Particular Significance of SRD2-Dependent snRNA Accumulation in Polarized Pattern Generation during Lateral Root Development of Arabidopsis

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Lateral root primordia are initiated by anticlinal division of cells in the pericycle and are constructed through an ordered set of cell divisions. At the completion of the development of the primordium, cell division ceases, after which the lateral root meristem is activated. In Arabidopsis, this course of lateral root morphogenesis was found to be significantly susceptible to srd2-1, a temperature-sensitive mutation of the SRD2 gene encoding an activator of small nuclear RNA (snRNA) transcription. The srd2-1 mutation altered the organization of cells of the root primordium and, importantly, maintained primordial cell division for a long period, resulting in the formation of abnormal hemispherical laterals. Expression patterns of various reporter genes suggested that both the apical–basal and radial axes were not well established in the lateral root primordia of the srd2-1 mutant. In the early stages of development of the primordium, the srd2-1 mutation reduced the amount of the auxin efflux facilitator PIN-FORMED (PIN) and, probably by this means, interfered with the generation of an auxin gradient. Spliceosomal snRNAs accumulated at high levels throughout young root primordia and then decreased in association with the arrest of cell division, and finally increased again when the apical meristem became activated. The accumulation of snRNAs was severely suppressed by the srd2-1 mutation, and this was detectable before any morphological defect became evident. These findings suggest that high-level accumulation of snRNA involving the SRD2 function is particularly important for expression of PINs in polarized pattern generation during the development of lateral root primordia.

Keywords: Arabidopsis • Auxin • Lateral root development • Polarized pattern generation • snRNA • SRD2.

Abbreviations: DIC, differential interference contrast; GFP, green fluorescent protein; GMA, germination medium A; PIN, PIN-FORMED; GUS, β-glucuronidase; RAM, root apical meristem; RIM, root-inducing medium; RT–PCR, reverse transcription–PCR; snRNA, small nuclear RNA.

Introduction

In animals, organogenesis is largely completed during embryogenesis. In contrast, organogenesis in plants continues over the entire lifetime of the organism. Post-embryonic plant organogenesis depends on the function of the apical meristems, which are located at both ends of the plant body axis. In the regular course of plant development, above-ground organs, such as stems, leaves and flowers, are derived from the shoot apical meristem, whereas underground organs (roots) are derived from the root apical meristem (RAM). Stems and roots newly formed on the pre-existing body axis have their own meristems at their apices. These meristems act to form branch axes, thereby resulting in the complex architecture that is characteristic of plants. Thus, the establishment of apical meristems on branches is very significant in the post-embryonic development of plants.

In the root system, branching starts with anticlinal division of cells in the pericycle in response to an auxin signal (De Smet et al. 2007, Fukaki et al. 2007). This is followed by a set of cell divisions to form lateral root primordia. In each root primordium, the RAM is constructed and activated for apical growth. Malamy and Benfey (1997) performed a histological analysis of the process of primordium development leading to the establishment of RAMs in Arabidopsis, and they defined various stages in the developmental process. In the early stages, ordered cell divisions build up a root primordium into a cone shape. During these stages, cells in the primordium gradually differentiate, as demonstrated by localized expression of several genes, such as SCARECROW (SCR) (Di Laurenzio et al. 1996). In the next stage, cell division ceases and the primordium grows solely by expansion for a short time. In the final stage, cell division.
resumes in the RAM region, thereby establishing the complete structure of the RAM.

Spatio-temporal control of cell division during development of the primordium is undoubtedly a key to lateral root morphogenesis. The molecular mechanism regulating cell division patterns in lateral root primordia is not currently well understood, but it is believed to involve polar transport of auxins as a vital component. Benková et al. (2003) obtained convincing evidence for this by demonstrating that PIN-FORMED (PIN) proteins, which play major roles as auxin efflux facilitators in auxin polar transport, are arranged during development of the root primordium so as to establish a gradient in auxin accumulation that is maximal at the primordium tip. When the formation of the auxin gradient was disrupted by a genetic lesion caused by multiple mutations in the PIN genes or by pharmacological disturbance of the relocation of the PIN proteins, unorganized massive division was induced instead of normal morphogenesis of the root primordia (Benková et al. 2003). These findings clearly demonstrated that patterned cell division during development of the root primordium is linked to the generation of an auxin gradient by means of a finely controlled transport process.

PUCHI, an AP2/EREBP transcriptional factor gene, has recently been implicated in the auxin-dependent regulation of morphogenesis of lateral root primordia (Hirota et al. 2007). Expression of the PUCHI gene is closely associated with the initiation of lateral root primordia and is responsive to the exogenous application of auxin. Loss-of-function mutations of this gene affected the ordered division of cells in the root primordia, resulting in excess cell division at the base of the primordium. Therefore, PUCHI probably acts downstream of auxins to tune the cell division pattern during the early stages of development of the root primordium.

Except for pin and puchi mutants, few materials are available for genetic studies on the control of cell division in lateral root primordia. We therefore utilized a temperature-sensitive mutant of Arabidopsis known as shoot redifferentiation defective 2-1 (srd2-1), which may provide new insights into the regulatory mechanism of cell division during root primordium morphogenesis. This mutant was isolated originally as being impaired in shoot regeneration (Yasutani et al. 1994), and was characterized for tissue culture responses and some aspects of post-embryonic development (Ozawa et al. 1998, Ohtani et al. 2008). The SRD2 gene was shown to act in upstream sequence element (USE)-dependent transcription of small nuclear RNAs (snRNAs), many of which are spliceosomal components that have essential roles in pre-mRNA splicing (Burge et al. 1999), and the srd2-1 phenotype has been attributed to a shortage of snRNA accumulation (Ohtani and Sugiyama 2005). Among a wide range of morphological abnormalities observed in the srd2-1 mutant under high temperature conditions (Sugiyama 2003, Ohtani and Sugiyama 2005), in the present work we focused on the deformation of lateral roots. A detailed analysis of developing root primordia of srd2-1 revealed alterations in cell division patterns and defective organization in the early stages; a decrease in the level of snRNA was detected at an even earlier stage. These results were combined to examine the significance of SRD2-dependent control of snRNA accumulation in morphogenesis of lateral root primordia.

**Results**

Temperature-dependent aberrations in lateral root morphogenesis in srd2-1

To examine the possible role of the SRD2 gene in root development, we first compared the morphology of primary and lateral roots in wild-type and srd2-1 mutant plants (Fig. 1). After the plants had been germinated and grown at 22°C (permissive temperature) for 12 d, half of them were transferred to 22°C or 28°C for a further 18 d. (A) Morphological appearance of the plants. (B) Higher magnification views of the lateral roots in A. Scale bars: 1 cm in A; 0.5 mm in B.

**Fig. 1** Effects of the srd2 mutation on lateral root formation. The wild type (WT) and srd2-1 mutant were grown at 22°C for 12 d and then at 22 or 28°C for a further 18 d. (A) Morphological appearance of the plants. (B) Higher magnification views of the lateral roots in A. Scale bars: 1 cm in A; 0.5 mm in B.
to a temperature of 28°C (restrictive temperature) for the observation of temperature-dependent defects, and the other half were kept at 22°C as a control. Under the control conditions, the srd2-1 plants were similar to the wild type in terms of the overall appearance of their root architecture, although the primary root was shorter in srd2-1 (Fig. 1A). When cultured at 28°C, however, the srd2-1 plants did not develop lateral roots normally, and thus had a very poorly branched root architecture (Fig. 1A). On closer observation, the primary roots of srd2-1 grown at 28°C were found to bear many hemispherical knobs that appeared to have resulted from incorrect development of the lateral roots (Fig. 1B).

In our previous studies, the peculiar defect of the srd2-1 mutant in lateral root morphogenesis was also seen in tissue cultures of the primary root segments (Sugiyama 2003, Ohtani and Sugiyama 2005). To investigate this effect of the srd2 mutation, we improved the culture protocol to develop an experimental system in which lateral roots formed with fairly good synchrony (see the Materials and Methods for details).

In wild-type explants cultured by this system, the development of lateral root primordia was initiated upon commencement of culture and was almost complete within 48–60 h, whereupon lateral roots emerged from the primary roots and began meristematic growth (Fig. 2A). When cultured at 22°C, explants of the srd2-1 mutant formed lateral roots of normal shape, following a similar time course to the wild-type explants. At 28°C, however, morphological disorders of the lateral root primordia in the srd2-1 mutant became evident after 60 h of culture, and these laterals continued to show apparently primordial growth during 4 d of culture (Fig. 2A). This abnormal development occurred in almost all of the srd2-1 laterals (97.9%, n = 146) under the present conditions.

Cell organization in the early phase of lateral root formation was examined by the method of Malamy and Benfey (1997), and the results were checked against their stage descriptions (Fig. 2B). When lateral root formation was induced from the wild-type explants at 28°C, newly formed primordia of lateral roots were clearly observed after 12 h in culture. As judged from

Fig. 2 Lateral root development in the semi-synchronous lateral root induction system. Explants of the wild type (WT) and srd2-1 mutant were cultured on RIM at 22 or 28°C, and samples were collected every 12 h during culture. Numbers above the micrographs represent the time of sampling. (A) DIC images of developing lateral roots cleared by chloral hydrate. (B) DIC images of early-stage lateral root primordia cleared by the method of Malamy and Benfey (1997). Arrowheads indicate the edges of newly formed primordia. (C) Schematic drawings traced from the 48 h primordia in B. Putative tissue derivations in the wild-type primordium according to Malamy and Benfey (1997) are shown. Scale bars: 100 µm in A; 50 µm in B.
the number of cell layers and the number of cells in the outermost layer, the root primordia were in stage II at this time. They reached stage V after 36 h of culture, and went beyond stage VII after 48 h. In the srd2-1 explants cultured at 28°C, lateral root primordia developed more slowly and they were still in stage IV at 36h of culture. Until this stage, there was no obvious difference in cell organization between the wild type and srd2-1. At later stages, however, aberrations of cell arrangement were recognizable in the srd2-1 laterals (Fig. 2B, C).

The density of lateral roots formed in the semi-synchronous system, which was defined as the number of lateral roots per 1 mm length of primary root, was determined from 15 explants after 10 d of culture; the values (average ±SD of three experiments) that were obtained were 0.84 ± 0.15 for the wild type and 0.81 ± 0.16 for srd2-1 at 22°C, and 0.83 ± 0.17 for the wild type and 0.89 ± 0.24 for srd2-1 at 28°C. Therefore, lateral roots were induced at almost the same density from the wild-type and the srd2-1 explants at both temperatures. These results show that the srd2 mutation does not block initiation of lateral root formation but does disturb its organization.

Effects of the srd2 mutation on cell proliferation during lateral root development

Cell proliferation during lateral root formation in the semi-synchronous system was monitored by using an M-phase marker gene, CYCB1;1::DB:GUS (Colón-Carmona et al. 1999). The β-glucuronidase (GUS) activity from this gene showed a marked change in the pattern of cell proliferation during lateral root formation in the wild type (Fig. 3). In the early stages, within 48 h of culture, cell division was very active throughout the developing lateral root primordia. At the time of root emergence, at around 60 h, the activity of cell division declined and, after a short period in a non-proliferative phase, cell division was reactivated exclusively in the apical region (Fig. 3). This pattern of cell proliferation is consistent with the histological observations made by Malamy and Benfey (1997). In a striking contrast to the wild type, GUS expression from CYCB1;1::DB:GUS in the abnormal laterals of srd2-1 did not disappear, but continued throughout the entire area (Fig. 3). This showed that the srd2 mutation interfered with the control of cell proliferation, including the developmental switch of the cell division pattern, required for proper development of lateral roots.

Effects of the srd2 mutation on spatial differentiation in lateral root primordia

As the lateral root primordium develops, cells inside it become differentiated in a position-dependent manner. Localized expression of SCR, a key regulator gene involved in radial organization and endodermal determination, is indicative of such spatial differentiation in the early stages of lateral root development (Di Laurenzio et al. 1996). The effects of srd2 mutation on the expression pattern of SCR were assessed with an enhancer trap line, En199, in which the GUS gene was inserted into the upstream region of the SCR gene (Malamy and Benfey 1997). During normal development of lateral roots in the semi-synchronous system, strong expression of GUS was gradually confined to the quiescent center and the endodermal cell files near the apex (Fig. 4). This pattern was detectable after 60 h of culture, when primordial development was almost complete (Fig. 4). In the abnormal laterals of the srd2-1 mutant, however, the SCR-directed GUS expression did not show clear patterns at any stages, although there was a tendency for the GUS expression to be relatively high in the basal region (Fig. 4). The aberrant expression of SCR observed in srd2-1 may reflect the fact that the srd2 mutation perturbs radial differentiation of lateral root primordia and prevents construction of the RAM.

DSR::GUS is a reporter gene that is designed to be expressed in response to auxin (Ulmasov et al. 1997). By using this gene as

![Fig. 3](image1.png)

**Fig. 3** Effect of the srd2 mutation on the pattern of cell proliferation in developing lateral roots. Explants of the wild type (WT) and srd2-1 mutant carrying the CYCB1;1::DB:GUS gene were cultured at 22 or 28°C on RIM and subjected to histochemical staining for GUS activity. The blue GUS signals show mitotic cells. Numbers represent the times of sampling. Scale bar: 50 μm.

![Fig. 4](image2.png)

**Fig. 4** Effect of the srd2 mutation on the expression pattern of SCR in developing lateral roots. Patterns of SCR-directed GUS expression in the wild-type (WT) and srd2-1 mutant explants cultured on RIM at 22 or 28°C. Numbers represent the times of sampling. Scale bars: 50 μm.
an indicator of auxin accumulation, we tested the possibility that the srd2 mutation might interfere with the generation of an auxin gradient in the root primordium, which is considered to be a positional cue for spatial differentiation leading to the establishment of the RAM (Benková et al. 2003). After 36 h of culture, a high level of DR5::GUS expression was observed in developing root primordia at stages IV–V. At these stages, no difference was found in the expression pattern of DR5::GUS between the wild type and srd2-1 (Fig. 5). Subsequently, DR5::GUS in normal primordia exhibited a graded pattern of expression with a maximum at the tip; this arose as the primordium developed into a cone-shaped form. After 72 h of culture or later, strong expression of DR5::GUS was restricted to the meristem region of apically growing lateral roots (Fig. 5). In contrast, DR5::GUS expression was uniformly high throughout the lateral root primordia of srd2-1 formed at 28°C, and it did not show graded or localized patterns even after the primordia had grown into large knobs (Fig. 5). This demonstrated that no auxin gradient was generated in the srd2-1 laterals that failed to develop into normal roots.

To obtain more information about the effects of srd2 mutation on the formation of an auxin gradient, we checked the expression and localization patterns of PIN auxin efflux facilitators. First, the levels of transcripts of PIN1, PIN2, PIN3, PIN4, PIN6 and PIN7, which have been reported to be expressed during lateral root primordium development (Benková et al. 2003), were examined by semi-quantitative reverse transcription–PCR (RT–PCR) analysis. The results showed that while the expression levels of these PIN genes were elevated or remained almost constant during RIM culture in the wild type at both 22 and 28°C, their expression was markedly reduced in srd2-1 after 72 h of culture at 28°C (Fig. 6A). This suggested a negative impact of the srd2 mutation on the expression of all of the PIN genes tested. On the electrophoretograms of the RT–PCR products either from the wild type or from srd2-1, no splicing variants were detected for any PIN genes, implying that splicing of the PIN transcripts is not altered by the srd2 mutation. Next, we examined spatial distributions of the PIN1 and PIN2 proteins by using green fluorescent protein (GFP) reporter genes PIN1::PIN1:GFP and PIN2::PIN2:GFP. The results obtained here with wild-type lateral roots agreed well with observations by Benková et al. (2003). After 24 h of culture, the fluorescence signal of PIN1–GFP was detected in almost all cells of the arising root primordia. As the primordia developed, the distribution of PIN1–GFP became limited to the inner region, and thereafter the signal became localized at the apical side (at the side toward the root apex) of each cell (Fig. 6B). PIN2–GFP signals appeared at the basal side of the outermost layer cells after 60 h of culture, and this confined and polarized pattern of distribution was maintained at later stages of development of the lateral roots (Supplementary Fig. S1). The srd2 mutation was found to have a considerable effect on these patterns. In lateral root primordia induced from the srd2-1 mutant at 28°C, the PIN1–GFP signals showed an almost normal pattern during the initial phase, but after 48 h of culture they declined gradually and finally fell to an undetectable level (Fig. 6B). The expression of PIN2–GFP was completely blocked in the srd2-1 mutant laterals at 28°C (Supplementary Fig. S1). These results suggest that the srd2 mutation inhibits expression of PIN proteins, and this may be responsible for the failure to develop an auxin gradient.

Changes in SRD2 expression and snRNA accumulation during lateral root development

The expression pattern of SRD2 during the development of lateral roots was examined by using SRD2::GUS as a reporter gene in the semi-synchronous system. In the wild type, the SRD2::GUS gene was uniformly expressed throughout the developing primordia of lateral roots. After 72 h in culture, when lateral roots were starting to undergo meristematic growth, the level of SRD2::GUS expression was elevated in the basal region. Thereafter, the zone of strong expression of SRD2::GUS was extended acropetally, and a high level of SRD2::GUS expression was observed in the subapical region and the central cylinder of elongated lateral roots (Fig. 7). This change in the expression pattern of SRD2::GUS was not present in the abnormal laterals of srd2-1. These results show that expression of SRD2 is regulated in association with SRD2-dependent development of lateral roots.

Our previous analysis of the srd2-1 phenotypes in tissue culture showed that SRD2 is involved in the control of cell proliferation competence (Ozawa et al. 1998, Ohtani and Sugiyama 2005). The expression level of CDKA;1, a gene encoding A class cyclin-dependent kinase, has also been implicated in the competence of cell proliferation (Hemerly et al. 1993). A comparison of the expression patterns of these two genes during lateral root development showed that they were linked in an interesting manner (Supplementary Fig. S2). Expression of CDKA;1::GUS followed almost the same pattern as that of
SRD2::GUS after the lateral roots had entered the meristematic growth phase. In addition, as in the case of SRD2::GUS, the spatial patterning of CDKA::GUS expression was severely disturbed by the srd2 mutation. Because SRD2 encodes an activator of snRNA transcription (Ohtani and Sugiyama 2005), the abnormal morphogenesis of lateral roots caused by the srd2 mutation and the expression of SRD2 in developing lateral roots suggested that active control of snRNA accumulation might be of particular significance in root formation. To test this possibility, we analyzed the accumulation patterns of U2 and U6 snRNAs during development of lateral roots in the semi-synchronous system. In situ hybridization revealed a noticeable change in the amount of U2 snRNA during the normal course of lateral root formation (Fig. 8A). In the early stages, large amounts of snRNA accumulated uniformly throughout the entire primordium. After 60 h

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**Fig. 6** Effects of the srd2 mutation on the expression patterns of PIN genes in developing lateral roots. (A) Total RNA was isolated from cultured root explants and examined by semi-quantitative RT–PCR for the mRNA amounts of PIN genes. RT–PCR amplification of the ACT2 mRNA is shown as an internal control. (B) Explants of the wild type (WT) and srd2-1 mutant carrying the PIN1::PIN1::GFP reporter gene were cultured at 22 or 28°C on RIM and examined for the fluorescence of GFP. Numbers represent the times of sampling. Scale bar: 50 µm.

**Fig. 7** Effects of the srd2 mutation on the expression pattern of SRD2 in developing lateral roots. Explants of the wild type (WT) and srd2-1 mutant carrying the SRD2::GUS reporter gene were cultured at 22 or 28°C on RIM and stained for GUS activity. Numbers represent the times of sampling. Scale bar: 50 µm.
in culture, the amount of snRNA dropped to an almost undetectable level, and then rose again (Fig. 8A). The decrease in snRNA coincided temporally with the completion of primordial development and the cessation of cell division, whereas the subsequent increase in snRNA appeared to be related to the start of meristematic growth and the reinforcement of SRD2 expression. In the srd2-1 explants cultured at 28°C, lateral root primordia contained a significant amount of snRNA only during the initial stage, and they rapidly lost the accumulated snRNA (Fig. 8A). These primordia continued to undergo unorganized growth for several days or longer, during which time the amount of snRNA remained very low (Fig. 8A). Within 48 h of culture at 28°C, a decrease in the amount of snRNA in the srd2-1 explants could be clearly detected by RNA gel-blot analysis (Fig. 8B); this process was concurrent with the development of the earliest signs of morphological abnormalities in the root primordia. Therefore, the developmental defect present in the root primordia of the srd2-1 mutant can be reasonably attributed to a deficiency in snRNAs.

### Effects of the srd2 mutation on RAM activation during lateral root development

The activation of the RAM is one of important checkpoints in the development of lateral roots (Zhang et al. 1999, De Smet et al. 2003). To test the possible involvement of SRD2 in this step, we carried out temperature shift experiments in which the srd2-1 explants were cultured on RIM at 22°C for 2 d and then at 28°C for another 2 d. Under these culture conditions, the mutant SRD2 protein was expected to lose its function only after cell organization of the lateral root primordia was almost complete. As shown in Fig. 9A (compare srd2-1 22°C→28°C with 28°C and 22°C, and with the wild type), the resultant morphology of the lateral roots of srd2-1 was relatively normal and indicative of meristematic growth, but the growth was considerably retarded. For quantitative evaluation of RAM activation, we focused on cells in the outermost layer behind the root cap, which correspond to the central cell region ‘OL1’, as designated by Malamy and Benfey (1997). According to Malamy and Benfey (1997), 8–10 cells in this region align in the median plane of the lateral root primordium at the stage of root emergence. After emergence, the number of the central cells of the OL1 that increase as a result of cell divisions depends on the RAM activity, and this number of cells can therefore serve as a useful index for RAM activation (Malamy and Benfey 1997, De Smet et al. 2003). The number of the OL1 central cells counted after 4 d in culture was >30 in the wild type for both temperature regimes. In the srd2-1 lateral roots, the number of OL1 central cells, which was about 25 at 22°C, fell to an average of 15 under the temperature shift condition (Fig. 9B), thereby indicating that, although RAM activation occurred even under the influence of the srd2-1 mutation, the RAM activity was much lower in srd2-1 than in the wild type. RNA gel-blot analysis revealed that the increase in temperature resulted in a significant decrease in the amounts of U2 and U6 snRNAs in the srd2-1 explants (Fig. 9C). From these results, it can be seen that the lowered level of snRNA accumulation caused by the srd2 mutation still allows the RAM to be activated during lateral root development but affects the RAM activity after the activation.

### Discussion

The srd2-1 mutant, which has a missense mutation in the gene encoding the snRNA transcription activator (Ohtani and Sugiyama 2005), exhibits a temperature sensitivity at several stages during development (Ozawa et al. 1998, Sugiyama 2003, Ohtani et al. 2008). One of the characteristic phenotypes of srd2-1 is the deformation of lateral roots when the temperature is increased (Fig. 1). We investigated this defect in detail by using a new experimental system in which de novo formation of lateral roots from cultured explants was induced with considerable synchrony. Histological observations showed the presence of disorders of cell patterning in lateral root primordia of the srd2-1 mutant at the restrictive temperature after 48 h of...
culture in the semi-synchronous system, at which time primordia
dvelopment would normally be almost complete and the
RAM structure would be recognizable (Fig. 2). In the morpho-
logically abnormal laterals of the srd2-1 mutant lacking the
RAM structure, cell division was not restricted to the apical
region (Fig. 3), and the SCR reporter gene was misexpressed
rather evenly or randomly (Fig. 4). These results show that
SRD2 has an important role in cell organization and specifi-
cation associated with the construction of the RAM in lateral root
primordia.

During the development of the lateral root primordium,
an auxin gradient with its maximum at the tip is established
with the aid of the PIN auxin efflux facilitators (Benková et al.
2003). The PIN-dependent system for generation of the auxin
gradient was shown to be very vulnerable to the srd2 mutation,
because expression of PINs was strongly inhibited in the
srd2-1 laterals formed at the restrictive temperature (Fig. 6)
and, probably as a result of this, auxin accumulated uniformly
(Fig. 5). These results show that SRD2 has an important role in
cell organization and specification associated with the construc-
tion of the RAM in lateral root primordia.

As SRD2 acts as an activator of snRNA transcription, and
srd2-1 is temperature sensitive for this function (Ohtani and
Sugiyama 2005), the primary defect of the srd2-1 mutant that
leads to deformation of lateral roots at higher temperatures is
likely to be insufficient production of snRNAs. Consistent with
this view, the accumulation of snRNA in root primordia of the
srd2-1 mutant decreased shortly after the initiation of pri-

tordial development at the restrictive temperature, whereas
snRNAs accumulated at high levels in all cells of the wild-type
root primordium throughout the early phase of development
(Fig. 8). Our results, taken together with the discussion in the
preceding paragraph, suggest that one of the key effects of
the srd2 mutation on lateral root development is to inhibit
the expression of PINs as a result of a deficiency in snRNA.

On the basis of their histological studies of lateral root
formation, Malamy and Benfey (1997) reported that cell divi-
sion is inactivated temporarily at the end of the primordial
stage and then reactivated in the RAM region after the lateral
root emerges from the primary root. This was demonstrated
more clearly in the current experiments by the analysis of the
expression of CYCB1;1::DB::GUS (Fig. 3). Interestingly, snRNAs
disappeared at maturity of the primordium concurrently with
the decay of the CYCB1;1::DB::GUS signal; when lateral roots
subsequently emerged, snRNAs increased again (Figs. 3, 8). We
have previously suggested that the snRNA level is limiting in
terms of the competence of cells to proliferate in some situa-
tions (Ohtani and Sugiyama 2005). In consideration of this fact,
the changing patterns of snRNA accumulation in root primor-
dia seem to imply that a decline in snRNA levels is involved

Fig. 9 Effects of the srd2 mutation on RAM activation in lateral roots. Explants of the wild type (WT) and srd2-1 mutant were cultured at 22°C
for 2 d and then at 22 or 28°C for a further 2 d on RIM. (A) DIC images of lateral root tips cleared by chloral hydrate. (B) Close-up observations.
The arrows indicate the boundaries between the basal and central cells of OL1, and the red broken lines represent the central cell regions
in OL1. Scale bars: 100 µm in A; 50 µm in B. (C) RNA gel-blot analysis of the accumulation of U2 and U6 snRNAs. 5.8S rRNA bands are shown
as a loading control.
in the down-regulation of cell division. However, lateral root primordia of srd2-1 formed at the restrictive temperature, where the snRNA content decreased much earlier than in normal primordia, did not show a transient inactivation of cell division. Instead, active and random cell division was maintained in the srd2-1 laterals throughout the culture, resulting in the formation of hemispherical knobs (Figs. 2, 3). These findings show that a deficiency of snRNA in developing root primordia does not simply cause a shutdown of cell division, but rather it interferes with the temporally programmed switching of cell division patterns.

The SRD2 promoter-driven expression of GUS was observed throughout lateral root primordia in their early stages (Fig. 7), suggesting the presence of a uniform abundance of the SRD2 protein and a uniformly high rate of SRD2-dependent snRNA transcription. This is in good agreement with snRNA accumulation in young root primordia and also with the effect of the srd2 mutation on the accumulation of snRNA in primordia. At later stages, however, a significant discrepancy was found between SRD2::GUS expression and snRNA accumulation. At the time of the suspension of cell division, the accumulation of snRNA was markedly reduced, whereas the expression of SRD2::GUS was not reduced. As the half-life of the GUS protein is known to be relatively long (Harkins et al. 1990), one possible explanation is that, despite the sustained activity of GUS expressed from SRD2::GUS, the actual amount of SRD2 protein may have fallen, leading to a loss in snRNA production. This possibility should be tested by further analysis of SRD2 at the protein level.

Note that the srd2 mutation did not have an equally strong effect on all processes of lateral root formation. The initiation of lateral root primordium development was almost normal in srd2-1, even at the restrictive temperature. Moreover, the temperature shift experiments showed that the activation of the RAM after lateral root emergence was not blocked by the srd2-1 mutation, despite a deficiency of snRNAs (Ohtani and Sugiyama 2005), a decrease in the amount of snRNA as a result of the srd2 mutation is expected to limit the capacity for pre-mRNA splicing. In the light of our knowledge that splicing efficiency varies considerably among pre-mRNA species and among splice sites (Smith et al. 2008), we presume that a situation that is unfavorable for splicing does not necessarily have an equal impact on all splicing events. Thus, if we assume that some genes directing the expression of PINs are hypersusceptible to a reduction in the splicing capacity, this would at least partly explain the unique effects of the srd2 mutation in lateral root primordia. Future studies taking this as a working hypothesis could shed light on the physiological significance of active control of snRNA transcription in plant development.

### Materials and Methods

#### Plant materials and growth conditions

The srd2-1 mutant was derived from an ethyl methanesulfonate-mutagenized population of the Landsberg erecta (Ler) strain of Arabidopsis thaliana (Yasutani et al. 1994). The GUS and GFP reporter lines were as follows. The DR5::GUS line was the Columbia (Col) strain carrying the DR5($\times$7)-GUS transgene (Ulmasov et al. 1997). For SCR reporters, the End199 line originating from Wassilewskija (Ws) (Malamy and Benfey 1997) was used. The PIN2::PIN2:GFP line corresponded to pPIN1::PIN1:EGFP described by Xu et al. (2006), whereas the PIN2::PIN2:GFP line corresponded to PIN2-EGFP described by Xu and Scheres (2005). The CYCB1;1::DB:GUS line, established from a Col strain plant transformed with pCDG (Colón-Carmona et al. 1999), and the CDKA1;1::GUS line, established from the Ler plant transformed with pVPC2AGUS (Hemerly et al. 1993), have been described previously (Huang et al. 2003, Ohtani et al. 2005). The SRD2::GUS line originating from Col has been described by Ohtani et al. (2008). The srd2-1 mutation was introduced into these lines by means of crossing. Resultant progeny lines that were homozygous for the reporter gene were used for the subsequent analyses.

Seedlings were grown aseptically on germination medium A (GMA) at 22°C under continuous light (10–15 μmol m$^{-2}$ s$^{-1}$). GMA consisted of Murashige–Skoog medium supplemented with 1.0% (w/v) sucrose, buffered to pH 5.7 with 0.05% (w/v) MES, and solidified with 1.5% (w/v) agar.

#### Semi-synchronous induction of lateral root formation

To induce the formation of lateral roots with good synchrony, tissue culture was performed as described below. Surface-sterilized seeds were imbibed in pure water, incubated at 4°C for 2 d, and then grown on GMA at 22°C. Shoot tips, including cotyledons, and root apices were removed from 4-day-old seedlings, and the remaining parts were cultured as explants...
on root-inducing medium (RIM) to induce lateral roots at 22 or 28°C under continuous light (15–25 μmol m−2 s−1). The RIM was B5 medium supplemented with 2.0% (w/v) glucose and 0.5 ml l−1 4-(1H-indol-3-yl)butanoic acid, buffered to pH 5.7, with 0.05% (w/v) MES, and solidified with 0.25% (w/v) gellan gum.

**Histological observations**

For microscopic examination of morphology, unless otherwise indicated, explants were fixed overnight at 4°C in 25 mM sodium phosphate buffer (pH 7.0) containing 2% (w/v) formaldehyde and 1% (w/v) glutaraldehyde, rinsed with 100 mM sodium phosphate buffer (pH 7.0), and cleared with an 8:1:2 (w/v/v) mixture of chloral hydrate, glycerin and water. In some experiments, the method of Malamy and Benfey (1997) was also employed for tissue fixation and clearing. For histochemical detection of GUS activity, samples were treated as described previously (Ohtani et al. 2008). Observations were made with a microscope equipped with Nomarski optics (BX50-DIC and BX51-DIC; Olympus) to obtain differential interference contrast (DIC) images.

To examine the fluorescence signals of GFP, samples of the GFP reporter lines were observed by using a confocal laser scanning microscope (FV-1000; Olympus).

**In situ hybridization analysis**

In situ hybridization analysis of snRNA accumulation was performed as described previously (Ohtani et al. 2008). Samples were fixed in phosphate-buffered saline containing 4% (w/v) paraformaldehyde. Paraffin sections were made from the fixed samples and hybridized with a digoxigenin-labeled probe for U2.2 snRNA. Hybridization signals were visualized by a detection system based on alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche).

**Semi-quantitative RT–PCR analysis and RNA gel-blot analysis**

Total RNA was extracted from root explants cultured on RIM, using TRizol reagent and the PureLink™ RNA Mini kit (Invitrogen) according to the manufacturer’s protocol. For semi-quantitative RT–PCR analysis, 1 μg of total RNA was reverse transcribed by M-MLV Reverse Transcriptase RNaseH Minus, Point Mutant (Promega) in 25 μl of reaction solution with oligo d(T)12–16 primer. Aliquots (1 μl) of the reaction solution containing the first-strand cDNA were used as templates for PCR amplification. The primer sets used were: 5′-ATGATTACGGCGCCGACTTCTACCC-3′ and 5′-CTTATGGAAGAATGATGAGG-3′ for PIN7; and 5′-CTGGATTCTGCGATTGGTG TGTC-3′ and 5′-CTTCTGCTCATACGGTGAGGG-3′ for ACT2. PCR was performed for 25 cycles (PIN1, PIN2, PIN3, PIN4, PIN7 and ACT2) or 30 cycles (PIN6). Each cycle of PCR was comprised of a 30 s denaturation phase (95°C), a 30 s annealing phase (56°C) and a 1 min extension phase (72°C). The PCR products were separated electrophoretically on agarose gels.

RNA gel-blot analysis of snRNA accumulation was performed as described previously (Ohtani et al. 2008). In brief, total RNA samples were separated by gel electrophoresis, blotted onto nylon membranes and then subjected to hybridization with digoxigenin-labeled probes specific for the U2.2 and U6-1 snRNA genes.

**Supplementary data**

Supplementary data are available at PCP online.

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**References**


