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Organogenic responses in tissue culture of *srd* mutants of *Arabidopsis thaliana*

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SUMMARY

In *Arabidopsis thaliana*, shoot redifferentiation and root redifferentiation can be induced at high frequency from hypocotyl and root explants by a two-step culture method. Tissues are precultured on callus-inducing medium and then transferred onto shoot-inducing medium for shoot redifferentiation or onto root-inducing medium for root redifferentiation. In an attempt to dissect these organogenic processes genetically, we characterized the responses in tissue culture of *srd1*, *srd2* and *srd3* mutants that were originally isolated as temperature-sensitive strains with defects in shoot redifferentiation (Yasutani, I., Ozawa, S., Nishida, T., Sugiyama, M. and Komamine, A. (1994) *Plant Physiol.* 105, 815-822). These mutants exhibited temperature sensitivity at different steps of organogenesis, which allowed the identification of three states associated with organogenic competence: IC (incompetent); CR (competent with respect to root redifferentiation); and CSR (competent with respect to shoot and root redifferentiation). Hypocotyl explants were shown to be in the IC state at the initiation of culture and to enter the CSR state, via the CR state, during preculture on callus-inducing medium, whereas root explants seemed to be in the CR state at the initiation of culture. The transition from IC to CR and that from CR to CSR appeared to require the functions of *SRD2* and *SRD3*, respectively. It appears that explants in the CSR state redifferentiate shoots with the aid of the products of *SRD1* and *SRD2* when transplanted onto shoot-inducing medium. Histological examination of the *srd* mutants revealed that the function of *SRD2* is required not only for organogenesis but also for the reinitiation of cell proliferation in hypocotyl explants during culture on callus-inducing medium. Linkage analysis using RFLP markers indicated that *SRD1*, *SRD2*, and *SRD3* are located at the lower region, the central region, and the upper region of chromosome 1, respectively.

Key words: *Arabidopsis*, Cell division, Organogenesis, Root redifferentiation, Shoot redifferentiation, Temperature-sensitive mutant, Tissue culture

INTRODUCTION

Organogenesis de novo in tissue cultures has provided useful systems for studying development of higher plants. One of the remarkable feats of physiological research on organogenesis in vitro was the identification of a predominant role of cytokinin and auxin as chemical determinants in plant development (Skoog and Miller, 1957). Screening of culture conditions for the induction of organogenesis has revealed various factors affecting plant development in addition to these phytohormones. How such factors, in particular phytohormones, act on cultured tissues to induce organogenesis is, however, still unknown. We have poor information about events following the application of phytohormones and preceding morphological changes. At present, it is difficult even to give a brief sketch of fundamental sequences of organogenesis.

As the first step to analyze organogenesis, it is very fruitful to subdivide organogenesis physiologically, genetically, or morphologically into several distinct phases. The characterization of each phase provides important information for understanding the organogenesis process. Such attempts were systematically made by Christianson and Warnick (1983, 1984, 1985) for shoot organogenesis from the leaf explants of *Convolvulus arvensis*. They carried out tissue-transfer experiments, and divided the process of organogenesis into three phases, based on the temporal requirement of explants for a specific balance of phytohormones in the control of organogenesis (Christianson and Warnick, 1983). In the first phase of organogenesis, explants acquire 'competence' which is defined as the ability (not capacity) to respond to signals of induction like phytohormones. These competent explants are canalized and determined for specific organ development by inductive signals through the second phase. Then the morphogenesis proceeds independently of the exogenously supplied phytohormones during the third phase.

The concept that organogenesis is composed of three phases, i.e., 'competence acquisition phase', 'induction (determination) phase', and 'morphological differentiation phase', may be applicable to organogenesis in various tissue culture systems, even though experimental identification of each phase is not easy in all cases.

In tissue culture of *Arabidopsis thaliana*, a model plant for molecular genetics, root and hypocotyl explants are induced to redifferentiate shoots by a procedure consisting of preculture on CIM (callus-inducing medium) and the subsequent culture on SIM (shoot-inducing medium) (Valvekens et al.1988; Akama et al., 1992). Since preculture on CIM is indispensable to effective induction of shoot redifferentiation, explants are supposed to acquire competence for shoot redifferentiation during the preculture. This allows us to distinguish the 'competence acquisition phase' from the following phases with the least ambiguity.

Recently, we isolated temperature-sensitive mutants of *Arabidopsis thaliana*, namely, *srd1*, *srd2* and *srd3*, that are impaired in the redifferentiation of shoots from root explants (Yasutani et al., 1994). *srd* mutants are very powerful tools for subdividing the process of organogenesis into genetically distinct phases. These phases can be related to physiologically identified phases by tissue-transfer experiments. In addition, such mutants help us to find elementary processes that are common to apparently different phenomena. This article describes the genetic dissection of organogenesis in *Arabidopsis thaliana* through the characterization of *srd* mutants in terms of their morphogenetic responses in tissue cultures.

MATERIALS AND METHODS

Plant materials and growth conditions

In the present study, the Landsberg *erecta* strain of *Arabidopsis thaliana* (L.) Heynh was used as the wild type. Mutant lines, namely, L1045, L131, and L1919, were derived from this strain and harbored the *srd1*, *srd2* and *srd3* mutations, respectively (Yasutani et al., 1994). For tissue culture experiments, mutant lines partially purified from these lines by self-pollination after two or three back crosses were used as *srd* mutants. Seeds were surface sterilized by agitation in a 1.2% solution of sodium hypochlorite supplemented with 0.1% Triton X-100 for 10 minutes. After rinsing several times with sterile water, seeds were sown on germination medium (GM) in a Petri dish (90 mm diameter, 15 mm or 20 mm in height). Plates were sealed with Micropore surgical tape (3M Health Care), and incubated at 22°C. Donor plants that were sources of root segments were grown for 18-21 days under continuous light at a fluence rate of 30-50 μmol/m^2/second, and sourses of hypocotyl segments were grown for 10-14 days under continuous light at a fluence rate of 8-14 μmol/m^2/second.

Tissue culture

The procedure for induction of the redifferentiation of shoots was based on the methods described by Valvekens et al. (1988) and Akama et al. (1992). Root segments of 5-10 mm in length were excised from 18- to 21-day-old donor plants, and hypocotyl segments of 5-7 mm in length were excised from 10- to 14-day-old donor plants which were slightly etiolated. Excised segments were precultured on callus-inducing medium (CIM) for 4 days and then transferred onto shoot-inducing medium (SIM) for the induction of shoot redifferentiation. In the case of induction of the redifferentiation of roots, hypocotyl explants cultured on CIM for 4 days were transferred onto root-inducing medium (RIM). On the 25th day of culture (3 weeks after inoculation onto SIM or RIM), the frequency of redifferentiation of shoots or roots was scored. Tissue culture was carried out under continuous light (fluence rate, 30-50 μmol/m^2/second) in Petri dishes or in multiwell plates sealed with surgical tape.

The composition of the various culture media was described previously (Yasutani et al., 1994). GM was MS medium (Murashige and Skoog, 1962) supplemented with 10 g/l sucrose. CIM was B5 medium (Gamborg et al., 1968) supplemented with 20 g/l glucose, 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.1 mg/l kinetin. SIM was B5 medium supplemented with 20 g/l glucose, 0.15 mg/l indole acetic acid (IAA), and 0.5 mg/l N^6-Δ^2-isopentenyladenine. RIM was B5 medium supplemented with 20 g/l glucose and 0.5 mg/l indole-3-butyric acid. All media were buffered with 0.5 g/l 2-(*N*-morpholino)ethanesulphonic acid (pH 5.7), and solidified with 2.0 or 2.5 g/l gellan gum.

Whole-mount preparations

Plant materials were fixed in a solution that contained 2% (v/v) formaldehyde, 1% (v/v) glutaraldehyde, and 0.025 M sodium-potassium (Na-K) phosphate buffer (pH 7.0) for 12 - 24 hours at 4°C, and then they were washed with 0.1 M Na-K phosphate buffer (pH 7.0) and mounted in a mixture of chloral hydrate/water/glycerol (8:2:1, w/w). Samples were observed with the light microscope which was equipped with Nomarski optics (U-DICT; Olympus).

Microautoradiography

Hypocotyl explants were cultured on CIM supplemented with 1 μCi/ml [methyl-^{3}H]thymidine (48 Ci/mmol; Amersham) for 4 days at 22°C or 27°C. Labeled explants were fixed as described above and then dehydrated in a graded ethanol series. Dehydrated samples were embedded in the Technovit 7100 resin (Heraeus Kulzer) and cut into 10 μm thick sections. Preparations were coated with a thin layer of NTB2 liquid nuclear emulsion (Eastman Kodak) that had been diluted to half the original concentration with distilled water. After exposure for 5-7 days in a refrigerator, microautoradiograms were developed and examined under the light microscope.

Linkage analysis

Linkage analysis and mapping were performed with TC_2 lines, exploiting restriction fragment length polymorphism (RFLP) between the Landsberg *erecta* and the Columbia strains as a source of genetic markers. Mutant plants of the Landsberg *erecta* strain that were homozygous for one of the mutations were test-crossed with F_1 plants from a cross between a wild-type plant of the Columbia strain and a mutant plant of the Landsberg *erecta* strain. TC_1 seeds from a test-cross were allowed to germinate, and resultant plants were grown and self-fertilized. TC_2 seeds were gathered from each individual TC_1 plant. The phenotype was scored for both plants of the TC_2 lines and their parental TC_1 plants. Total DNA was extracted from a minimum of 20 plants for each TC_2 line by the CTAB method, as described by Rogers and Bendich (1985). DNA was digested with *Eco*RI and subjected to Southern hybridization with RFLP markers as probes. RFLP markers, cloned in bacteriophage λ (Chang et al., 1988), were kindly donated by Prof. Y. Komeda (Hokkaido University, Sapporo, Japan). The *Arabidopsis thaliana* RFLP Mapping Set (ARMS) developed by Fabri and Schäffner (1994) was obtained from the Arabidopsis Biological Resource Center, Ohio State University (Columbus, OH).

RESULTS

Redifferentiation of shoots from root explants

The mutant lines, L131, L1045 and L1919, were characterized

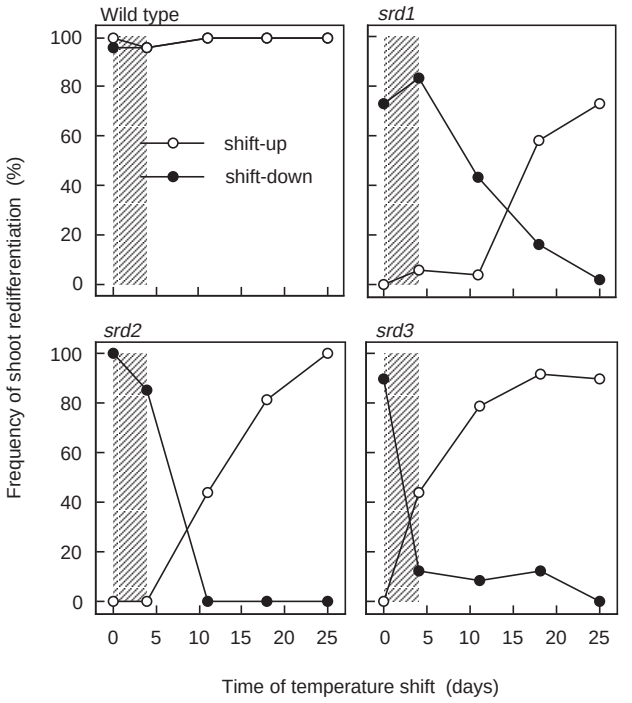

Fig. 1. Determination of the phenocritical periods for *srd* mutants during the redifferentiation of shoots from root explants. Explants of the wild type, and the *srd1*, *srd2*, and *srd3* mutants were cultured on CIM for 4 days and then on SIM for 21 days. Cultures were initiated at 22°C or 27°C, and the temperature was raised from 22°C to 27°C (○) or lowered from 27°C to 22°C (●) at various times during culture. The number of explants with redifferentiated shoots was scored after 25 days in culture. Data were collected from at least 48 explants for each point. The shaded portion indicates the period of preculture on CIM.

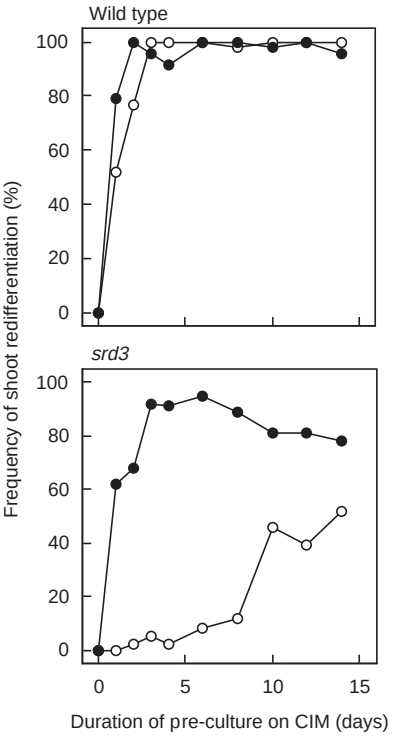

Fig. 2. Effects of the duration of preculture on the redifferentiation of shoots from root explants of *srd3* and the wild type. Explants were precultured on CIM for various periods of time at 22°C (●) or 27°C (○) and then cultured on SIM at 22°C. The number of explants with redifferentiated shoots was scored 21 days after transfer to SIM. Data were collected from at least 48 explants for each point.

by a temperature-dependent defect in the redifferentiation of shoots from root explants (Yasutani et al., 1994). Root explants of these mutants redifferentiated shoots at 22°C (permissive temperature) but not at 27°C (restrictive temperature). Such traits in L1045, L131, and L1919 were shown by genetic tests to be the results of recessive mutations at three different loci, namely, *SRD1*, *SRD2* and *SRD3*, respectively. In an attempt to examine the temporal requirements of shoot redifferentiation for the products of the three *SRD* genes, temperature-shift experiments were performed with *srd* mutants (Fig. 1). Root explants were incubated on CIM for 4 days and then transferred to SIM. Cultures were initiated at 22°C or 27°C, and the temperature was raised from 22°C to 27°C or lowered from 27°C to 22°C at various times during culture. The number of explants that formed shoots was scored 21 days after transfer to SIM. All the strains used, when cultured at 22°C, produced shoots from some root explants 7-11 days after transfer onto SIM, and most of explants formed shoots within 17 days after transfer (data not shown).

The frequency of shoot redifferentiation from wild-type explants was greater than 90% under all culture conditions and there were no significant changes after shifts of temperature at any time (Fig. 1). In the case of *srd1*, shoot redifferentiation was severely inhibited by an increase in temperature on the 11th day (Fig. 1). When the explants of *srd1* were cultured on SIM at the permissive temperature for longer periods, shoot redifferentiation was less affected by subsequent exposure to the restrictive temperature. In shift-down experiments, a shift on the 11th day or later resulted in a significant reduction in the frequency of shoot redifferentiation (Fig. 1). In summary, the redifferentiation of shoots from root explants of the *srd1* mutant was most sensitive to the restrictive temperature when explants were exposed to this temperature within 2 weeks after transfer onto SIM. A similar tendency was observed in the temperature-shift experiments with root explants of *srd2*, but the sensitive period of this strain was restricted to the first week after transfer (Fig. 1). The third mutant line, *srd3*, was distinctly different from *srd1* and *srd2*. Shoot redifferentiation in *srd3* was markedly inhibited only when explants were exposed to the restrictive temperature during preculture on CIM (Fig. 1). The temperature during the subsequent culture on SIM did not have much effect on the frequency of shoot redifferentiation. The results can be summarized as follows. *srd1* and *srd2* each have a temperature-dependent defect in the process of shoot redifferentiation that is induced by incubation on SIM whereas *srd3* has such a defect in the part of the redifferentiation process that occurs during preculture on CIM.

Attempts were made to identify culture conditions that could induce shoot redifferentiation from root explants of the mutants even at the restrictive temperature. Alterations in the concentrations of phytohormones in CIM and SIM yielded no positive results for any of the mutants (data not shown). In *srd3*, extension of the preculture on CIM was effective in overcoming the impairment in shoot redifferentiation (Fig. 2).

Redifferentiation of shoots from hypocotyl explants

Shoot redifferentiation can be induced from hypocotyl explants, as well as from root explants, by culture on SIM after preculture on CIM (Akama et al., 1992). The temporal requirements of shoot redifferentiation from hypocotyl

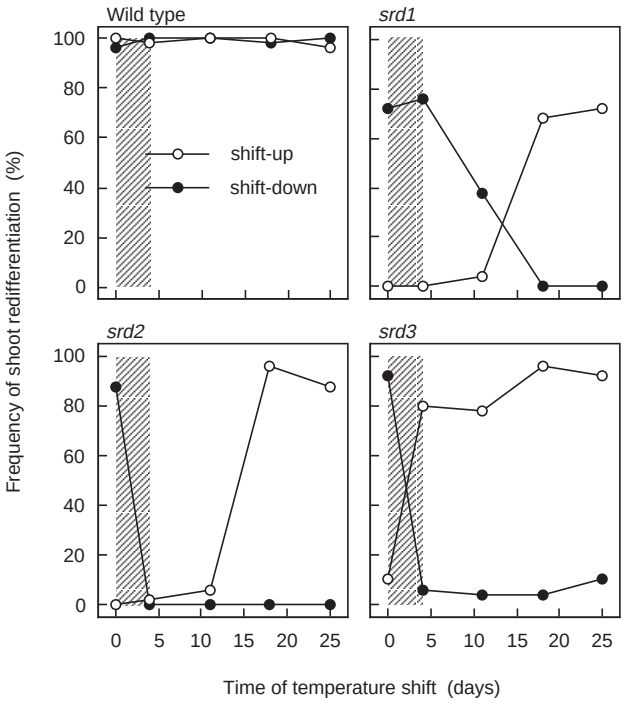

Fig. 3. Determination of the phenocritical periods for *srd* mutants during the redifferentiation of shoots from hypocotyl explants. Explants of the wild type, and the *srd1*, *srd2* and *srd3* mutants were cultured on CIM for 4 days and then on SIM for 21 days. Cultures were initiated at 22°C or 27°C, and the temperature was raised from 22°C to 27°C (○) or lowered from 27°C to 22°C (●) at various times during culture. The number of explants with redifferentiated shoots was scored 21 days after transfer onto SIM. Data were collected from 50 explants for each point. The shaded portion indicates the period of preculture on CIM.

explants for the product of each *SRD* gene were examined by characterizing the temperature sensitivity of hypocotyl explants of *srd* mutants. Hypocotyl segments of *srd1, srd2* and *srd3* were precultured on CIM for 4 days at 22°C or 27°C, and then they were cultured on SIM. During culture, the temperature was raised from 22°C to 27°C or lowered from 27°C to 22°C at various times. Explants that redifferentiated shoots were scored 21 days after transfer onto SIM (Fig. 3). Shoot redifferentiation from hypocotyl explants was a little faster than that from root explants. In most of hypocotyl explants of every strain cultured at 22°C, shoots were formed within 14 days after transfer onto SIM (data not shown).

Hypocotyl explants of *srd1* and *srd3* were prevented from forming shoots by exposure to the restrictive temperature (27°C) during culture on SIM and during preculture on CIM, respectively (Fig. 3). These effects of temperature on shoot redifferentiation were the same as those observed with root explants.

In the case of *srd2*, hypocotyl explants were clearly different from root explants. Shoot redifferentiation from hypocotyl explants of this mutant was inhibited by exposure to the restrictive temperature at either of the two culture steps whereas the temperature-sensitive period of root explants corresponded to the culture period after transfer onto SIM (Fig. 3).

Table 1. Effects of temperature on the redifferentiation of roots from hypocotyl explants of *srd* mutants and the wild type

Temperature*		Frequency of root redifferentiation†			
CIM	RIM	*srd1*	*srd2*	*srd3*	Wild type
22˚C	22˚C	30‡ / 30§	27 / 30	29 / 30	30 / 30
22˚C	27˚C	29 / 30	25 / 30	27 / 30	30 / 30
27˚C	22˚C	25 / 30	4 / 30	29 / 30	30 / 30
27˚C	27˚C	27 / 30	2 / 30	30 / 30	30 / 30

*Explants were cultured on CIM for 4 days at 22˚C or 27˚C, and then they were cultured on RIM at 22˚C or 27˚C.
†Explants that formed roots were scored 21 days after transfer to RIM.
‡Number of hypocotyl explants with redifferentiated roots.
§Total number of hypocotyl explants.

Redifferentiation of roots from hypocotyl and root explants

When hypocotyl explants were transferred to RIM instead of to SIM after precultured on CIM, vigorous formation of adventitious roots was induced on the explants. Culture of hypocotyl explants on RIM without preculture on CIM resulted in the formation of a few roots only at the proximal ends. Exploiting the former result, we examined the temperature sensitivity of root redifferentiation with each *srd* mutant. The temperature-sensitive redifferentiation of roots was found only with *srd2* explants (Table 1). Exposure of *srd2* explants to the restrictive temperature during preculture on CIM resulted in inhibition of root redifferentiation, and the temperature during culture on RIM had much less of an effect on the formation of roots. However, the growth of redifferentiated roots appeared to be suppressed by the restrictive temperature given after transfer onto RIM in *srd2* explants (data not shown).

When root segments were inoculated onto RIM, a large number of roots was formed over the explants in response to auxin (indole-3-butyric acid). Such formation of roots from root explants was not sensitive to the restrictive temperature in any of *srd* mutants (data not shown).

Formation of callus from hypocotyl explants

Visible callus is formed from root and hypocotyl explants when they are cultured on CIM without transfer onto SIM. Callus formation from root explants was shown previously to be insensitive to the restrictive temperature in all the *srd* mutant lines (Yasutani et al., 1994). In the present experiments, the temperature sensitivity of callus formation from hypocotyl explants was examined for each *srd* mutant. Among three mutant lines, inhibition of callus formation at the restrictive temperature was observed only with *srd2*.

For quantitative characterization of the effect of the *srd2* mutation on callus formation, hypocotyl explants of the wild type and the *srd2* mutant were cultured on CIM under various temperature conditions and fresh weights were measured every 2 days. After a lag period of 10 days, the fresh weight of wild-type explants increased at both 22°C and 27°C (Fig. 4A). A similar pattern of increases in fresh weight was observed with hypocotyl explants of the *srd2* mutant cultured at 22°C, even though the growth rate of *srd2* explants was lower than that of wild-type explants (Fig. 4C). By contrast, when hypocotyl explants of the *srd2* mutant were cultured at 27°C, no apparent

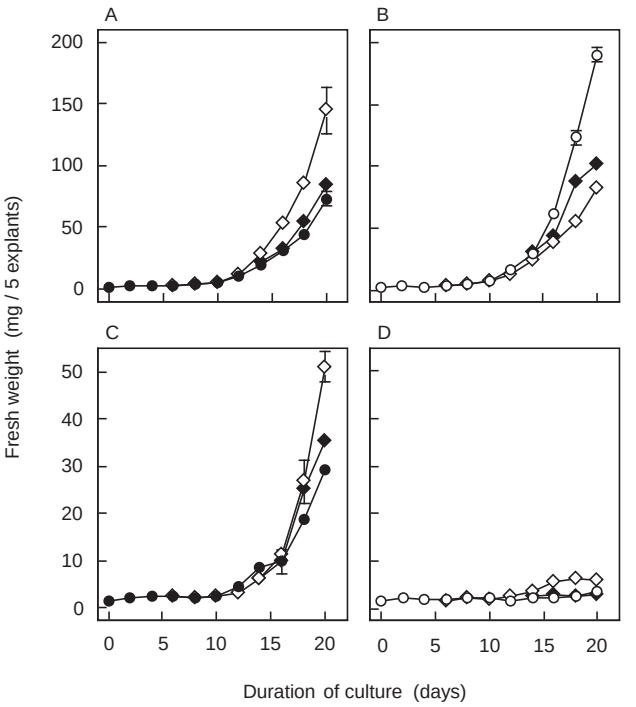

Fig. 4. Effects of temperature on the growth of callus from hypocotyl explants. Explants of the wild type (A, B) and the *srd2* mutant (C,D) were cultured on CIM for 21 days, and the fresh weight of 5 explants was determined every 2 days. (A,C) Cultures were initiated at 22°C and then the temperature was raised from 22°C to 27°C on the 4th day (◇) or the 12th day (◆), or explants were cultured at 22°C without a temperature-shift (●). (B,D) Cultures were initiated at 27°C and then the temperature was lowered from 27°C to 22°C on the 4th day (◇) or the 12th day (◆), or explants were cultured at 27°C without a temperature-shift (○). The mean values for three individual specimens are plotted with s.d. shown as error bars. When bars are absent, s.d. falls within the symbol.

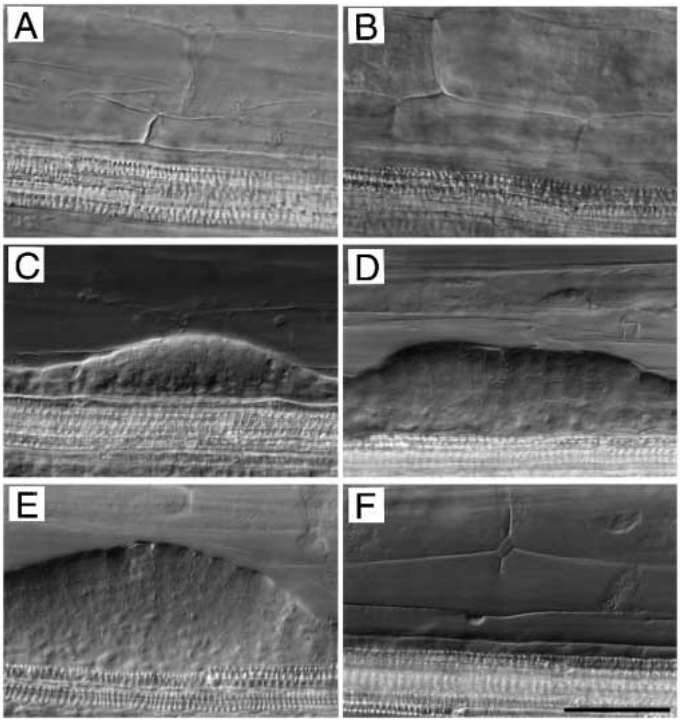

Fig. 5. Nomarski micrographs of hypocotyl explants that had been cultured on CIM. Explants of (A) the wild type and (B) the *srd2* mutant just after excision. Explants of the wild type (C,E) and the *srd2* mutant (D,F) after culture on CIM for 4 days at 22°C (C, D) or 27°C (E, F). Bar = 20 μm.

increase in fresh weight could be detected during a 20-day culture (Fig. 4D).

Effects of temperature shifts on the growth of callus were also examined. Cultures were initiated at 22°C or 27°C, and then the temperature was raised from 22°C to 27°C or lowered from 27°C to 22°C after 4 days or 12 days in culture. In the wild type, the growth pattern was hardly affected by such temperature shifts (Fig. 4A, B). In the case of the *srd2* mutant, a shift to 22°C after 4 days or 12 days in culture at 27°C resulted in severe suppression of callus growth (Fig. 4D), whereas a shift to 27°C after 4 days or 12 days in culture at 22°C did not inhibit callus growth (Fig. 4C). Thus, hypocotyl explants of the *srd2* mutant were sensitive to exposure to the restrictive temperature during the first 4 days in culture with respect to the capacity for formation of callus. This result implies that the *srd2* mutation influenced the early process of callus formation from hypocotyl explants rather than the growth rate of callus.

For histological characterization of the effect of the *srd2* mutation on cell proliferation, samples were examined under a light microscope equipped with Nomarski optics. Whole-mount samples were prepared for hypocotyl explants of the wild type and the *srd2* mutant that had been cultured on CIM for 4 days at 22°C or 27°C. In wild-type explants, proliferating cells formed small clusters inside the cortex (Fig. 5C,E), and the number of such clusters was greater in explants cultured at 27°C than in those cultured at 22°C (data not shown). In hypocotyl explants of the *srd2* mutant, clusters of proliferating cells were formed at 22°C (Fig. 5D) but not at 27°C (Fig. 5F). These observations indicate that *SRD2* is involved in the reinitiation of cell proliferation in hypocotyl explants, a possibility that is consistent with the results of temperature-shift experiments (Fig. 4).

Effect of the *srd2* mutation on DNA synthesis in hypocotyl explants

The effect of the *srd2* mutation on DNA synthesis was investigated by microautoradiography after labeling with [^{3}H]thymidine. Hypocotyl explants of the wild type and the *srd2* mutant were cultured for 4 days on CIM that contained [^{3}H]thymidine at 22°C or 27°C and then subjected to microautoradiography (Fig. 6). In the wild type, clusters of cells with heavily labeled nuclei were found inside the cortex, regardless of culture temperature. Such cell clusters were also detected in hypocotyl explants of *srd2* muatnt that had been cultured at 22°C but there were few labeled nuclei in explants that had been cultured at 27°C. The remarkable decrease in the number of labeled nuclei in hypocotyl explants of the *srd2* mutant cultured at 27°C suggests that reinitiation of the progression of the cell cycle, leading to DNA synthesis in the first S phase, was sensitive to the restrictive temperature in this mutant.

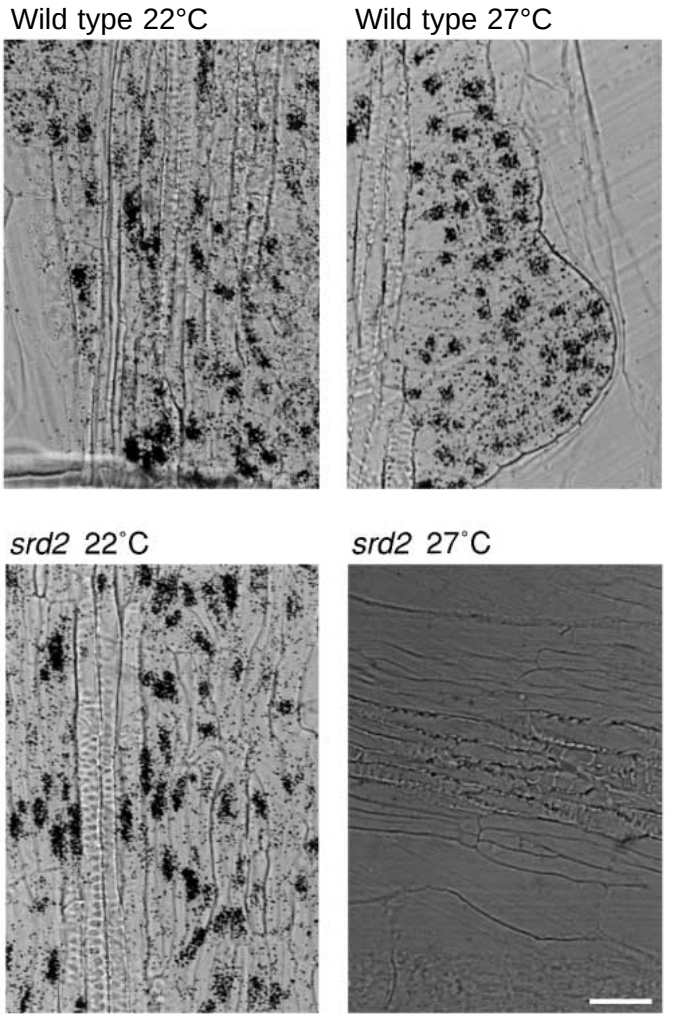

Fig. 6. Autoradiograms of hypocotyl explants of the wild type and the *srd2* mutant after culture on CIM that contained [^{3}H]thymidine for 4 days at 22°C and 27°C. Bar = 20 μm.

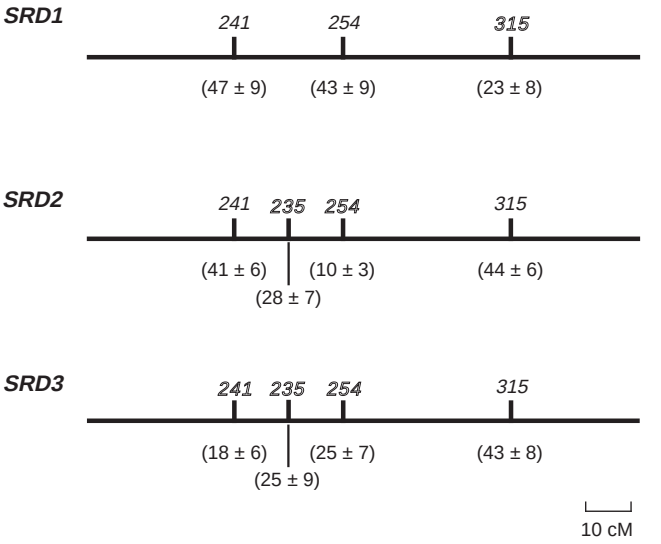

Fig. 7. Linkage between *SRD* loci and each of RFLP markers located on chromosome 1. The italicized numbers indicate clone numbers of RFLP (Chang et al., 1988), and the outlined numbers represent the RFLPs that exhibited significant linkage to each of *SRD* loci. Chromosomal positions of RFLP markers are according to Hauge et al. (1993). The frequency (%) of recombination between each of the *SRD* loci and the specified RFLP marker is shown with the expected s.d. in parenthesis.

Linkage analysis

The loci of *SRD1*, *SRD2*, and *SRD3* were mapped by linkage analysis using restriction fragment length polymorphism (RFLP) markers. Segregation of RFLP and the *srd* phenotype was scored for approximately 30 TC_2 lines of *srd1*, 80 TC_2 lines of *srd2*, and 40 TC_2 lines of *srd3*. As shown in Fig. 7, significant linkage was detected by application of the χ^2-test between the *SRD1* locus and RFLP marker 315 ($P<0.05$); between the *SRD2* locus and markers 235 ($P<0.05$) and 254 ($P<0.001$); between the *SRD3* locus and markers 241 ($P<0.001$), 235 ($P<0.02$) and 254 ($P<0.01$). No significant linkage was found between any of *SRD* loci and any of RFLP markers that are located on chromosomes 2, 3, 4 and 5 (data not shown). Thus, these RFLP analyses localized all of *SRD* loci on chromosome 1.

DISCUSSION

Genetic dissection of shoot and root redifferentiation

Temperature-sensitive phenotypes are, in most cases, attributable to the temperature-dependent loss of or reduction in function of the products of mutated genes. Thus, the phenocritical periods (temperature-sensitive periods) of *srd* mutants during organogenesis are expected to reflect aspects of organogenesis that require functional SRD proteins. Shoot redifferentiation from root explants of *srd1*, *srd2* and *srd3* was shown, by temperature-shift experiments (Fig. 1), to be highly sensitive to the restrictive temperature when explants were exposed to the restrictive temperature within 2 weeks after transfer onto SIM, within a week after transfer, and during preculture on CIM, respectively. These results suggest that *SRD1* and *SRD2* play essential roles in the process of shoot redifferentiation induced by culture on SIM, while *SRD3* is involved in some process that occurs during preculture on CIM and that is required prior to shoot formation.

According to the terms of Christianson and Warnick (1983), the preculture corresponds to the ‘competence acquisition phase’, and the subsequent culture on SIM includes the ‘induction phase’ and the ‘morphological differentiation phase’. *SRD3* seems to be involved in the ‘competence acquisition phase’, while *SRD1* and *SRD2* participate in the ‘induction phase’ and/or the ‘morphological differentiation phase’. The inhibitory effect of the restrictive temperature on shoot redifferentiation in *srd3* was reduced by prolonging preculture on CIM (Fig. 2). This finding suggests that the mutant protein is less effective than the wild-type SRD3 protein but is still partially functional at the restrictive temperature in the process of acquisition of organogenic competence.

It is of great importance to clarify the common and different aspects of the organogenic responses of various organs. To this end, we compared shoot redifferentiation from root explants with that from hypocotyl explants in terms of requirements for

products of *SRD* genes. All the *srd* mutants had temperature-sensitive defects in the redifferentiation of shoots from hypocotyl explants, as well as from root explants (Fig. 3). Thus, shoot redifferentiation from root explants and that from hypocotyl explants share elementary processes that involve *SRD* genes. However, a discrepancy between root and hypocotyl explants was found in the timing of the requirement for the protein encoded by *SRD2* during shoot redifferentiation. While the phenocritical time for root explants fell within the period of culture on SIM, shoot redifferentiation from hypocotyl explants of this strain was severely inhibited by exposure to the restrictive temperature either during preculture on CIM or after transfer onto SIM. Thus, hypocotyl explants required *SRD2* in addition to *SRD3*, while root explants required only *SRD3*, in the 'competence acquisition phase'.

Hypocotyl explants also differed from root explants in root-forming ability. Pre-culture on CIM prior to culture on RIM was very effective in the induction of roots from hypocotyl explants. Culture of hypocotyl explants on RIM after preculture on CIM induced redifferentiation of many roots on the explants. However, only a few roots formed near the proximal ends of hypocotyl explants when they were cultured on RIM without preculture. Thus, the hypocotyl seems to be rather ineffective for root redifferentiation, becoming competent only during preculture on CIM. In contrast to hypocotyl explants, root segments inoculated directly onto RIM formed a large number of roots. This implies that roots are equipped with the machinery for differentiation of roots de novo.

Out of the three *srd* mutants only *srd2* showed a defect in root differentiation. Root redifferentiation from hypocotyl explants of *srd2* was inhibited by exposure to the restrictive temperature during preculture on CIM (Table 1), and auxin-induced formation of roots from root explants was not temperature-sensitive (data not shown). Taking this result together with the different root-forming ability of root and hypocotyl explants, as discussed above, we can reasonably postulate that *SRD2* is involved in the acquisition of competence for root redifferentiation. In *srd3*, shoot redifferentiation from hypocotyl explants was sensitive to the restrictive temperature during preculture on CIM while root redifferentiation was insensitive, a result that indicates that *SRD3* participates in the acquisition of competence for shoot redifferentiation but not in the acquisition of competence for root redifferentiation. Thus, in view of the requirement for *SRD2*, in addition to SRD3, for shoot redifferentiation from hypocotyl explants during preculture on CIM, the 'competence acquisition phase' of hypocotyl explants can be presumed to consist of two sub-phases. During the first subphase, explants acquire competence for root redifferentiation with the aid of *SRD2*, and during the second subphase, they acquire competence for shoot redifferentiation with the aid of *SRD3*. It should be noted that competence for shoot redifferentiation is assumed always to be acquired additively, over and above the competence for root redifferentiation.

Here, we propose a hypothetical scheme (Fig. 8) for the process of organogenesis in tissue cultures of *Arabidopsis thaliana*, which accounts for the above mentioned phenotypes of the *srd* mutants. In this scheme, three physiological states of explants are postulated: IC (incompetent); CR (competent with respect to root redifferentiation); and CSR (competent with respect to shoot and root redifferentiation). Root segments are equipped for de novo differentiation of roots, being in the CR state at the beginning of culture. Root explants acquire the competence for shoot redifferentiation and enter the CSR state during preculture on CIM. By contrast, hypocotyl segments just after excision are incompetent and are in the IC state. Hypocotyl explants acquire competence for root redifferentiation and subsequently acquire the competence for shoot redifferentiation during preculture on CIM. That is to say, the physiological state of explants moves from IC to CR and then from CR to CSR. The transition from IC to CR and that from CR to CSR require the functions of *SRD2* and *SRD3*, respectively. Explants in the CSR state can redifferentiate shoots with the aid of *SRD1* and *SRD2* when transplanted onto SIM. Explants in either the CR or the CSR state can redifferentiate roots when transplanted onto RIM. The transition from the IC to the CSR state corresponds to the 'competence acquisition phase' described by Christianson and Warnick (1983). Thus, the present hypothetical scheme assumes that the 'competence acquisition phase' consists of two subphases: the first subphase from the IC to the CR state and the second subphase from the CR to the CSR state.

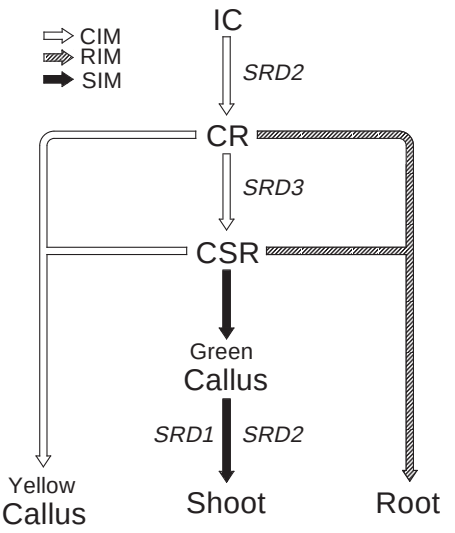

Fig. 8. Hypothetical scheme for in vitro organogenesis of *Arabidopsis thaliana*. IC, incompetent; CR, competent with respect to the redifferentiation of roots; CSR, competent with respect to the redifferentiation of shoots and roots.

Role of *SRD2* in cell proliferation

Culture of root and hypocotyl explants on CIM, without transfer to SIM or RIM, resulted in the visible formation of yellow callus. The effects of *srd* mutations on the formation of such callus were examined with hypocotyl explants of each of the *srd* mutants. Temperature-sensitive formation of callus was observed only on the *srd2* explants. Since callus formation from root explants was not inhibited by exposure to the restrictive temperature in any of mutants (Yasutani et al., 1994), the *SRD* genes appear not to be involved in fundamental events required for the progression of the cell cycle in proliferating cells.

Whole-mount observations revealed that cell proliferation was reinitiated in the stele tissue of hypocotyl explants during

a 4-day culture on CIM (Fig. 5C,E). In explants of the *srd2* mutant, such cell proliferation was not induced at the restrictive temperature (Fig. 5F). Furthermore, microautoradiography of *srd2* explants that had been incubated with [^{3}H]thymidine for 4 days indicated that cells in the stele did not enter the first S phase under the restrictive condition (Fig. 6). However, once the cell division cycle had been reinitiated at the permissive temperature, it appeared not to be arrested by exposure to the restrictive temperature as callus growth continued during the temperature-shift experiments (Fig. 4). Thus, it can be concluded that the reinitiation of cell proliferation in hypocotyl explants is mediated by the function of the SRD2 protein. This *SRD2*-mediated process seems to be specific to hypocotyls since formation of callus on root explants of the *srd2* mutant was insensitive to the restricitve temperature.

What events take place at the cellular level in association with the transition from IC to CR during preculture on CIM? Histological observations revealed that the proliferation of cells was induced in the stele (pericycle and parenchyma of vascular cylinder) during the 4 days of preculture and that meristems formed in the resultant mass of stele-derived cells after transfer of explants onto SIM or RIM (data not shown). As discussed above, *SRD2*, which might mediate the transition from IC to CR, was shown to be involved in the reinitiation of cell proliferation in the stele tissues of the hypocotyl. The possibility then arises that cell proliferation is essential for the acquisition of organogenic competence. However, this possibility does not seem likely because explants that had been precultured for 1 day on CIM, in which no proliferation of cells had yet been detected, could redifferentiate shoots upon transfer to SIM (data not shown). It seems more likely that a prerequisite for organogenesis is not cell proliferation itself but competence for proliferation and that *SRD2* is necessary for incompetent hypocotyl explants to become competent for proliferation. If the root is competent for cell proliferation, this idea is supported by the previous observation (Yasutani et al., 1994) that callus formation from root explants of *srd2* was not inhibited at the restrictive temperature, indicating the independence in root explants of both the reinitiation of cell proliferation and proliferation itself from *SRD2*. We must note here that competence for cell proliferation in this context cannot be distinguished by any aspects of the *srd2* phenotype from competence for root redifferentiation. This idea is included in Fig. 8.

The Ser/Thr protein kinase p34^{cdc2} is known to play a central role in the regulation of the eukaryotic cell cycle (Nurse, 1990). Genes for p34^{cdc2} (homologs of the *cdc2* gene of fission yeast), *CDC2a* and *CDC2b* have been isolated from *Arabidopsis thaliana* and characterized (Ferreira et al., 1991; Hirayama et al., 1991). An analysis of spatial patterns of gene expression revealed that *CDC2a* is expressed not only in dividing cells but also in non-dividing cells of root tissues, such as the pericycle and parenchyma of the vascular cylinder (Martinez et al., 1992; Hemerly et al., 1993). From these findings, Hermerly et al. (1993) proposed a close correlation between the expression of *CDC2a* and competence for cell proliferation. This opinion coincides with our views described in the previous paragraph, and leads to the working hypothesis that *SRD2* might mediate the induction of *CDC2a* expression in incompetent tissues, such as the hypocotyl stele. We are now examining the regulation of cell division and the expression of *CDC2a* in the *srd2* mutant.

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