niches. (B) Auxin accumulation/signaling pattern in root meristems of the DR5rev::GFP background line. White arrow indicated the quiescent center, while blue arrow indicates the tier of distal stem cells. Boxed areas were magnified in the subsequent images. Insects were enlargements of root stem cell niches. (C) Promoter activity of PIN1. (D-F) Tissue-specific expression of SAUR41 from the PIN1 promoter led to auxin retention in stele initials transporting auxin (pink arrow), additional tiers of distal stem cells (blue arrow), and supernumerary cell layers (white bracket). (H, L) Differentiation status of columella cells in control roots (H) and PIN1::SAUR41 roots (L) determined by Lugol’s solution staining. (I) Promoter activity of PIN2. (J-K) Tissue-specific expression of SAUR41 from the PIN2 promoter did not induce auxin retention but caused irregular cell expansion in the corresponding cells.

LRC, lateral root cap; Ep, epidermis; C, Cortex; En, endodermis. Bars: 25 µm.
Fig. 5. Tissue-specific expression of SAUR41 and IAA2P65S from promoters of root meristem patterning genes. Green EGFP (enhanced green fluorescent protein) signals and red PI (propidium iodide, staining cell wall here) signals were viewed by confocal microscopy. Boxed areas were magnified in the subsequent images. Insects were enlargements of root stem cell niches. (A) Auxin accumulation/signaling pattern in root meristems of the DR5rev::GFP background line (left) and the promoter activity of WOX5 (right). (B-F) Tissue-specific expression of SAUR41 from the WOX5 promoter. (G, H) Tissue-specific expression of IAA2P65S from the WOX5 promoter. (I) Promoter activity of ACR4. (J, K) Tissue-specific expression of SAUR41 from the ACR4 promoter. (L) Tissue-specific expression of IAA2P65S from the ACR4 promoter. (M) Promoter activity of PLT2. (N, O) Tissue-specific expression of SAUR41 from the PLT2 promoter. (P) Tissue-specific expression of IAA2P65S from the PLT2 promoter. Arrow indicated the division of endodermal cells. < indicated additional auxin accumulation/signaling peaks; * indicated the quiescent center; + indicated tiers of distal stem cells; and [ indicated the supernumerary cell layers. Bars: 25 µm.
Fig. 6. Ectopic SAUR41 proteins differentially regulated root cell expansion and root gravitropic growth. (A) Cell areas of stele initial cells, quiescent center cells and distal stem cells in roots of 5-d-old seedlings. (B) Root gravitropic responses in 6-d-old seedlings. *, P<0.05, t-test.
AUTHOR/EDITOR QUERIES

Please tick all check box to confirm that you have agreed or have made relevant changes for each query

ARTICLE ID : PCP-pct028

Please respond to all queries and send any additional proof corrections. Failure to do so could result in delayed publication

<table>
<thead>
<tr>
<th>Query no</th>
<th>Section</th>
<th>Paragraph</th>
<th>Query</th>
<th>Checked</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q1</td>
<td>Author names</td>
<td></td>
<td>Please check that all names have been spelled correctly and appear in the correct order. Please also check that all initials are present. Please check that the author surnames (family name) have been correctly identified by a pink background. If this is incorrect, please identify the full surname of the relevant authors. Occasionally, the distinction between surnames and forenames can be ambiguous, and this is to ensure that the authors’ full surnames and forenames are tagged correctly, for accurate indexing online. Please also check all author affiliations.</td>
<td>□</td>
</tr>
<tr>
<td>Q2</td>
<td></td>
<td></td>
<td>Please check that the text is complete and that all figures, and their legends are included.</td>
<td>□</td>
</tr>
<tr>
<td>Q3</td>
<td>Funding</td>
<td></td>
<td>Please provide a Funding statement, detailing any funding received. Remember that any funding used while completing this work should be highlighted in a separate Funding section. Please ensure that you use the full official name of the funding body, and if your paper has received funding from any institution, such as NIH, please inform us of the grant number to go into the funding section. We use the institution names to tag NIH-funded articles so they are deposited at PMC. If we already have this information, we will have tagged it and it will appear as coloured text in the funding paragraph. Please check the information is correct.</td>
<td>□</td>
</tr>
<tr>
<td>Q4</td>
<td>Figures</td>
<td>Figures have been placed as close as possible to their first citation. Please check that they have no missing sections and that the correct figure legend is present.</td>
<td>☐</td>
<td></td>
</tr>
</tbody>
</table>
Making corrections to your proofs

- Please use the tools in the Annotation and Drawing Markups toolbars to correct your proofs. To access these, press ‘Comment’ in the top right hand corner.
- If you would like to save your comments and return at a later time, click Publish Comments.
- If applicable, to see new comments from other contributors, press Check for New Comments.
- Once you have finished correcting your article, click Finalize/Finalize PDF Comments.
- The Publish Comments and Finalize/Finalize PDF Comments options are available either as:
  1. A pop up JavaScript Window, shown right, or
  2. By clicking ‘Tools’ in the top right hand corner, then clicking ‘JavaScript Window’, shown right.
  3. Do not close the Javascript window.

Annotation tools

- Insert text at cursor: click to set the cursor location in the text and start typing to add text. You may cut and paste text from another file into the commenting box.
- Replace (Insert): click and drag the cursor over the text then type in the replacement text. You may cut and paste text from another file into the commenting box.
- Strikethrough (Delete): click and drag over the text to be deleted then press the delete button on your keyboard for text to be struck through.

Comments list This provides a list of all comments and corrections made to the article. It can be sorted by date or person.

Drawing tools There is the ability to draw shapes and lines, if required.

- Underline: click to indicate text that requires underlining.
- Add sticky note: click to add a comment to the page. Useful for layout changes.
- Add note to text: click to add a note. Useful for layout changes.
- Highlight text: click to highlight specific text and make a comment. Useful for indicating font problems, bad breaks, and other textual inconsistences.

Attach file, Record audio, and Add stamp are not in use.
Tissue-Specific Expression of SMALL AUXIN UP RNA41 Differentially Regulates Cell Expansion and Root Meristem Patterning in Arabidopsis

Yingying Kong1,2,3, Yubin Zhu1,2,3, Chen Gao1, Wenjing She1, Weiqiang Lin1, Yong Chen1, Ning Han1, Hongwu Bian1, Muyuan Zhu1 and Junhui Wang1,2,*

1Institute of Genetics, College of Life Sciences, Zhejiang University, Zijingang Campus, Hangzhou 310058, China
2Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou 310016, China
3These authors contributed equally to this work.

*Corresponding author: E-mail, junhuiwang@zju.edu.cn; Fax: +86-571-88206535.

Introduction


AUXIN regulates cell division, differentiation and elongation in part by changing gene expression (Woodward and Bartel 2005, Paponov et al. 2008, Santner and Estelle 2009, Vanneste and Friml 2009, Hayashi 2012). Primary auxin response genes consist of members of three gene families (Hagen and Guilfoyle 2002): Aux/IAA (Auxin/Indole-3-Acetic Acid), GUS (Gretchen Hagen3) and SMALL AUXIN UP RNA (SAUR), the function of SAUR genes remains unclear. Arabidopsis SAUR genes have been phylogenetically classified into three clades. Recent work has suggested that SAUR19 (clade II) and SAUR63 (clade I) promote cell expansion through the modulation of auxin transport. Herein, we present our work on SAUR41, a clade III SAUR gene with a distinctive expression pattern in root meristems. SAUR41 was normally expressed in the quiescent center and cortex/endodermis initials; upon auxin stimulation, the expression was provoked in the endodermal layer. During lateral root development, SAUR41 was expressed in prospective stem cell niches of lateral root primordia and in expanding endodermal cells surrounding the primordia. SAUR41–EGFP (enhanced green fluorescent protein) fusion proteins localized to the cytoplasm. Overexpression of SAUR41 from the Cauliflower mosaic virus 35S promoter led to pleiotropic auxin-related phenotypes, including long hypocotyls, increased vegetative biomass and lateral root development, expanded petals and twisted inflorescence stems. Ectopic SAUR41 proteins were able to promote auxin transport in hypocotyls. Tissue-specific expression of SAUR41 from the PIN2 and PLT2 promoters enhanced root gravitropic growth.

Results

SAUR41 had a distinctive expression pattern in Arabidopsis root meristems

Previously, to counteract possible position effects in plant promoter analysis, we used the gypsy-Su(Hw) system of Drosophila in a novel approach that facilitated high and precise expression of reporter genes (She et al. 2010). Using this system, together with the GATEWAY recombination approach, we generated sets of promoter reporter lines for the PIN (PINFORM) gene family encoding auxin carriers and the TIR1/AFB gene family encoding auxin receptors (She et al. 2010). We extended this system to promoter analysis of certain SAUR genes, and found that the SAUR41 (At1g16510) gene showed a distinctive expression pattern in Arabidopsis root meristems.

The SAUR41 subfamily contained four members: SAUR41 (At1g16510), SAUR40 (At1g79130), SAUR71 (At1g56150) and SAUR72 (At3g12830). Their amino acid sequences differed from those of other SAUR families in the N-terminus. In previous microarray experiments, the expression of SAUR41 has been reported to be regulated by circadian rhythm (Mazzella et al. 2005, Darrah et al. 2006), biotic stress (Zhang et al. 2007, Peltier et al. 2011), and mitochondrial dysfunction and reactive oxygen species (Carrie et al. 2010, Gleason et al. 2011). The expression of SAUR40 and SAUR71 was responsive to ABA signaling (Leonardt et al. 2004, Zeng et al. 2012) and the functional status of chloroplasts (Bosco et al. 2004, Estavillo et al. 2011). SAUR71 and SAUR72 were expressed in vascular development (Nagawa et al. 2006, Shirakawa et al. 2009).

We found that SAUR41 was normally expressed in the quiescent center and cortex/endodermis initials, but it was provoked in the endodermal layer upon an auxin or gravitropic stimulation. During lateral root development, SAUR41 was distinctively expressed in prospective stem cell niches of lateral root primordia and in expanding endodermal cells surrounding the primordia. SAUR41-enhanced green fluorescent protein (EGFP) fusion proteins localized to the cytoplasm, unlike EGFP–SAUR19 and SAUR63–EGFP which localized predominantly to the plasma membrane. Interestingly, although the gene expression pattern and the protein localization pattern of SAUR41 were different from those of SAUR19 and SAUR63, the phenotypes resulting from overexpression of SAUR41 driven by the Cauliflower mosaic virus (CaMV) 35S promoter shared many similarities with those of SAUR19 and SAUR63 subfamily genes. Tissue-specific expression of SAUR41 from promoters of auxin transporter genes and root meristem patterning genes differentially modulated root meristem development, root cell expansion and root gravitropic growth. The distinctive expression pattern of the SAUR41 gene and the explicit function of ectopic SAUR41 proteins implied that further investigations on the loss-of-function phenotypes of this gene family in root development are of great interest.

Recent studies have begun to address the function of SAUR proteins in rice (Oryza sativa) and Arabidopsis. OsSAUR39 has been identified as a negative regulator of auxin synthesis and transport, since rice plants overexpressing OsSAUR39 exhibit reduced lateral root development and shoot and root length (Kant et al. 2009). The SAUR19 subfamily proteins (SAUR19–SAUR24) of Arabidopsis have been reported to be highly unstable; however, the addition of an N-terminal tag increases the stability of these proteins (Spartz et al. 2012). Arabidopsis plants overexpressing these stabilized SAUR fusion proteins exhibited increased hypocotyl elongation and larger leaf size, while seedlings expressing an artificial microRNA targeting the SAUR19 subfamily genes exhibit opposite phenotypes (Franklin et al. 2011, Spartz et al. 2012). In another report on Arabidopsis, transgenic plant lines overexpressing SAUR63:GFP or SAUR63:GUS displayed long hypocotyls, petals and stamen filaments; in contrast, transgenic plants expressing artificial microRNA constructs targeting the SAUR63 subfamily genes (SAUR61–SAUR68 and SAUR75) had slightly reduced hypocotyl and stamen filament elongation (Chae et al. 2012). The SAUR19 subfamily genes were expressed in elongating tissues, including the root elongation zone (Spartz et al. 2012), while the SAUR63 subfamily genes were not expressed in roots (Chae et al. 2012). As far as the mechanism is concerned, Arabidopsis SAUR proteins have been proposed to promote cell expansion through the modulation of auxin transport (Chae et al. 2012, Spartz et al. 2012).

The SAUR gene family could be phylogenetically classified into three clades (Kodaira et al. 2011). The genes in clades I and II had a tendency to display higher expression levels in leaves and lower expression in roots, while the genes in clade III demonstrated opposite expression patterns (Kodaira et al. 2011). The SAUR63 subfamily and the SAUR19 subfamily are clade I and clade II SAUR genes, respectively (Kodaira et al. 2011). Herein, we present our work on SAUR41, a clade III SAUR gene displaying a distinctive expression pattern in Arabidopsis root meristems.
In generating promoter reporter lines for SAUR41, an ~1,800 bp DNA fragment upstream of the ATG start codon of SAUR41, as predicted by AtcisDB (Davuluri et al. 2003), was fused with the EGFP-GUS reporter gene. Viewed by confocal microscopy, SAUR41 was found to be specifically expressed in the quiescent center and cortex/endodermis initials of root stem niches (Fig. 1A). Considering that SAUR genes had been proposed to function in auxin-mediated growth events, we examined the expression of SAUR41 under gravitropic stimulation and auxin treatment. Gravitropism allows plant roots to grow directionally, whereas auxin is an essential regulator in this process (Harrison and Masson 2008). During the gravitropic response, the expression of SAUR41 was provoked on both sides of the endodermis at the proximal meristem region and on the upper side of the endodermis at the distal elongation zone, indicating that this gene might act to coordinate root elongation rather than simply respond to auxin redistribution (Fig. 1B). SAUR41 was specifically induced in the endodermis with 1 h of auxin [10 μM 1-naphthalene acetic acid (NAA)] treatment (Fig. 1C). Since the endodermis was recently reported as the primary responsive tissue for gibberellins to coordinate root growth (Ubeda-Tomás et al. 2008, Ubeda-Tomás et al. 2008), we examined the expression of SAUR41 under gibberellin stimulation. SAUR41 was normally restricted to the quiescent center and cortex/endodermis initials at the root meristems (A). Upon a gravitropic (B) or auxin (C) stimulation, SAUR41 was provoked in the endodermis layer. (D) SAUR41 was unresponsive to gibberellin treatment. After extended auxin treatment, SAUR41 was induced in multiple cell layers at the root meristem (E); however, in the elongation zone, it was specifically induced in the endodermal cells (F). During lateral root development, SAUR41 was expressed in the prospective quiescent center and initial cells of lateral root primordia (G, H) and in the endodermal cells surrounding the lateral root primordia (G). Insets are enlargements of lateral root primordia. In a new lateral root, SAUR41 was strongly expressed in the quiescent center and initial cells (I). SAUR41 was predominantly expressed in the vascular tissues of hypocotyls (J), petioles (K) and cotyledons (L). SAUR41–EGFP fusion proteins localized to the cytoplasm of epidermal cells and cortical cells in hypocotyls (M, N) and to the cytoplasm of quiescent center cells, cortex/endodermis initial cells and epidermal cells in root tips (O, P). LRC, lateral root cap; Ep, epidermis; C, cortex; En, endodermis. Bars: 25 μm.
et al. 2009, Ubeda-Tomás et al. 2012), we then tested whether or not SAUR41 was a gibberellin-responsive gene. As shown in Fig. 1D, SAUR41 was unresponsive to gibberellin treatment. This result was in agreement with the results of a microarray experiment (Joss et al. 2011).

Extending the duration of auxin treatment to 12 h, and using detection by overnight histochemical staining, SAUR41 was found to be induced in multiple cell layers at the root meristem and transition zone (Fig. 1E). However, in the elongation zone, it was specifically induced in the endodermal cells (Fig. 1F).

During lateral root development, SAUR41 was expressed in the prospective quiescent center of lateral root primordia (Fig. 1G, H). Interestingly, SAUR41 was also specifically expressed in the endodermal cells surrounding the lateral root primordia during the process of the lateral root primordia breaking through the endodermis (Fig. 1G). In the newly formed lateral roots, SAUR41 was strongly expressed in the quiescent center and initial cells, and weakly expressed in the endodermis (Fig. 1I). In hypocotyls, petioles and cotyledons, SAUR41 was predominantly expressed in the vascular tissues (Fig. 1J–L).

**SAUR41–EGFP fusion protein accumulated in the cytoplasm**

To check the subcellular localization of the SAUR41 protein, we generated a transgenic construct in which the CaMV 35S promoter drove a C-terminal translational fusion between the full-length SAUR41 and the EGFP protein. Location of the fusion protein in hypocotyls and root tips of stably transformed Arabidopsis plants was examined by confocal microscopy. Results showed that the EGFP fluorescence was identified at the cytoplasm of epidermal and cortical cells in hypocotyls (Fig. 1M, N), and at the cytoplasm of all types of cells in root tips, including quiescent center cells, cortex/endodermis initial cells and epidermal cells (Fig. 1O, P).

**Overexpression of SAUR41 conferred pleiotropic auxin-related phenotypes**

To explore potential roles for SAUR41 in Arabidopsis growth and development, we first screened T-DNA and transposon insertion lines from the Arabidopsis Biological Resource Center (ABRC) and the Rikagaku Kenkyusho Bioresource Center (RIKEN-BRC), respectively. The line SALK_056968 from the ABRC stocks contained a T-DNA at the promoter region of SAUR41. However, reverse transcription–PCR (RT–PCR) analysis revealed that this insertion did not impair the expression of SAUR41 (data not shown). In three additional lines (SALK_121397, PST_11030 and PST_17947), we failed to identify DNA insertions inside the SAUR41 gene.

We then used a gain-of-function approach and generated transgenic Arabidopsis plants overexpressing the untagged (35S::SAUR41) or MYC-tagged (35S::SAUR41-MYC) SAUR41 gene, in addition to the 35S::SAUR41-EGFP plants described above. More than 20 independent lines were obtained for each transgenic construct. Plants overexpressing untagged SAUR41 had strong phenotypes, similar to that of MYC-tagged SAUR41. In contrast, plants overexpressing EGFP-tagged SAUR41 had the weakest phenotypes. We chose 35S::SAUR41 plants for detailed study.

The 35S::SAUR41 transgenic lines displayed pleiotropic auxin-related phenotypes. Light-grown seedlings exhibited 1.7- to 2.0-fold longer hypocotyls than wild-type controls (Fig. 2A, B, E; P < 0.01, t-test). Surprisingly, these seedlings also displayed 1.3- to 1.5-fold longer primary roots compared with wild-type controls (Fig. 2C, D, F; P < 0.05, t-test). In addition, overexpression of SAUR41 increased root waving on vertically oriented agar plates (Fig. 2D). After 10 d of growth, 35S::SAUR41 seedlings had a 1.5- to 1.9-fold increase in lateral root numbers (P < 0.05, t-test) and a 20–30% increase in vegetative biomass as measured by the fresh weight of shoots (Fig. 2G, H; P < 0.05, t-test).

Adult 35S::SAUR41 plants had twisted inflorescence stems (Fig. 2I). Furthermore, the petals of transgenic flower organs were overexpanded and defective in opening completely (Fig. 2J–L), resulting in reduced seed setting in many siliques (Fig. 2I).

To determine if the elongated hypocotyl phenotype induced by ectopic SAUR41 was due to increased cell expansion, we measured hypocotyl epidermal cell length in 6-day-old seedlings. The change in hypocotyl epidermal cell length was parallel to the change in hypocotyl length (Figs. 3A, 2E). We then directly measured [3H]IAA transport in hypocotyls and detected a 40–70% increase in basipetal IAA transport in hypocotyls of 35S::SAUR41-EGFP, 35S::SAUR41-MYC and 35S::SAUR41 seedlings (Fig. 3B).

**Tissue-specific expression of SAUR41 from the PIN1 promoter induced alterations in root meristem patterning**

As SAUR41 had a distinctive expression pattern in Arabidopsis root meristems, and rice and Arabidopsis SAUR proteins have been proposed to modulate auxin transport (Kant et al. 2009, Chae et al. 2012, Spartz et al. 2012), we next implemented tissue-specific expression of SAUR41 from promoters of auxin transporter genes and root meristem patterning genes. To facilitate the examination of auxin signaling and distribution, all transgenic plants were generated in a DR5rev::GFP background (Friml et al. 2003; Fig. 4B).

We first expressed SAUR41 from the PIN1 and PIN2 promoters. In Arabidopsis roots, PIN1 promoter activity was strong in stele cells and weak in endodermis and the quiescent center (She et al. 2010; Fig. 4C). Ectopic expression of SAUR41 driven by the PIN1 promoter led to auxin retention in stele initials transporting auxin, resulting in a large auxin accumulation/signaling peak (Fig. 4D–G). In addition, PIN1::SAUR41 roots had additional tiers of distal stem cells (Fig. 4E, G), while wild-type roots typically had one tier of distal stem cells (Ding and Friml 2010; Fig. 4A, B). Non-differentiated distal stem cells below the quiescent center were characterized by...
Fig. 2 Overexpression of SAUR41 from the CaMV 3SS promoter conferred pleiotropic auxin- or cell expansion-related phenotypes. (A–E) Light-grown 6-day-old seedlings. (F–H) Light-grown 10-day-old seedlings. (I–L) Adult plants. Hypocotyls of wild-type controls (A) and 3SS::SAUR41 seedlings (B). Roots of wild-type controls (C) and 3SS::SAUR41 seedlings (D). (E–H) Statistical comparison of hypocotyl length (E), primary root length (F), lateral root number (G) and vegetative biomass (H) of wild-type controls with 3SS::SAUR41 seedlings. (I–L) Adult 3SS::SAUR41 plants had twisted inflorescence stems (I) and overexpanded petals (J–L). Roots or flowers marked by arrows were enlarged in the subsequent images. **P < 0.01; *P < 0.05, t-test.
Elongated (of epidermal and cortical cells, but the auxin accumulation/signalings in roots displayed abnormalities in root meristem patterning (Fig. 4E, G). Finally, PIN1::SAUR41 roots had supernumerary cell layers (Fig. 4C, D, F), like those observed in PIN1::SAUR41 roots (Fig. 4E, G). As expected, tissue-specific expression of IAA2P65S driven by the WOX5 promoter inhibited auxin accumulation/signaling in the quiescent center (Fig. 5G, H). Interestingly, it also inhibited auxin accumulation/signaling in the stele initials (Fig. 5G, H), opposite to the effects observed in tissue-specific expression of SAUR41 from the WOX5 promoter (Fig. 5B–F). ACR4 was expressed in the root stem cell niche and its surrounding cells, including young epidermal cells, cortical cells and columella cells (De Smet et al. 2008; Fig. 5I). Ectopic expression of SAUR41 from the ACR4 promoter led to the expansion of cells expressing ACR4 (Fig. 5J, K). Formation of auxin accumulation/signaling peaks in stele initials was visible (Fig. 5J, K), but not as dramatically as that in the WOX5::SAUR41 roots (Fig. 5B–F), indicating that the balanced expression of SAUR41 in the entire root stem cell niche attenuated the effects of ectopic SAUR proteins on auxin transport, compared with the selected expression of SAUR41 in the quiescent center by the WOX5 promoter. On the other hand, tissue-specific expression of IAA2P65S from the ACR4 promoter had a more remarkable impact on root meristem development compared with that seen in WOX5::IAA2P65S roots. As shown in Fig. 5, ACR4::IAA2P65S roots had two separate auxin accumulation/signaling maxima: one in the quiescent center and the other in the young columella cells (Fig. 5L).

Comparison of tissue-specific expression of SAUR41 and IAA2P65S from promoters of root meristem patterning genes

We also expressed SAUR41 from the promoters of root meristem patterning genes. Three promoters were chosen: WOX5 (WUSCHEL-related homebox 5), ACR4 (ARABIDOPSIS CRINKLY4) and PLT2 (PLETHORA2). WOX5 encoded a homeodomain transcription factor (Sarkar et al. 2007). PLT2 encoded an AP-2 type transcription factor (Aida et al. 2004, Galinha et al. 2007) and ACR4 encoded a receptor-like kinase (De Smet et al. 2008). They were three of the major regulators of root stem cell activity (Aichinger et al. 2012, Perilli et al. 2012, Petricka et al. 2012). To compare the effects between tissue-specific expression of a SAUR protein and a stabilized AUX/IAA protein, we introduced IAA2P65S that harbored a site-directed mutation in the proline residue of conserved domain II. As one of 29 Aux/IAA genes of Arabidopsis, IAA2 was highly auxin inducible and expressed in vascular tissues and auxin accumulation/signaling peaks (Swarup et al. 2001).

WOX5 was expressed in the quiescent center of root stem cell niches (Sarkar et al. 2007; Fig. 5A). Tissue-specific expression of SAUR41 from the WOX5 promoter induced the formation of additional auxin accumulation/signaling peaks in stele initials above the quiescent center (Fig. 5B–F). In addition, WOX5::SAUR41 roots had supernumerary cell layers (Fig. 5C, D, F), like those observed in PIN1::SAUR41 roots (Fig. 4E, G). As expected, tissue-specific expression of IAA2P65S driven by the WOX5 promoter inhibited auxin accumulation/signaling in the quiescent center (Fig. 5G, H). Interestingly, it also inhibited auxin accumulation/signaling in the stele initials (Fig. 5G, H), opposite to the effects observed in tissue-specific expression of SAUR41 from the WOX5 promoter (Fig. 5B–F). ACR4 was expressed in the root stem cell niche and its surrounding cells, including young epidermal cells, cortical cells and columella cells (De Smet et al. 2008; Fig. 5I). Ectopic expression of SAUR41 from the ACR4 promoter led to the expansion of cells expressing ACR4 (Fig. 5J, K). Formation of auxin accumulation/signaling peaks in stele initials was visible (Fig. 5J, K), but not as dramatically as that in the WOX5::SAUR41 roots (Fig. 5B–F), indicating that the balanced expression of SAUR41 in the entire root stem cell niche attenuated the effects of ectopic SAUR proteins on auxin transport, compared with the selected expression of SAUR41 in the quiescent center by the WOX5 promoter. On the other hand, tissue-specific expression of IAA2P65S from the ACR4 promoter had a more remarkable impact on root meristem development compared with that seen in WOX5::IAA2P65S roots. As shown in Fig. 5, ACR4::IAA2P65S roots had two separate auxin accumulation/signaling maxima: one in the quiescent center and the other in the young columella cells (Fig. 5L).

PLT2 was gradiently expressed in root meristems and lateral cap cells (Aida et al. 2004, Galinha et al. 2007; Fig. 5M). Tissue-specific expression of SAUR41 from the PLT2 promoter exhibited triple effects as addressed above: additional auxin accumulation/signaling peaks in stele initials and supernumerary cell layers in the proximal meristem zone (Fig. 5N, O). On the other hand, tissue-specific expression of IAA2P65S from the PLT2 promoter inhibited auxin accumulation/signaling in the quiescent center, promoted auxin accumulation/signaling in stele initials and led to establishment of new auxin accumulation/signaling peaks in the adjacent provascular cells (Fig. 5P), similar to that seen for relocalization of auxin accumulation/signaling peaks upon polar auxin transport inhibitor treatment (Sabatini et al. 1999).
Ectopic SAUR41 proteins differentially regulated root cell expansion and root gravitropic growth

We measured cell areas of stele initial cells, quiescent center cells and distal stem cells in root meristems of transgenic seedlings expressing SAUR41 from promoters of auxin transporter genes and root meristem patterning genes. The results showed that PIN1::SAUR41, WOX5::SAUR41 and PLT2::SAUR41 roots had a 30–60% increase in cell areas of stele initial cells and quiescent center cells, while ACR4::SAUR41 roots had enlarged quiescent center cells and distal stem cells (Fig. 6A; P < 0.05, t-test).

The root gravitropic responses in these transgenic seedlings were also analyzed. ACR4::SAUR41 roots showed delayed gravitropic growth (Fig. 6B), coinciding with their higher auxin accumulation/signaling in young columella cells but lower auxin accumulation/signaling in LRC cells (Fig. 5I). PIN2::SAUR41 roots had advanced gravitropic growth 2 h after the gravitropic stimulation, while PLT2::SAUR41 roots displayed enhanced gravitropic growth 3 h after the gravitropic stimulation (Fig. 6B). It has been suggested that auxin transport in the lower side of lateral cap cells and auxin accumulation in the lower side of epidermal and cortical cells were essential for the root gravitropic response (Ottenschläger et al. 2003). Our results were consistent with the expression pattern of PIN2 and PLT2 promoters. PIN2 was expressed in both lateral cap cells and epidermal and cortical cells (Fig. 4I), while PLT2 was expressed in lateral cap cells (Fig. 5M).

Discussion

The expression pattern of SAUR41 in root meristems was distinctive. SAUR19 subfamily genes were expressed in growing hypocotyls in response to shade avoidance, and in root...
elongation zones in response to auxin treatment (Spartz et al. 2012). SAUR63 and members of its clade were expressed in growing regions of hypocotyls, cotyledons, petiole, young rosette leaves and inflorescence stems, but not in roots (Chae et al. 2012). Herein, we found that the expression of SAUR41 was normally restricted to the quiescent center and cortex/endo
dermis initials of root meristems; upon an auxin or gravitropic stimulation, it was provoked in the endodermis at the proximal meristem region and transition zone of Arabidopsis roots (Fig. 1A–F). Furthermore, SAUR41 was differentially expressed

Fig. 5 Tissue-specific expression of SAUR41 and IAA2P65S from promoters of root meristem patterning genes. Green EGFP (enhanced green fluorescent protein) signals and red PI (propidium iodide, staining the cell wall here) signals were viewed by confocal microscopy. Boxed areas were magnified in the subsequent images. Insets were enlargements of root stem cell niches. (A) Auxin accumulation/signaling pattern in root meristems of the DR5rev::GFP background line (left) and the promoter activity of WOX5 (right). (B–F) Tissue-specific expression of SAUR41 from the WOX5 promoter. (G, H) Tissue-specific expression of IAA2P65S from the WOX5 promoter. (I) Promoter activity of ACR4. (J, K) Tissue-specific expression of SAUR41 from the ACR4 promoter. (L) Tissue-specific expression of IAA2P65S from the ACR4 promoter. (M) Promoter activity of PLT2. (N, O) Tissue-specific expression of SAUR41 from the PLT2 promoter. (P) Tissue-specific expression of IAA2P65S from the PLT2 promoter. The arrow indicates the division of endodermal cells. < indicates additional auxin accumulation/signaling peaks; * indicates the quiescent center; + indicates tiers of distal stem cells; and [indicates the supernumerary cell layers. Bars: 25 μm.
during lateral root development, as manifested by β-glucuronidase (GUS) activity in prospective stem cell niches of lateral root primordia, and in expanding endodermal cells surrounding the lateral root primordia (Fig. 1G–I). In previous microarray experiments, the expression of SAUR41 has been reported to be regulated by multiple environmental signals (Mazzella et al. 2005, Darrah et al. 2006, Zhang et al. 2007, Peltier et al. 2011). Taken together, it seemed that SAUR41 was actively expressed in response to both developmental and environmental cues.

SAUR41–EGFP fusion proteins localize to the cytoplasm (Fig. 1M–P), while EGFP–SAUR19 and SAUR63–EGFP localize predominantly to the plasma membrane (Chae et al. 2012, Spartz et al. 2012). Although the protein localization pattern and the gene expression pattern of SAUR41 were different from those of SAUR19 and SAUR63, the phenotypes resulting from overexpression of SAUR41 driven by the CaMV 35S promoter shared many similarities with those of SAUR19 and SAUR63 subfamily genes. In all three cases, transgenic seedlings had elongated hypocotyls, expanded hypocotyl epidermal cells and enhanced hypocotyl basipetal IAA transport compared with the wild type (Figs. 2A, B, E, 3; Chae et al. 2012, Spartz et al. 2012). In the cases of SAUR41 and SAUR19, transgenic seedlings displayed waving roots and increased vegetative biomass (Fig. 2C, D, H; Spartz et al. 2012); while in the cases of SAUR41 and SAUR63, transgenic plants had expanded petals and twisted inflorescence stems (Fig. 2I–L; Chae et al. 2012). Interestingly, only in the case of SAUR41 did the transgenic seedlings have increased primary root length and lateral root development compared with wild-type controls (Fig. 2F, G). Thus, it seems likely that Arabidopsis SAUR proteins have some similarities but also specificity in terms of molecular functions. Alternatively, SAUR proteins may share a similar molecular function, but different SAUR proteins require different tissue-specific partners.

Tissue-specific expression of SAUR41 from PIN1, WOX5, ACR4 or PLT2 promoters caused new auxin accumulation/signaling peaks in stele initial cells transporting auxin (Figs. 4D–G, 5B–F, N, O). Roots of PIN1::SAUR41 seedlings had additional tiers of distal stem cells below the quiescent center and super-numerary cell layers in root meristems (Fig. 4D–H, I, Q). It has been reported that auxin regulates distal stem cell differentiation in Arabidopsis roots, and defects in auxin transport would lead to additional tiers of distal stem cells (Ding and Friml 2010). Thus, it seemed likely that SAUR41 induced perturbation of auxin transport in root meristems as it was expressed above the stem cell niches (from the PIN1 promoter). In contrast, tissue-specific expression of SAUR41 from the PIN2 promoter did not induce alterations in root meristem patterning but caused alterations in cell expansion in the corresponding cell lineages (Fig. 4I, K). In addition, PIN2::SAUR41 and PLT2::SAUR41 roots had enhanced gravitropic growth (Fig. 6B), indicating that ectopic SAUR41 proteins promoted root basipetal auxin transport for root gravitropic responses. Taken together, it seems likely that ectopic SAUR41 proteins retard auxin transport in root stem cell niches, but promote auxin transport in LRC cells and epidermal and cortical cells.

Previously, it has been proposed that rice SAUR39 acted as a negative regulator of organ growth and auxin transport (Kant et al. 2009), while Arabidopsis SAUR19 and SAUR63 acted as positive regulators of cell expansion and auxin transport (Chae et al. 2012, Spartz et al. 2012). Herein, in terms of cell expansion, we found that SAUR41 promoted cell expansion, as it was constitutively expressed from the CaMV 35S promoter (Figs. 2, 3). In addition, stele initial cells, quiescent center cells and distal stem cells in root meristems of transgenic seedlings expressing SAUR41 from promoters of auxin transporter genes and root meristem patterning genes were differentially enlarged (Fig. 6A). Thus, similarly to SAUR19 with N-terminal tags and SAUR63 with C-terminal tags, untagged SAUR41 promoted cell expansion, as it was ectopically expressed. However, in terms of auxin transport, the functions of SAUR proteins appeared to be more complicated. The observed higher flow rate of labeled IAA in hypocotyls could be an indirect effect of SAUR protein over-expression. Two questions, why ectopic SAUR41 proteins retarded auxin transport in root stem cell niches but promoted basipetal auxin transport, and why rice OsSAUR39 (analogous to Arabidopsis SAUR63, clade I) inhibited auxin transport but Arabidopsis SAUR63 promoted auxin transport, remained unanswerable. Further studies would be needed to investigate the roles of SAUR proteins in regulating cell expansion and auxin transport.
unanswered. It should be noted that the DR5::GFP marker basically indicated the status of auxin signaling but not the auxin transport. Currently, direct measurement of auxin transport in root stem cell niches is unavailable. It was tempting to speculate that SAUR41 proteins used different mechanism to regulate auxin transport for cell elongation and for root meristem patterning.

Tissue-specific expression of IAA296SS from WOX5, ACR4 and PLT2 promoters displayed fundamentally different effects on root meristem patterning compared with that observed for SAUR41 (Fig. 5). The mechanism of stabilized Aux/IAA proteins is clear. They impaired the SCFTIR1 pathway of auxin signaling to regulate cell division, differentiation and elongation. They also disturbed auxin transport by transcriptional modification of the auxin export machinery (Hayashi 2012, Scherer et al. 2012). In contrast, the precise mechanism by which SAUR proteins regulate cell expansion and auxin transport remains unclear. It will be interesting to learn whether there exist epistatic interactions between the IAA2 and the SAUR41 gain-of-function phenotypes. We are currently crossing the IAA296SS lines with the corresponding SAUR41 lines to answer this question.

The SAUR41 function reported here was solely dependent on the ectopic expression data, while its endogenous role in stem cell maintenance remained unclear. The gene could be involved in the regulation of cell sizes of quiescent center and cortex/endodermis initials, and/or in the modulation of auxin transport in these cells. In addition, the SAUR41 subfamily contains four members: SAUR40, SAUR41, SAUR71 and SAUR72. Further investigations on promoter activity and protein localization patterns of other SAUR41 subfamily members, as well as on loss-of-function phenotypes of the SAUR41 gene family, are required and of great interest.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia-0 and the DR5rev:GFP background (Friml et al. 2003) were used as sources of wild-type plant materials. Promoter reporter lines for the PIN gene family and the TIR1/AFB gene family (She et al. 2010) have been donated to ABRC. Seeds were surface sterilized and cultured aseptically on 9 cm Petri dishes containing Gamborg’s B5 medium with 1% (w/v) sucrose and 1% (w/v) agar. The plates were maintained at 4°C for 2 d, and then transferred to a culture room (23°C, 80 μmol m⁻² s⁻¹ irradiance with a 16 h photoperiod and 30–40% relative humidity).

Vector construction and plant transformation

We used the GATEWAY™ system for vector construction. Entry vectors were created using the pENTR™/D-TOPO kits (Invitrogen). The PCR primers for construction of entry vectors for the coding region of SAUR41 and for promoter regions of SAUR41, WOX5, ACR4 and PLT2 are listed in Supplementary Table S1. IAA296SS was generated by overlapping PCR using the primers given in Supplementary Table S1. Each entry clone was confirmed by DNA sequencing. GATEWAY™-compatible destination vectors for protein subcellular localization, overexpression, MYC tagging and promoter analysis were used (Karimi et al. 2002, Earley et al. 2006, She et al. 2010). The LR reaction was conducted to generate different expression vectors.

To facilitate tissue-specific gene expression from various promoters, the CaMV 3S promoter in the overexpression constructs (35S:SAUR41 and 35S:IAA296SS) was replaced with ccdB fragment by a method described previously (Yang et al. 2012). Briefly, the ccdB fragment was PCR amplified using pH7FWG2.0 as a template, with the primers ccdB-Up and ccdB-Dn, containing an introduced HindIII and SpeI site, respectively. The ccdB fragment was then digested to replace the 35S promoter sequence, thus forming new destination vectors for tissue-specific expression.

All of the expression vectors were electroporated into Agrobacterium tumefaciens strain GV3101. Plants were transformed using the vacuum infiltration method (Bechtold et al. 1993). Transgenic plants were selected on B5 plates with 12.5 μg ml⁻¹ hygromycin or 25 μg ml⁻¹ kanamycin depending on the selection markers. Single-locus and homozygous transgenic lines were characterized as we described previously (She et al. 2010).

Microscopic analysis and histochemical detection

For histochemical detection of GUS activities, young seedlings at different developmental stages and different parts from transgenic plants were collected. They were stained at 37°C overnight in 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc), 1 mM potassium ferricyanide, 0.1% Triton X-100 and 0.1 M sodium phosphate buffer, pH 7.0 with 10 mM EDTA. Samples were washed in 70% ethanol to remove Chl. Differential interference contrast (DIC) images were visualized using a microscope (Nikon Eclipse 80i) with a Dxm1200 CCD camera and EclipseNet software. For the localization of fluorescence fusion proteins, a confocal microscope system (Zeiss LSM510) was used. Without specificiation, 5-day-old seedlings were mounted in water. Starch grains in columella cells were stained with I₅–KI as described previously (Ding and Friml 2010).

Hypocotyl IAA transport assay

IAA transport in hypocotyls was measured as previously described (Chae 2012). [³H] IAA was a product of American Radiolabeled Chemicals, Inc. The radioisotope counts of [³H] IAA were detected using a low-noise scintillation counter (MicroBeta 2, Perkin Elmer).

Growth and cell measurement, statistical analysis and image processing

After incubations for the durations indicated in the text, the plates were digitally photographed. Root and hypocotyl length
was measured using magnified images. Lateral root (>1 mm)
numbers were counted using each seedling as an individual
sample. For hormone treatment, seedlings were transferred
onto a medium containing 10 μM NAA or GA₃ for 1 or 12 h.
For gravitropism assays, the protocol of Weijers et al. (2005)
was adopted. The mean hypocotyl epidermal cell length and
the cell area of root stem cell niches were measured as
described by Spartz et al. (2012). Each treatment contained
30–50 seedlings and was replicated three times. Statistical ana-
lysis of the data was performed using Microsoft Excel and
Student’s t-test. Images were processed using Adobe Photoshop CS2.

Supplementary data

Supplementary data are available at PCP online.

Funding

This work was supported by the National Natural Science
Foundation of China [grant No. 31170211 to J.W.]

Acknowledgments

We are grateful to ABRC and RIKEN-BRC for the distribution of
Arabidopsis materials. No conflict of interest is declared.

References

Aida, M., Beis, D., Heidstra, R., Willemsen, V., Blilou, I., Galinha, C. et al.
Agrobacterium-mediated gene transfer by infiltration of adult
1194–1199.
Bosco, C.D., Lezhneva, L., Biehl, A., Leister, D., Strotmann, H.,
Wanner, G. et al. (2004) Inactivation of the chloroplast ATP syn-
thase gamma subunit results in high non-photochemical fluores-
cence quenching and altered nuclear gene expression in
Carrie, C., Giraud, É., Duncan, O., Xu, L., Wang, Y., Huang, S. et al.
(2010) Conserved and novel functions for Arabidopsis thaliana
MIA40 in assembly of proteins in mitochondria and peroxisomes.
Chae, K., Isacs, C.G., Reeves, P.H., Maloney, G.S., Muday, G.K.,
Nagpal, P. et al. (2012) Arabidopsis SMALL AUXIN UP RNA63 pro-
motes hypocotyl and stamen filament elongation. Plant J. 71:
684–697.
Darrah, C., Taylor, B.L., Edwards, K.D., Brown, P.E., Hall, A. and
reveals novel circadian quantitative trait loci in Arabidopsis. Plant
Physiol. 140: 1464–1474.
Davuluri, R.V., Sun, H., Palaniswamy, S.K., Matthews, N., Molina, C.,
Information Server, an information resource of Arabidopsis
cis-regulatory elements and transcription factors. BMC Bioinform.
4: 25.
De Smet, I., Vassileva, V., De Rybel, B., Levesque, M.P., Gruenwald, W.,
Van Damme, D. et al. (2008) Receptor-like kinase ACR4 restricts
formative cell divisions in the Arabidopsis root. Science 322:
594–597.
Ding, Z. and Friml, J. (2010) Auxin regulates distal stem cell differen-
12046–12051.
Earley, K.W., Haag, J.R., Pontes, O., Oppen, K., Juehne, T., Song, K. et al.
Estavillo, G.M., Crisp, P.A., Pornsiriwong, W., Wirtz, M., Collinge, D.,
Carrie, C. et al. (2011) Evidence for a SAL1–PAP chloroplast retro-
grade pathway that functions in drought and high light signaling.
(2011) PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) regulates
auxin biosynthesis at high temperature. Proc. Natl Acad. Sci. USA
(2003) Efflux-dependent auxin gradients establish the apical-basal
Galinha, C., Hofhuis, H., Luijten, M., Willemsen, V., Blilou, I., Heidstra, R.,
Estelle, M. et al. (2007) PLETHORA proteins as dose-dependent master regu-
Gleason, C., Huang, S., Thatcher, L.F., Foley, R.C., Anderson, C.R.,
Carroll, A.J. et al. (2011) Mitochondrial complex II has a key role
in mitochondrial-derived reactive oxygen species influence on plant
stress gene regulation and defense. Proc. Natl Acad. Sci. USA 108:
10768–10773.
Auxin regulates SCL-independent degradation of AUX/IAA pro-
genomes, promoters and regulatory factors. Plant Mol. Biol. 49:
373–385.
Harrison, B.R. and Masson, P.H. (2008) ARL2, ARG1 and PIN3 define a
gravity signal transduction pathway in root statocytes. Plant J.
evolutionary expansion, and expression of early auxin-responsive
Josse, E.-M., Gan, Y., Bou-Torrent, J., Stewart, K.L., Gilday, A.D.,
Jeffere, C.E. et al. (2011) A DELLA in disguise: SPATULA restrains
the growth of the developing Arabidopsis seedling, Plant Cell 23:
1337–1351.
Kant, S., Bi, Y.M., Zhu, T. and Rothstein, S.J. (2009) SAUR39, a small
auxin-up RNA gene, acts as a negative regulator of auxin synthesis


Table S1. Primer sequences used in this study

<table>
<thead>
<tr>
<th>Primers</th>
<th>Primer Sequence 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>generating entry clone for coding sequence</strong></td>
<td></td>
</tr>
<tr>
<td>SAUR41-Up</td>
<td>CACCATGAAGCATCTCATCCGC</td>
</tr>
<tr>
<td>SAUR41-Dn1</td>
<td>CTACTCTGTAGTTTCAGGTATC (for overexpression)</td>
</tr>
<tr>
<td>SAUR41-Dn2</td>
<td>CTCTGTAGTTTCAGGTATC (for protein fusion)</td>
</tr>
<tr>
<td>IAA2-Up</td>
<td>CACCATGGCGTACGAGAAAGTC</td>
</tr>
<tr>
<td>IAA2-P65S-Dn</td>
<td>AGATCTCACTGGTGAACCAAC</td>
</tr>
<tr>
<td>IAA2-P65S-Up</td>
<td>AGATCTTCCCCTGGTAAAGAACA</td>
</tr>
<tr>
<td>IAA2-Dn</td>
<td>TCATAAGGAAGAGTCTAGAG</td>
</tr>
<tr>
<td><strong>generating entry clone for promoter region</strong></td>
<td></td>
</tr>
<tr>
<td>SAUR41P-Up</td>
<td>CACCTAGTTAGCGTATACATGAGA</td>
</tr>
<tr>
<td>SAUR41P-Dn</td>
<td>GGTTTAAAACTAATGATAGA</td>
</tr>
<tr>
<td>WOX5P-Up</td>
<td>CACCTTGGACCATTTTCGTCTTGCTTA</td>
</tr>
<tr>
<td>WOX5P-Dn</td>
<td>TCAACTGTGTGTTAAGGCTCT</td>
</tr>
<tr>
<td>PLT2P-Up</td>
<td>CACCGTTATTTCCTTTCTTTCTTCGTCTC</td>
</tr>
<tr>
<td>PLT2P-Dn</td>
<td>CCACTTTCCCTGGGAAATCAANAGCTTA</td>
</tr>
<tr>
<td>ACR4P-Up</td>
<td>CACCTTGATTGTATTTACATCTGTCC</td>
</tr>
<tr>
<td>ACR4P-Dn</td>
<td>CACACGGCTTTCTAGTCTAGCTCT</td>
</tr>
<tr>
<td><strong>generating destination clone for tissue-specific expression</strong></td>
<td></td>
</tr>
<tr>
<td>ccdB-Up</td>
<td>GGACTAGTCCACCACTTTGTACAAGAAGGCTGAAC</td>
</tr>
<tr>
<td>ccdB-Dn</td>
<td>CCAAGCTTGG ACAAGTCTATCAAGAAAAGGCTGAAC</td>
</tr>
</tbody>
</table>
Supplemental Information

Supplemental Experimental Procedures

GFP lines

The GFP lines shown in Figure 2 and S1 are the following (except for H1-GFP lines described below): HTR12/CENH3 lines: pHTR12::HTR12-GFP (Fang and Spector, 2005), pCENH3::GFP-CENH3 (Ravi et al., 2011). TFL2/LHP1 line: pLHP1::LHP1-GFP (Nakahigashi et al., 2005). pHTR5::HTR5-GFP, pHTR8::HTR8-CFP (Ingouff et al., 2010). pHTA11::HTA11-GFP (Kumar and Wigge, 2010).

Generation of GFP and RFP -tagged H1 variants

To generate promH1.1::H1.1-EGFP vector, we amplified the promoter (1982 bp) with coding sequence of H1.1 (except the termination codon) with the following primers [5' - GC GTGCAGCTCATCTGATAGGGATGG-3'; 5' - GCGGATCCCTTCTTAACCCTAGAAGAAGC-3'] and subcloned the fragment in the pCambia1390 vector (carrying a 35S:Hygromycin resistance for in planta selection) using the SalI and BamHI restriction sites introduced in the forward and reverse primers, respectively (underlined), giving pCambia1390/promH1.1::H1.1 vector. The EGFP sequence was subcloned with the BamHI and EcoRI restriction sites into pCambia1390/promH1.1::H1.1 vector, giving pCambia1390/promH1.1::H1.1-EGFP vector. The termination sequence of H1.1 (873 bp) was amplified with the following primers [5' - GCCCATGGCTCTCATAAGGTAGTTTGTAGTAGGTTTAG-3'; 5' - GCCCATGGCTCTCATAAGGTAGTTTGTAGTAGGTTTAG-3'] and subcloned in pCambia1390/promH1.1::H1.1-EGFP vector using the EcoRI and NcoI restriction sites introduced in the forward and reverse primers, respectively, giving the final vector promH1.1::H1.1-EGFP.

To generate promH1.2::H1.2-EGFP vector, we amplified the promoter (2191 bp) with coding sequence of H1.2 (except the termination codon) with the following primers [5' - GCCCTGAGTTGCATAAGATGGGAAACAA-3'; 5' - GCCGTGACCTCTTCTAGCCTTGATAAGCTGAA-3'] and subcloned the fragment in the pCambia1390 vector using the PstI and SalI restriction sites introduced in the forward and reverse primers, respectively, giving pCambia1390/promH1.2::H1.2 vector. By using the BamHI and EcoRI restriction sites the EGFP sequence was subcloned in pCambia1390/promH1.2::H1.2 vector, giving pCambia1390/promH1.2::H1.2-EGFP vector. The termination sequence of H1.2 (1187 bp) was amplified with the following primers [5' - GCCGAATTCCTGAGATTGTTTCTGGAG-3'; 5' - GCCGAATTCCTGAGATTGTTTCTGGAG-3'] and subcloned in pCambia1390/promH1.2::H1.2-EGFP vector using the EcoRI and NheI restriction sites introduced in the forward and reverse primers, respectively, giving the final vector promH1.2::H1.2-EGFP.

To verify that the expression pattern observed was not influenced by the long-range effect of the 35S enhancer, we generated new fusions in a vector carrying a NOS::BAR selection cassette for BASTA selection in planta. The NOS::BAR selection cassettes was obtained from pGreenII0229 plasmid (Bendel-Stenzel et al., 1998) using the EcoRV restriction sites and inserted into XmnI-
digested plasmid pCambia0390, giving pCambia0390/NOS::BAR plasmid. Then the whole promH1.1::H1.1-EGFP fragment from pCambia1390 (35S::HYG) was subcloned in pCambia0390 (NOS::BAR) using the AscI and NcoI restriction sites and similarly promH1.2::H1.2-EGFP fragment was subcloned using the AscI and NheI restriction sites. An additional promH1.1::H1.1-RFP vector was generated by swapping the EGFP with the RFP-T (Maheshwari, 1950) using EcoRI and BamHI restriction sites.

To generate the promH1.1::EGFP-H1.1 vector, we amplified the promoter (2006 bp) with the following primers [5’-GCGTCACTTGGGGGAAGTAAATCCAA-3’; 5’-GCGGATCCCATCTTCTCTGAACCTAAAGTC-3’] and subcloned the fragment in the pCambia1390 vector using the Sall and BamHI restriction sites introduced in the FWD and REV primers, respectively, giving pCambia1390/promH1.1 vector. The EGFP sequence was subcloned into pCambia1390/promH1.1 vector by using the BamHI and EcoRI restriction sites, giving pCambia1390/promH1.1::EGFP vector. The coding sequence of H1.1 (except the start codon) with its termination sequence (1510 bp) was amplified with the following primers [5’-GCGAATTCATAGGGGAATAGAGACG-3’; 5’-GCCCATGTGGTGGTACATCCACAACAACTG-3’] and subcloned in pCambia1390/promH1.1::EGFP vector using the EcoRI and NcoI restriction sites introduced in the forward and reverse primers, respectively, giving the final vector promH1.1::EGFP-H1.1.

To generate the promH1.1::RFP-H1.1 vector, the GFP sequence from the promH1.1::EGFP-H1.1 vector was replaced using the RFP-T sequence amplified from the pRFP-T_tag plasmid (Shaner et al., 2008) using the following primers [5’-TTAGGATTCCTGCTCTTAAGGGCCGAAAGGC-3’; 5’-ATTAGAATTCCTCTGTAAGCTCGTGAGTCCCTCAGAAGG-3’] and subcloned with the respective BamHI and EcoRI restriction sites.

To generate promH1.2::EGFP-H1.2 vector, we amplified the promoter (2191 bp) with the following primers [5’-GCCTGAGGCGATTCGTAATGATGG-3’; 5’-GCCTGAGGCGATTCGTAATGATGG-3’] and subcloned the fragment in the pCambia1390 vector using the PstI and Sall restriction sites introduced in the FWD and REV primers, respectively, giving pCambia1390/promH1.2 vector. The EGFP sequence was subcloned into pCambia1390/promH1.2 vector using the BamHI and EcoRI restriction sites, giving pCambia1390/promH1.2::EGFP vector. The coding sequence of H1.2 (except the start codon) with its termination sequence (1044 bp) was amplified with the following primers [5’-GCCCAATTTGCTATAGAGGGAAGAAAAGCCT-3’; 5’-GCCCAATTTGCTATAGAGGGAAGAAAAGCCT-3’], digested with the MnlI and NheI restriction sites introduced in the FWD and REV primers, respectively and inserted into EcoRI-NheI-pCambia1390/promH1.2::EGFP vector, giving the final vector promH1.2::EGFP-H1.2.

The constructs were introduced into Agrobacterium tumefaciens (GV3101) which were then used to transform Arabidopsis (Col-0) plants with the floral dip method (Seisenberger et al., 2012). Seeds of transformed plants were selected on ½ MS agar plates containing 30 mg/l hygromycin or were grown in soil and selected by spraying with Basta solution (0.05 mg/ml). Plants were selected for single insertion lines and homozygous lines from T3 or T4 generation were eventually used for analysis. Expression of EGFP-tagged H1 was confirmed by fluorescence analysis.
Immunostaining in whole-mount ovule primordia

Immunostaining of active polII was done as described in Autran et al (2011). Below is described the method for immunostaining of H3 and H3 modifications as shown in Figure 3, 5. Young carpels were collected and fixed in freshly made BVO fixation buffer (2mM EGTA pH7.5, 1% Formaldehyde, 10% DMSO, and 0.1% Tween in 1xPBS) at room temperature for 30min. After fixation, the carpels were kept in PBT (1x PBS with 0.1% tween-20) on ice. The ovules were then dissected and embedded in 5% acrylamide + 1.2% APS (ammonium persulfate) + 1.2% NaPS (sodium sulfite) in 1x PBS, and covered with a 20mm x 20mm coverslip. The coverslip was removed after 60min using a razor blade. All subsequent steps are made in coplin jars, under gentle shaking, unless indicated. The samples were treated as follows: 5min methanol, 5min ethanol, 30min ethanol:xylene (1:1), 5min ethanol, 5min methanol, and 15min methanol:PBT(1:1) + 2.5% Formaldehyde. Following 2x10 min PBT washes, the samples were incubated with an enzyme mix for cell wall digest [0.5% (w/v) cellulose, 1% (w/v) driselase, 0.5% (w/v) pectolyase in PBS], 100µL was applied per slide with a coverslip, for 1-2hrs at 37°C in a moist-chamber (the incubation time has to be determined empirically for each batch of enzyme mix). After 2x5min PBT washes, the slides were incubated with RNaseA 100µg/ml in PBS + 1% Tween-20 at 37°C for 1hr. The slide were then washed with 2x5 min PBT and fixed with PBT-F (PBT+ formaldehyde 2.5% (v/v) for 20min. Following 10min PBT wash, the samples were permeabilized in PBS + 2% Tween-20 at 4°C for 2hrs. After 2x5 min PBT washes, the samples were incubated with 100µL primary antibody dilution (Table S6) in PBS + 0.2% Tween-20 at 4°C for 16-21hrs in a moist chamber. The slide was then rinsed in PBT for 2-4 hrs and incubated with the secondary antibody 1:200 in PBS + 0.2% Tween-20 at 4°C for 2days as before. Following 1hr PBT wash, the samples were counterstained with Propidium Iodide at 10µg/ml in PBS for 15min, washed in PBS for 10min and mounted in Prolong Gold (Invitrogen) supplemented with 10µg/ml PI.
Figure S1. Developmental dynamics of GFP-tagged histone variants and LHP1 during MMC differentiation

The expression dynamics of GFP-tagged histone variants is shown along the stages of ovule primordia development 1-0 until 2-III (onset Prophase I) and in the functional megaspore.
Figure S2. H1 Immunostaining with a novel, plant specific antibody confirms H1 depletion in MMCs

(A) H1 is depleted in MMC (dashed line) at stage 2-I, as revealed by whole-mount immunostaining in wild type ovule primordia.
(B) Reloading of H1 in MMC at stage 2-II in wild-type ovule primordia (faint signals).
(C) H1 is undetectable in the ovule primordia of h1.1; h1.2,h1.3 triple mutant. The green signals arise from non-specific secondary antibody binding.
Figure S3. GFP-tagged H1 dynamics is influenced by an inhibitor of the proteasome
Whole inflorescences were incubated in water (Mock) or water containing 100nM Syringolin A (Groll et al., 2008) for 48h before imaging (CSLM, green: GFP, red: FM4-64, grey: DIC). Yellow arrows point to the MMCs where H1 signals clearly remained detectable.
Figure S4. Selected candidate modifiers do not contribute to chromatin dynamics in the MMC

A. H3K27me3 levels in the MMCs relative to the nucellus are stable in the ref6 mutant (Lu et al., 2011), compared to that in wild type. Representative image is shown for the overlay of antibody (green), DNA (propidium iodide, red) and transmission light (DIC, grey). Detailed quantification is provided in Table S6. B. Decreased levels of H3K27me3 in wild-type MMCs relative to the nucellus is visible both after normalization against the DNA content (left graph as in Figure 3) or in absolute values of immunostaining signals (right graph) suggesting both a replication-coupled
passive dilution and a probable active demethylation process. C. H3K4me3 levels in MMCs which lack ATX1 activity (atx1-1 (Alvarez-Venegas et al., 2003)) are not increased, compared to that in col. Representative images show the antibody (green), DNA (propidium iodide, red), transmission light (DIC, grey), and overlay of antibody (green) with DNA (red). C. H3K9me2 immunosignals are drastically decreased in kyp-2 (Lindroth et al., 2004) ovule primordia both in the MMC and nucellus, yet MMC and gametophyte differentiation proceeds normally. D. H1.1-GFP is normally depleted in mutant MMCs of the quadruple mutant lacking NAP1;1-4 activity (Liu et al., 2009) or the double mutant lacking NRP1;NRP2 activity (Zhu et al., 2006) and reloaded at prophase I. Scale bar: 10µm.
Figure S5. Developmental dynamics of changes in chromatin modification levels in MMCs relative to nucellus’ cells.

Graphs represent a MMC/nucellus ratio of relative chromatin modification levels (measured as antibody signal intensity/DNA signal intensity). The stars indicate the level of significance in a Welch's t-test done as for Figure 3 (MMC vs nucellus). Most euchromatin marks are significantly altered in MMC only at stage 2-I or 2-II, while H3K9me2 enrichment is typically measured at stage 1-II.
Figure S6. H1.1 is a stable chromatin component during mitotic S-phase

In mitotically active zones of the seedling’ root, H1.1::RFP (red) remains incorporated in the chromatin of nuclei engaged in the S-phase as revealed by EdU incorporation (green). (A) confocal section of a root tip (B) close-up on one S-phase nucleus with EdU incorporation in euchromatin. After 2hrs pulse, nuclei showed EdU incorporation in euchromatin only, heterochromatin only, or both. 21/21 nuclei with EdU incorporation in euchromatin only as in (B) showed H1.1-RFP signals. Scale Bar: 10µm.

Figure S7. Meiosis entails a novel dynamics in histone modifications

During meiosis I, the dynamic trends for H3K27me3 and H3K9me2 seen during MMC differentiation become more pronounced with a near-loss and high enrichment of immunosignals, respectively, while the trend seems to reverse for H3K27me1 with apparent higher signals than in differentiated MMCs (Figure 3). H3K4me3 and H4K16Ac are well detected, yet the rapid evolution of meiotic chromosomes does not allow precise quantifications for those marks. The upper panel shows immunostaining signals of the indicated histone modifications in primordia tips at stage 2-III (dotted contours: MMC in prophase I). Scale Bar: 10 µm. Lower panel: close-up on MMC nuclei . Red, Propidium iodide, green, antibody signal.
Figure S8. Replication and transcriptional status in the FM

(A) The selected product of female meiosis, the functional megaspore, rapidly engages in mitosis for gametophytic development, with EdU incorporation. (B) Active PolII is detected at low and variable levels by immunodetection, probably due to the rapid transition into the gametophytic stage.

<table>
<thead>
<tr>
<th>Occurrence phenotype</th>
<th>Wild-type</th>
<th>ago9-3/+</th>
<th>ago9-4/+</th>
<th>rdr6-11/+</th>
<th>sgs3-11/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class A</td>
<td>~ 5%</td>
<td>26%</td>
<td>31.5%</td>
<td>30.3%</td>
<td>26.3%</td>
</tr>
<tr>
<td>Class B</td>
<td>-</td>
<td>26%</td>
<td>21%</td>
<td>15%</td>
<td>23.6%</td>
</tr>
<tr>
<td>Class C</td>
<td>-</td>
<td>-</td>
<td>11.5%</td>
<td>15%</td>
<td>2.7%</td>
</tr>
<tr>
<td>n</td>
<td>100</td>
<td>30</td>
<td>57</td>
<td>33</td>
<td>38</td>
</tr>
</tbody>
</table>

Figure S9. Loss of H1.1/GFP is a hallmark of multiple MMCs in ago9, rdr6 and sgs3 mutants

The number of primordia showing more than one enlarged cell (MMC and MMC-like) was scored in wild-type or mutant primordia with the indicated genotypes. The expression pattern of H1.1-GFP in these MMC and MMC-like cells was scored: class A, primordia with only one H1.1-GFP negative cell (MMC); class B, primordia where all MMC’s are H1.1-GFP negative; class C: primordia with one H1.1-GFP negative MMC and reduced H1.1-GFP levels in the other(s).
Figure S10. Female meiosis in sdg2

Ovule primordia lacking SDG2 activity (Berr et al., 2010) undergo proper meiosis as assessed by clearing and DNA staining and despite reduced H3K4me3 levels compared to that in wild type (Figure 6, this Figure). The lower panel shows deconvolved, 3D-reconstruction of MMC nuclei at different stages of prophase I corresponding to the primordia on the upper panel. Chromosome condensation is initiated during early prophase I (pre-leptotene/leptotene), and becomes more visible at the late prophase during which bivalent are formed. Images in the lower panel were deconvolved using Huygens (SVI).
Figure S11. Chromatin reprogramming in plant MMCs
Table S1. Detailed quantification of nuclear size and heterochromatin content in MMC and nucellus cells during ovule primordia development

<table>
<thead>
<tr>
<th>Nuclear Size (μm³)</th>
<th>MMC</th>
<th></th>
<th></th>
<th>nucellus</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>stage</td>
<td>average</td>
<td>s.d.</td>
<td>n</td>
<td>average</td>
<td>s.d.</td>
<td>n</td>
</tr>
<tr>
<td>Stage 1-II</td>
<td>44.2 ± 16.8</td>
<td>(n=10)</td>
<td>36.698 ± 11.46</td>
<td>(n=46)</td>
<td>P=0.206</td>
<td></td>
</tr>
<tr>
<td>Stage 2-I</td>
<td>76.127 ± 33.66</td>
<td>(n=13)</td>
<td>43.072 ± 15.4</td>
<td>(n=63)</td>
<td>P=0.0042</td>
<td></td>
</tr>
<tr>
<td>Stage 2-II</td>
<td>90.869 ± 25.2</td>
<td>(n=10)</td>
<td>47.382 ± 13.92</td>
<td>(n=50)</td>
<td>P=0.0003</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>RHF</th>
<th>MMC</th>
<th></th>
<th></th>
<th>nucellus</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>stage</td>
<td>average</td>
<td>s.d.</td>
<td>n</td>
<td>average</td>
<td>s.d.</td>
<td>n</td>
</tr>
<tr>
<td>Stage 1-I</td>
<td>24.87 ± 6.06</td>
<td>(n=10)</td>
<td>31.517 ± 3.58</td>
<td>(n=30)</td>
<td>P=0.007</td>
<td></td>
</tr>
<tr>
<td>Stage 1-II</td>
<td>21.24 ± 6.08</td>
<td>(n=10)</td>
<td>31.85 ± 4.5</td>
<td>(n=30)</td>
<td>P=0.0002</td>
<td></td>
</tr>
<tr>
<td>Stage 2-I</td>
<td>18.38 ± 7.84</td>
<td>(n=10)</td>
<td>31.38 ± 4.44</td>
<td>(n=30)</td>
<td>P=0.0004</td>
<td></td>
</tr>
<tr>
<td>Stage 2-II</td>
<td>10.51 ± 4.4</td>
<td>(n=10)</td>
<td>32.3 ± 5.7</td>
<td>(n=30)</td>
<td>P&lt;0.0001</td>
<td></td>
</tr>
</tbody>
</table>

RHF: Relative Heterochromatin Fraction calculated as a % ratio of fluorescence intensity (propidium iodide) in heterochromatic foci over intensity in the whole nucleus. s.d., standard deviation (note that the graphs show the standard error to mean= s.d/√n). P-value: Welch's t-test (2 tails)
Table S2: Detailed quantifications of relative nuclear immunostaining in MMC and nucellus cells of primordia at stage 2-II

(a) Antibody signal over propidium iodide signal, relative to the nucellus

<table>
<thead>
<tr>
<th></th>
<th>MMC % ± s.d</th>
<th>n</th>
<th>nucellus % ± s.d</th>
<th>n</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3</td>
<td>102.51 ± 33.48</td>
<td>(n= 9)</td>
<td>100 ± 36.46</td>
<td>(n= 43)</td>
<td>0.84</td>
</tr>
<tr>
<td>H3K9me1</td>
<td>73.54 ± 14.23</td>
<td>(n= 11)</td>
<td>100 ± 20.74</td>
<td>(n= 51)</td>
<td>3.57E-06</td>
</tr>
<tr>
<td>H3K9me2</td>
<td>171.44 ± 47.72</td>
<td>(n= 11)</td>
<td>100 ± 31.21</td>
<td>(n= 45)</td>
<td>1.69E-05</td>
</tr>
<tr>
<td>H3K27me1</td>
<td>43.57 ± 8.25</td>
<td>(n= 6)</td>
<td>100 ± 20.23</td>
<td>(n= 26)</td>
<td>6.70E-12</td>
</tr>
<tr>
<td>H3K27me3</td>
<td>53.51 ± 15.16</td>
<td>(n= 7)</td>
<td>100 ± 19.02</td>
<td>(n= 28)</td>
<td>7.58E-08</td>
</tr>
<tr>
<td>H3K4me2</td>
<td>74.56 ± 17.28</td>
<td>(n= 19)</td>
<td>100 ± 28.88</td>
<td>(n= 88)</td>
<td>1.74E-06</td>
</tr>
<tr>
<td>H3K4me3</td>
<td>270.17 ± 263.77</td>
<td>(n= 12)</td>
<td>100 ± 24.30</td>
<td>(n= 60)</td>
<td>0.03</td>
</tr>
<tr>
<td>H4KAc16</td>
<td>74.18 ± 10.63</td>
<td>(n= 10)</td>
<td>100 ± 17.30</td>
<td>(n= 46)</td>
<td>1.10E-07</td>
</tr>
<tr>
<td>RNA PolII</td>
<td>61 ± 23.00</td>
<td>(n= 7)</td>
<td>100 ± 19.00</td>
<td>(n= 25)</td>
<td>2.81E-04</td>
</tr>
</tbody>
</table>

(b) Antibody signal over propidium iodide signal- absolute ratios in the nucellus

<table>
<thead>
<tr>
<th></th>
<th>Ab/PI ± s.d</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3</td>
<td>0.98664 ± 0.36</td>
<td>(n= 43)</td>
</tr>
<tr>
<td>H3K9me1</td>
<td>0.72185 ± 0.15</td>
<td>(n= 51)</td>
</tr>
<tr>
<td>H3K9me2</td>
<td>0.53354 ± 0.17</td>
<td>(n= 45)</td>
</tr>
<tr>
<td>H3K27me1</td>
<td>0.91792 ± 0.19</td>
<td>(n= 26)</td>
</tr>
<tr>
<td>H3K27me3</td>
<td>0.83598 ± 0.16</td>
<td>(n= 28)</td>
</tr>
<tr>
<td>H3K4me2</td>
<td>0.75001 ± 0.22</td>
<td>(n= 88)</td>
</tr>
<tr>
<td>H3K4me3</td>
<td>0.71775 ± 0.17</td>
<td>(n= 60)</td>
</tr>
<tr>
<td>H4KAc16</td>
<td>0.81903 ± 0.14</td>
<td>(n= 46)</td>
</tr>
</tbody>
</table>

The relative immunostaining signals are calculated as fluorescence intensity ratios of Antibody (Ab) signals over Propidium Iodide (PI) signals. (a) The ratios in nucellus cells are averaged across n samples and set as 100%. Ab/PI ratio in the MMC relative to that in nucellus cells. (b) absolute ratios in nucellus cells. s.d., standard deviation. (note that the graphs show the standard error to mean= s.d/√n). P-value: Welch's t-test (2 tails)
Table S3: Detailed quantifications of relative nuclear immunostaining in MMC and nucellus cells of primordia at stage 1-II and 2-I

A. stage 1-II

(a) Antibody signal over propidium iodide signal, relative to the nucellus

<table>
<thead>
<tr>
<th></th>
<th>MMC</th>
<th></th>
<th>nucellus</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>s.d.</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>H3K9me2</td>
<td>150 ± 36.14</td>
<td>(n=8)</td>
<td>100 ± 22.43</td>
<td>(n=35)</td>
</tr>
</tbody>
</table>

(b) Antibody signal over propidium iodide signal- absolute ratios in the nucellus

<table>
<thead>
<tr>
<th></th>
<th>Ab/PI</th>
<th>s.d.</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3K9me2</td>
<td>0.58919</td>
<td>± 0.59</td>
<td>(n=35)</td>
</tr>
</tbody>
</table>

B. stage 2-I

(a) Antibody signal over propidium iodide signal, relative to the nucellus

<table>
<thead>
<tr>
<th></th>
<th>MMC</th>
<th></th>
<th>nucellus</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>s.d.</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>H3</td>
<td>95.45 ± 13.17</td>
<td>(n=10)</td>
<td>100 ± 19.71</td>
<td>(n=48)</td>
</tr>
<tr>
<td>H3K9me1</td>
<td>99.37 ± 31.34</td>
<td>(n=10)</td>
<td>100 ± 71.85</td>
<td>(n=48)</td>
</tr>
<tr>
<td>H3K9me2</td>
<td>138.91 ± 30.08</td>
<td>(n=10)</td>
<td>100 ± 23.65</td>
<td>(n=44)</td>
</tr>
<tr>
<td>H3K27me3</td>
<td>85.56 ± 20.26</td>
<td>(n=9)</td>
<td>100 ± 21.12</td>
<td>(n=37)</td>
</tr>
<tr>
<td>H3K4me2</td>
<td>86.49 ± 32.27</td>
<td>(n=9)</td>
<td>100 ± 25.30</td>
<td>(n=37)</td>
</tr>
<tr>
<td>H3K4me3</td>
<td>338.32 ± 364.38</td>
<td>(n=6)</td>
<td>100 ± 32.22</td>
<td>(n=29)</td>
</tr>
<tr>
<td>H4KAc16</td>
<td>74.34 ± 17.87</td>
<td>(n=12)</td>
<td>100 ± 30.25</td>
<td>(n=58)</td>
</tr>
</tbody>
</table>

(b) Antibody signal over propidium iodide signal- absolute ratios in the nucellus

<table>
<thead>
<tr>
<th></th>
<th>Ab/PI</th>
<th>s.d.</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3</td>
<td>0.621242</td>
<td>± 0.06</td>
<td>(n=34)</td>
</tr>
<tr>
<td>H3K9me1</td>
<td>0.49682</td>
<td>± 0.18</td>
<td>(n=48)</td>
</tr>
<tr>
<td>H3K9me2</td>
<td>0.555674</td>
<td>± 0.07</td>
<td>(n=44)</td>
</tr>
<tr>
<td>H3K27me3</td>
<td>0.928</td>
<td>± 0.10</td>
<td>(n=37)</td>
</tr>
<tr>
<td>H3K4me2</td>
<td>0.640063</td>
<td>± 0.08</td>
<td>(n=37)</td>
</tr>
<tr>
<td>H3K4me3</td>
<td>0.666477</td>
<td>± 0.11</td>
<td>(n=29)</td>
</tr>
<tr>
<td>H4KAc16</td>
<td>0.802813</td>
<td>± 0.12</td>
<td>(n=58)</td>
</tr>
</tbody>
</table>

legend: as for Table S2
Table S4. Quantifications of DNA content increase and EdU incorporation in MMCs

(a) Quantification of DNA content (PI fluorescence intensity) in MMC relative to the averaged content in L1-1, L1-0 and L1-2 nucellus cells during ovule primordia development

<table>
<thead>
<tr>
<th>stage</th>
<th>average</th>
<th>s.d.</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-I</td>
<td>1.01</td>
<td>± 0.12</td>
<td>10</td>
</tr>
<tr>
<td>1-II</td>
<td>1.24</td>
<td>± 0.32</td>
<td>10</td>
</tr>
<tr>
<td>2-I</td>
<td>1.59</td>
<td>± 0.37</td>
<td>10</td>
</tr>
<tr>
<td>2-II</td>
<td>1.96</td>
<td>± 0.40</td>
<td>10</td>
</tr>
</tbody>
</table>

s.d., standard deviation. (note that the graphs show the standard error to mean= s.d/√n).

(b) Quantification of MMCs with distinct EdU incorporation patterns (2h pulse)

<table>
<thead>
<tr>
<th>EdU signal</th>
<th>Stages 1-I + 1-II</th>
<th>Stage 2-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Euchromatin only (1)</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>Heterochromatin only (2)</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>Euchromatin and heterochromatin</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>12</strong></td>
<td><strong>17</strong></td>
</tr>
</tbody>
</table>
Table S5: Detailed quantifications of relative nuclear immunostaining in functional megaspore and nucellus cells

(a) Antibody signal over propidium iodide signal, relative to the nucellus

<table>
<thead>
<tr>
<th></th>
<th>MMC s.d.</th>
<th>n</th>
<th>nucellus s.d.</th>
<th>n</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3</td>
<td>101.24 ± 17.40 (n=6)</td>
<td>100 ± 20.18 (n=24)</td>
<td></td>
<td>0.881</td>
<td></td>
</tr>
<tr>
<td>H3K9me1</td>
<td>77.72 ± 29.65 (n=10)</td>
<td>100 ± 45.06 (n=50)</td>
<td></td>
<td>0.054</td>
<td></td>
</tr>
<tr>
<td>H3K9me2</td>
<td>76.13 ± 17.86 (n=6)</td>
<td>100 ± 28.69 (n=25)</td>
<td></td>
<td>0.015</td>
<td></td>
</tr>
<tr>
<td>H3K27me1</td>
<td>66.96 ± 15.59 (n=7)</td>
<td>100 ± 25.21 (n=32)</td>
<td></td>
<td>7.12E-05</td>
<td></td>
</tr>
<tr>
<td>H3K4me2</td>
<td>50.34 ± 18.05 (n=11)</td>
<td>100 ± 20.62 (n=54)</td>
<td></td>
<td>2.29E-11</td>
<td></td>
</tr>
<tr>
<td>H3K4me3</td>
<td>64.74 ± 10.40 (n=12)</td>
<td>100 ± 15.19 (n=51)</td>
<td></td>
<td>8.84E-14</td>
<td></td>
</tr>
<tr>
<td>H4KAc16</td>
<td>56.96 ± 13.66 (n=12)</td>
<td>100 ± 35.57 (n=58)</td>
<td></td>
<td>1.19E-09</td>
<td></td>
</tr>
</tbody>
</table>

(b) Antibody signal over propidium iodide signal- absolute ratios in the nucellus

<table>
<thead>
<tr>
<th></th>
<th>Ab/PI s.d.</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3</td>
<td>0.699 ± 0.14 (n=24)</td>
<td></td>
</tr>
<tr>
<td>H3K9me1</td>
<td>0.673 ± 0.30 (n=50)</td>
<td></td>
</tr>
<tr>
<td>H3K9me2</td>
<td>0.663 ± 0.19 (n=24)</td>
<td></td>
</tr>
<tr>
<td>H3K27me1</td>
<td>0.982 ± 0.25 (n=32)</td>
<td></td>
</tr>
<tr>
<td>H3K4me2</td>
<td>1.157 ± 0.24 (n=54)</td>
<td></td>
</tr>
<tr>
<td>H3K4me3</td>
<td>0.920 ± 0.14 (n=51)</td>
<td></td>
</tr>
<tr>
<td>H4KAc16</td>
<td>1.330 ± 0.47 (n=58)</td>
<td></td>
</tr>
</tbody>
</table>

s.d., standard deviation. (note that the graphs show the standard error to mean= s.d/√n).
Table S6: Detailed quantifications of relative nuclear immunostaining in ago9-4 and sdg2 mutant megaspore mother cells relative to nucellus cells

General legend: Ab/PI, Fluorescence intensity sum of the antibody (Ab) relative to the DNA (PI) signals. s.d., standard deviation. (note that the graphs show the standard error to mean= s.d/√n). n, number of cells quantified, P-value: Welch’s t-test (2 tails)

A. H3K27me1 relative levels in ago9-4 MMCs

(a) Antibody signal over propidium iodide signal, relative to the nucellus in ago9-4/ago9-4 mutant

<table>
<thead>
<tr>
<th>Ab/PI</th>
<th>s.d.</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmc1</td>
<td>38.43 ± 11.61</td>
<td>(n= 5)</td>
<td>0.7</td>
</tr>
<tr>
<td>mmc2</td>
<td>39.65 ± 8.31</td>
<td>(n= 5)</td>
<td></td>
</tr>
<tr>
<td>nucellus</td>
<td>100 ± 30.24</td>
<td>(n= 24)</td>
<td></td>
</tr>
</tbody>
</table>

In each primordia, the mmc with the highest intensity was called mmc1, while the one with the lowest was called mmc2. The distribution of fluorescence intensity in individual mmc’s (white and black dot) per ovule primordia is as follows:

(b) Antibody signal over propidium iodide signal- absolute ratios in the nucellus ago9-4/ago9-4 mutant

<table>
<thead>
<tr>
<th>Ab/PI</th>
<th>s.d.</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>nucellus</td>
<td>0.997043 ± 0.3016</td>
<td>(n= 24)</td>
</tr>
</tbody>
</table>

B. H3K27me3 relative levels in ago9-4 MMCs

(a) Antibody signal over propidium iodide signal, relative to the nucellus in ago9-4/ago9-4 mutant

<table>
<thead>
<tr>
<th>Ab/PI</th>
<th>s.d.</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmc1</td>
<td>62.82 ± 38.01</td>
<td>(n= 10)</td>
<td>0.38</td>
</tr>
<tr>
<td>mmc2</td>
<td>48.63 ± 32.7</td>
<td>(n= 10)</td>
<td></td>
</tr>
<tr>
<td>nucellus</td>
<td>100 ± 48.25</td>
<td>(n= 50)</td>
<td></td>
</tr>
</tbody>
</table>

In each primordia, the mmc with the highest intensity was called mmc1, while the one with the lowest was called mmc2. The distribution of fluorescence intensity in individual mmc’s (white and black dot) per ovule primordia is as follows:
She et al. Chromatin reprogramming during the somatic-to-reproductive cell fate transition in plants

black dot) per ovule primordia is as follows:

![Graph](image_url)

(b) Antibody signal over propidium iodide signal- absolute ratios in the nucellus of the *ago9-4/ago9-4* mutant

<table>
<thead>
<tr>
<th></th>
<th>Ab/PI</th>
<th>s.d.</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>nucellus</td>
<td>1.9505</td>
<td>± 0.89</td>
<td>(n= 50)</td>
</tr>
</tbody>
</table>

C. H3K4me3 relative levels in *sdg2* vs wild-type (Col) MMCs

(a) Antibody signal over propidium iodide signal, relative to the nucellus in *sdg2/sdg2* mutant

<table>
<thead>
<tr>
<th>MMC</th>
<th>nucellus</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>s.d.</td>
</tr>
<tr>
<td>163.29</td>
<td>± 48.59</td>
</tr>
<tr>
<td>100</td>
<td>± 54.16</td>
</tr>
</tbody>
</table>

(b) Antibody signal over propidium iodide signal- absolute ratios in the nucellus of the *sdg2/sdg2* mutant

<table>
<thead>
<tr>
<th></th>
<th>Ab/PI</th>
<th>s.d.</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.26</td>
<td>± 0.68</td>
<td>(n=59)</td>
<td></td>
</tr>
</tbody>
</table>

(c) Comparison of H3K4me3 relative levels (MMC/nucellus) in *sdg2* and wild-type (col) primordia

<table>
<thead>
<tr>
<th></th>
<th>average</th>
<th>s.d.</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col</td>
<td>270.17</td>
<td>131.89</td>
<td>12</td>
</tr>
<tr>
<td><em>sdg2</em></td>
<td>163.29</td>
<td>48.59</td>
<td>12</td>
</tr>
</tbody>
</table>

D. H3K27me3 relative levels in *ref6* vs wild-type (Col) MMCs

(a) Antibody signal over propidium iodide signal, relative to the nucellus of the *ref6/ref6* mutant

<table>
<thead>
<tr>
<th>MMC</th>
<th>nucellus</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>s.d.</td>
</tr>
<tr>
<td>59.77</td>
<td>± 16.3</td>
</tr>
<tr>
<td>100</td>
<td>± 17.15</td>
</tr>
</tbody>
</table>

P= 0.0001
(b) Antibody signal over propidium iodide signal- absolute ratios in the nucellus of the ref6/ref6 mutant

<table>
<thead>
<tr>
<th>Ab/PI</th>
<th>s.d.</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.79</td>
<td>± 0.14</td>
<td>(n=50)</td>
</tr>
</tbody>
</table>

(c) Comparison of H3K4me3 relative levels (MMC/nucellus) in ref6 and wild-type (col) primordia

<table>
<thead>
<tr>
<th></th>
<th>average</th>
<th>s.d.</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col</td>
<td>63.16</td>
<td>30.8</td>
<td>10</td>
</tr>
<tr>
<td>ref6</td>
<td>59.77</td>
<td>32.6</td>
<td>10</td>
</tr>
</tbody>
</table>

legend: as for Table S2
Table S7. List of antibodies and test of immunostaining signals’ reliability in whole-mount ovule primordia in serial dilutions

<table>
<thead>
<tr>
<th>Antibody Target</th>
<th>Provider</th>
<th>Cat#</th>
<th>(concentration)</th>
<th>1/1000</th>
<th>1/500</th>
<th>1/200</th>
<th>1/100</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3</td>
<td>Abcam</td>
<td>ab1791</td>
<td>(1mg/ml)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>nd</td>
</tr>
<tr>
<td>H3K27me1</td>
<td>Upstate</td>
<td>07-448</td>
<td>(1mg/ml)</td>
<td>nd</td>
<td>-</td>
<td>+</td>
<td>nd</td>
</tr>
<tr>
<td>H3K27me3</td>
<td>Upstate</td>
<td></td>
<td>(1mg/ml)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>H3K4me2</td>
<td>Abcam</td>
<td>ab32356</td>
<td>(0.04mg/ml)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>nd</td>
</tr>
<tr>
<td>H3K4me3</td>
<td>Upstate</td>
<td>07-473</td>
<td>(0.1-0.5 mg/ml)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>H3K9me1</td>
<td>Upstate</td>
<td>07-450</td>
<td>(1mg/ml)</td>
<td>nd</td>
<td>-</td>
<td>+</td>
<td>nd</td>
</tr>
<tr>
<td>H3K9me2</td>
<td>Upstate</td>
<td>07-441</td>
<td>(1mg/ml)</td>
<td>nd</td>
<td>-</td>
<td>+</td>
<td>nd</td>
</tr>
<tr>
<td>H4K16Ac</td>
<td>Millipore</td>
<td>07-329</td>
<td>(1mg/ml)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>nd</td>
</tr>
<tr>
<td>H1</td>
<td>Agrisera</td>
<td>AS111801</td>
<td>(1mg/ml)</td>
<td>nd</td>
<td>nd</td>
<td>+</td>
<td>nd</td>
</tr>
</tbody>
</table>

Immunostaining results on whole-mount primordia: +, stable signal. -: unstable signals (not reproducible across replicates). Grey: dilutions used for the quantifications in Figure 3, 5, 6, S2, Table S2, S3, S5, S6.
References related to Supplemental Information


