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Isolation of Temperature-Sensitive Mutants of *Arabidopsis thaliana* That Are Defective in the Redifferentiation of Shoots[1]

Izumi Yasutani[2], Shoichi Ozawa, Toshiaki Nishida[3], Munetaka Sugiyama*, and Atsushi Komamine[4]

Biological Institute, Faculty of Science, Tohoku University, Aoaba-yama, Sendai 980, Japan

Three temperature-sensitive mutants of *Arabidopsis thaliana* that were defective in the redifferentiation of shoots were isolated as tools for the study of organogenesis. M_3 lines were constructed by harvesting M_3 seeds separately from each M_2 plant. Comparative examination of shoot redifferentiation in root explants of 2700 M_3 lines at 22°C (permissive temperature) and at 27°C (restrictive temperature) led to the identification of seven temperature-sensitive mutant lines. Genetic tests of three of the seven mutant lines indicated that temperature-sensitive redifferentiation of shoots in these three lines resulted from single, nuclear, recessive mutations in three different genes, designated *SRD1*, *SRD2*, and *SRD3*. The morphology of root explants of *srd* mutants cultured at the restrictive temperature suggests that the products of these *SRD* genes function at different stages of the redifferentiation of shoots.

Plasticity is a key feature of cytodifferentiation in higher plants. In response to phytohormones and/or wounding, various types of mature, differentiated plant cells are reactivated so that they acquire organogenic competence and then proliferate to form shoots, roots, or somatic embryos. Physiological, biochemical, and molecular biological studies of this process have provided information important for an understanding of the differentiation of plant cells. However, in spite of many efforts, the key to organogenic competence and the molecular mechanisms of organogenesis is still unknown. Genetic analysis, which has not been exploited extensively in this field, can be expected to improve the present situation.

With respect to organogenesis, dependence on genotype has been practically the only focus of genetics to date. Organogenic responses in tissue culture often vary, depending on genotype. The genetic basis of such variations has been studied in several crop species (Ohki et al., 1978; Reich and Bingham, 1980; Frankenberger et al., 1981; Tomes and Smith, 1985; Mathias et al., 1986; Koornneef et al., 1987; Agache et al., 1989; Nadolska-Orczyk and Malepszy, 1989; Langridge et al., 1991; Yu and Pauls, 1993). From these studies, a locus, designated *Rg-1*, that controls shoot regeneration in tomato was recently identified and mapped (Koornneef et al., 1993). This result is undoubtedly a remarkable feat of genetic analysis but is still unable to provide adequate clues about the nature of the entire process of organogenesis, since organogenesis must be composed of many events driven by different genes.

For a genetic dissection of organogenesis, mutants are necessary that are defective at various stages of organogenesis. In particular, conditional mutants, such as temperature-sensitive mutants, would be very useful because they would provide information about the functions of mutated genes during organogenesis; for example, the critical timing of certain functions during organogenesis and pleiotrophic functions involved in physiological phenomena other than organogenesis. In addition, conditional mutations allow us to study genes that are essential for plants, if inactivation of gene function is lethal.

Temperature-sensitive mutations have been successfully exploited in research into somatic embryogenesis of cultured cells of carrot (Breton and Sung, 1982; Giuliano et al., 1984; Lo Schiavo et al., 1988, 1990; Schnall et al., 1988). Although cultures of carrot cells provide a unique experimental system for studies of somatic embryogenesis, which is a straightforward example of the plasticity of plant cells, further genetic analysis of mutants may be difficult because of the poorly characterized genetic background of carrot.

Arabidopsis thaliana is a model plant suitable for molecular and classical genetic studies (Meyerowitz, 1989). Construction of detailed chromosome maps, including both molecular and classical genetic markers (the most recent map was reported by Hauge et al., 1993), has allowed map-based isolation of genes of *Arabidopsis* (e.g. Leyser et al., 1993). Furthermore, a procedure for redifferentiation of shoots at quite high efficiency has been developed using *Arabidopsis* (Valvekens et al., 1988) and can be employed in studies of organogenesis. We recently screened mutagenized populations of *Arabidopsis* to isolate temperature-sensitive mutants, impaired in the redifferentiation of shoots, in an attempt to obtain tools for a genetic analysis of organogenesis. In this report we describe the isolation of a novel class of mutants

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[2] Present address: Bio Research Laboratory, Toyota Motor Corporation, Toyota 471, Japan.

[3] Present address: Research Institute of Development, Aging, and Cancer, Tohoku University, Sendai 980, Japan.

[4] Present address: Department of Chemical and Biological Sciences, Faculty of Science, Japan Women's University, Bunkyo-ku, Tokyo 112, Japan.

* Corresponding author; fax 81–022–263–9206.

Abbreviations: CIM; callus-inducing medium; GM, germination medium; SIM, shoot-inducing medium.

that are temperature sensitive for the redifferentiation of shoots.

MATERIALS AND METHODS

Plant Material

All the plants used in the present study were derived from the Landsberg *erecta* strain of *Arabidopsis thaliana* L. Heynh. This strain is referred to as the wild type in this paper. Mutagenesis was carried out with ethyl methanesulfonate as described by Estelle and Somerville (1987). After two cycles of self-fertilization, M_3 seeds were collected from individual M_2 plants for construction of M_3 lines (L1–L2700). Each M_3 line was derived from seeds harvested from only a single M_2 plant. Wild-type seeds and some batches of M_2 seeds were kindly provided by Prof. N. Goto (Miyagi College of Education, Sendai, Japan).

Tissue Culture

The procedure for induction of the redifferentiation of shoots has been described by Valvekens et al. (1988). Donor plants were grown aseptically at 22°C on GM for approximately 3 weeks. Root segments of 5 to 10 mm in length were excised and placed on CIM. After 4 d of culture on CIM, root explants were transferred to SIM. On the 25th d of culture (3 weeks after inoculation onto SIM), the frequency of redifferentiation of shoots was determined. Tissue culture was carried out in continuous light in Petri dishes or in multiwell plates sealed with Micropore surgical tape (3M Health Care, St. Paul, MN).

Culture media were slightly modified versions of media reported by Valvekens et al. (1988). GM was MS medium (Murashige and Skoog, 1962) supplemented with 10 g/L Suc. CIM was B5 medium (Gamborg et al., 1968) supplemented with 20 g/L Glc, 0.5 mg/L 2,4-D, and 0.1 mg/L kinetin. SIM was B5 medium supplemented with 20 g/L Glc, 0.15 mg/L IAA, and 0.5 mg/L N^6-(2-isopentenyl)adenine. A reduction in the concentration of N^6-(2-isopentenyl)adenine improved the stability of response in tissue culture. All media were buffered with 0.5 g/L Mes (pH 5.7) and solidified with 0.2% or 0.25% gellan gum.

Screening for Temperature-Sensitive Mutants

Six root segments were excised from three plants of each M_3 line and used for the first screening for mutants. Three segments were cultured at 22°C and another three were cultured at 27°C. M_3 lines in which redifferentiation of shoots occurred at 22°C and no redifferentiation occurred at 27°C were selected as candidates for temperature-sensitive mutants. In the second screening, temperature sensitivity of shoot redifferentiation was re-examined with many more explants for each candidate line. Subsequently, heritability was tested with M_4 plants produced by self-pollination of M_3 plants of putative mutant lines.

RESULTS

Isolation of Temperature-Sensitive Mutants

Mutagenized populations were screened to isolate mutants temperature sensitive for the redifferentiation of shoots in a study of root explants of 2700 M_3 lines, as described in "Materials and Methods." Repeated examination of shoot redifferentiation led to the identification of seven mutant lines: L131, L1045, L1047, L1919, L1266, L1393, and L2098. The frequency of redifferentiation of shoots from root explants of these mutants was significantly lower at 27°C (restrictive temperature) than at 22°C (permissive temperature), whereas shoots redifferentiated from nearly 100% of the root explants of the wild-type plants at both temperatures.

Four of the mutant lines (L131, L1045, L1047, and L1919) were used for an examination of the dependence on temperature of shoot redifferentiation (Fig. 1). Shoot redifferentiation of all mutant lines tested was sensitive to temperatures higher than 25°C, whereas wild-type explants exhibited a high frequency of shoot redifferentiation between 20°C and 27°C. Shoot redifferentiation was apparently also suppressed at 20°C in each mutant line, but this result does not imply true cold sensitivity of shoot redifferentiation. The low frequency of shoot redifferentiation at 20°C may have been due to the slow progress of the organogenic process, since after prolonged culture the frequency increased significantly (data

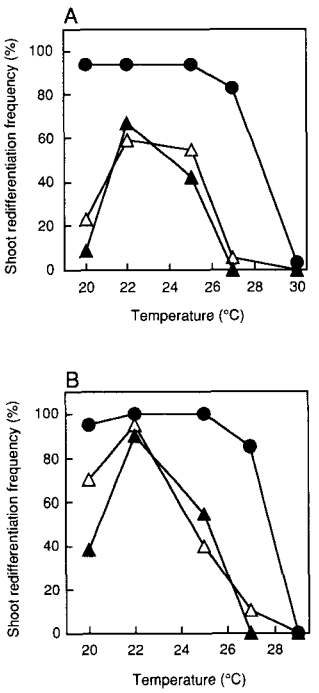

Figure 1. Effects of temperature on the frequency of redifferentiation of shoots from root explants. A, Root segments of the wild type (●), L1045 (▲), and L1047 (△) were cultured at various temperatures for 4 d on CIM and for 3 weeks on SIM. More than 17 segments were used for each treatment. B, Root segments of the wild type (●), L131 (▲), and L1919 (△) were cultured at various temperatures for 4 d on CIM and for 3 weeks on SIM. Forty-eight segments were used for each treatment. Shoot redifferentiation frequency was defined as the percentage of the number of explants forming shoots.

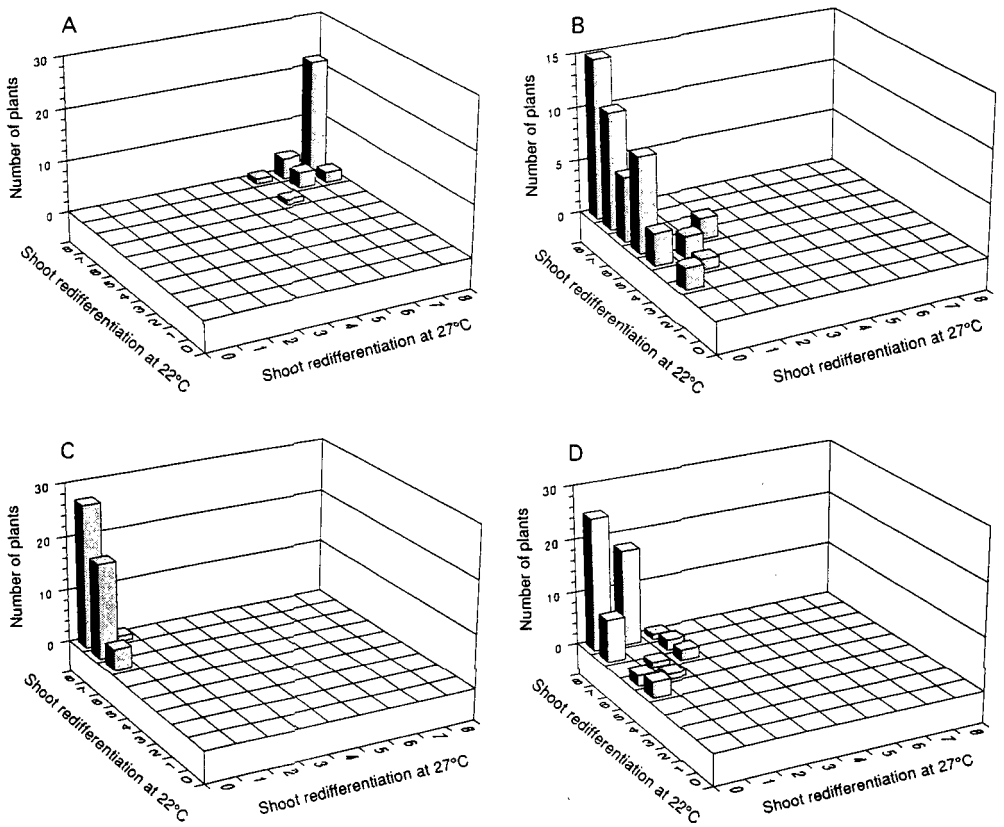

Figure 2. Temperature sensitivity of shoot redifferentiation from root explants of the wild type (A), L1045 (B), L131 (C), and L1919 (D). Sixteen root segments were excised from each individual plant of these lines. Eight explants were cultured at 22°C and another eight were cultured at 27°C. On the 25th d of culture (3 weeks after inoculation onto SIM), numbers of explants with redifferentiated shoots were scored for classification of individual plants. The numbers along horizontal axes indicate the numbers of explants forming shoots.

not shown). By contrast, inhibition of shoot redifferentiation at 27°C could not be overcome by prolonged culture of any of the mutant lines.

For demonstration of the ranges of tissue culture responses among plants of the same lines, temperature sensitivity of shoot redifferentiation was scored for individuals in three mutant lines (L131, L1045, and L1919), and the results were arranged as histograms (Fig. 2).

Genetic Analysis

Mutant lines L131, L1045, and L1919 were crossed with the wild type, with the mutant plants as the female parents. Root explants of several F_1 plants from each cross were subjected to an examination of shoot redifferentiation at 22°C and 27°C (Table I). In the case of the L131 × wild type and the L1919 × wild type crosses, the F_1 plants did not exhibit temperature-sensitive redifferentiation of shoots. F_1 plants generated by crossing L1045 and the wild type showed variable responses in culture, but the frequency of shoot redifferentiation was always higher in the F_1 plants than in the parental L1045 plants. Thus, the phenotype of the F_1 plants from the L1045 × wild type cross was intermediate but was closer to that of the wild type.

Temperature sensitivity of shoot redifferentiation was scored for individuals in F_2 generations produced by self-fertilization of F_1 plants. The results are shown as histograms in Figure 3. In all cases, although it was less clear for the L1045 × wild type cross, F_2 plants could be classified into two groups: one that was temperature insensitive for shoot redifferentiation, and the other that was temperature sensitive. The segregation corresponded approximately to a ratio of three insensitive plants to one sensitive plant. These data indicate that temperature sensitivity in shoot redifferentiation was caused by a single recessive mutation in each mutant line.

Complementation tests were performed by cross-pollination among three mutant lines. Each cross resulted in recovery from the mutant phenotype in the F_1 generation (Table II), indicating that mutations in these lines were located at different loci. In view of the characteristics of the mutations that affected shoot redifferentiation, the genes defined by mutations in L1045, L131, and L1919 were designated *SRD1*, *SRD2*, and *SRD3*, respectively.

Morphology of Mutant Explants

Morphological changes in root explants of mutant plants during culture were monitored by serial observations in a

Table I. *Temperature sensitivity of shoot redifferentiation in root explants of F_1 plants*

Crossing	F_1 plant	Shoot Redifferentiation	
		22°C	27°C
L1045 × wild type	1	8[a]/8[b]	7/8
	2	8/8	2/8
	3	8/8	0/8
	4	8/8	3/8
	5	8/8	2/8
	6	8/8	4/8
L131 × wild type	1	8/8	7/8
	2	7/8	7/8
	3	8/8	8/8
	4	8/8	8/8
L1919 × wild type	1	7/8	8/8
	2	8/8	7/8
	3	8/8	8/8
	4	8/8	8/8

[a] Number of root explants with redifferentiated shoots. [b] Total number of root explants.

comparison with root explants of wild-type plants (Fig. 4). In the wild type, callus became visible after transfer of the explant onto SIM and the callus gradually turned green. Dark purple spots, probably due to the accumulation of anthocyanin, appeared later in the development of the callus. Shoots emerged from the purple spots. These changes in morphology were always observed at both 22°C and 27°C in wild-type explants, and they were also observed at 22°C in explants of mutants.

When root explants of mutants were cultured at a restrictive temperature on SIM for 3 weeks after 4 d of culture on CIM, their morphology was similar to the wild-type morphology at intermediate stages of the above-mentioned process of shoot redifferentiation (Fig. 5). In most root explants of L1045 (*srd1*), green callus, sometimes associated with purple spots, developed similarly to that on wild-type explants. Explants of L131 (*srd2*) formed homogeneously green callus without purple spots. On explants of L1919 (*srd3*), development of callus was limited, and no remarkable changes in morphology were observed during culture on SIM.

Cell Growth of Mutants

When root explants from wild-type plants are cultured continuously on CIM for a long period, yellow, rapidly growing callus is formed. The temperature sensitivity of such formation of callus was examined with each mutant to determine whether *srd* mutations affected fundamental activities required for cell proliferation. As shown in Figure 6, no obvious differences were found in terms of callus growth at 22°C and 27°C between any of the mutants and the wild type. In addition, exposure of redifferentiated shoots of mutants to the restrictive temperature did not suppress subsequent development (data not shown). Accordingly, it is concluded that *srd* mutations do not affect fundamental aspects of cell growth.

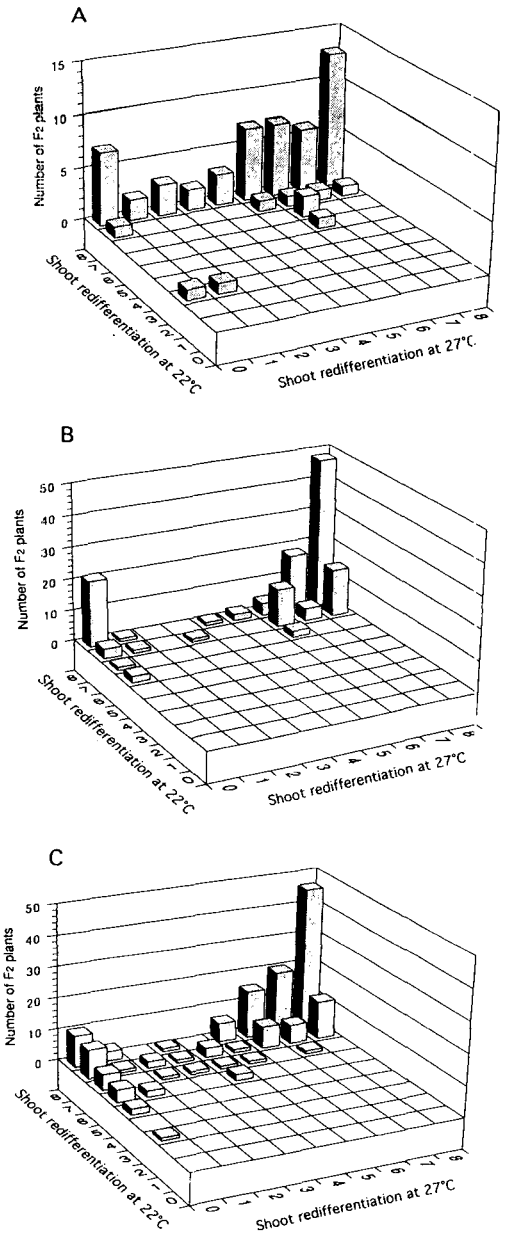

Figure 3. Temperature sensitivity of shoot redifferentiation from root explants of F_2 plants obtained by self-fertilization after crosses between L1045 and the wild type (A), L131 and the wild type (B), and L1919 and the wild type (C). Sixteen root segments were excised from each F_2 plant. Eight explants were cultured at 22°C and another eight were cultured at 27°C. On the 25th d of culture (3 weeks after inoculation onto SIM), numbers of explants with redifferentiated shoots were scored for classification of F_2 plants. The numbers along horizontal axes indicate the numbers of explants forming shoots. When F_2 plants, from which fewer than four explants formed shoots at 27°C, were judged as phenotypically temperature sensitive, segregation ratios of insensitive to sensitive plants were 43:17 (A), 106:30 (B), and 123:41 (C). χ^2 values calculated for an expected segregation of three insensitive plants to one sensitive plant are 0.35 (A), 0.63 (B), and 0 (C). Probability values are larger than 0.3 in all three cases.

Table II. *Complementation analysis of mutant lines temperature sensitive for shoot redifferentiation*

Crossing	F_1 Plant	Shoot Redifferentiation	
		22°C	27°C
L1045 × L131	1	7[a]/8[b]	8/8
	2	8/8	8/8
	3	5/8	6/8
	4	8/8	8/8
	5	7/8	8/8
	6	8/8	8/8
	7	8/8	8/8
	8	4/8	7/8
	9	8/8	8/8
	10	6/8	7/8
	11	7/8	7/8
L131 × L1919	1	8/8	8/8
	2	7/8	6/8
	3	8/8	6/8
	4	8/8	4/8
L1045 × L1919	1	5/8	5/8
	2	7/8	6/8
	3	7/8	7/8
	4	6/8	3/8
	5	7/8	0/8
	6	5/8	4/8

[a] Number of root explants with redifferentiated shoots. [b] Total number of root explants.

Phenotypes of Mutants at the Whole Plant Level

No major aberrations were found in mutant plants grown on soil at 22°C. Exposure to the restrictive temperature interfered with the growth of seedlings of L131 (*srd2*) and L1919 (*srd3*). Severe suppression of the growth was observed only when seedlings of these mutant lines were exposed to the restrictive temperature during the first 7 d after sowing (Fig. 7). Otherwise, mutant plants looked almost normal in morphology even at the restrictive temperature. Germination was not temperature sensitive in any of the mutant lines tested.

DISCUSSION

Temperature-sensitive mutations have not been extensively exploited in studies of plant physiology, although they are recognized as powerful tools for the genetic dissection of various physiological phenomena. In this report we describe the isolation of temperature-sensitive mutants of a model plant, *A. thaliana*, that are defective in the redifferentiation of shoots. Seven mutant lines were selected from 2700 M_3 lines, and three of them, L131, L1045, and L1919, were preliminarily characterized. Genetic analysis of these three mutant lines led to the identification of three genes, *SRD1*, *SRD2*, and *SRD3*, which appear to be involved in shoot redifferentiation.

SRD genes seem to play a role not in the fundamental events that are essential for cell proliferation, but in more specific aspects of organogenesis, since *srd* mutations did not result in temperature-sensitive growth of callus or of redifferentiated shoots. Comparative observations of root explants of mutants cultured at the restrictive temperature provided suggestive information about differences in the functions of *SRD* genes. Green callus developed on the explants of L1045 (*srd1*) and L131 (*srd2*), and very little callus was formed on the explants of L1919 (*srd3*). These terminal phenotypes of mutant explants at the restrictive temperature were almost equivalent to the intermediate morphology observed at different stages of the normal redifferentiation of shoots.

Morphological changes in the normal course of shoot redifferentiation can be summarized as follows. Callus develops along root segments (stage 1), turns green (stage 2), and then turns purple locally (stage 3), and finally, shoots form at the sites of purple spots (stage 4). Our present working hypothesis is that *SRD3* plays a critical role prior to stage 1 and *SRD2* and *SRD1* take part in the progression of the redifferentiation process from stage 2 to stage 3. Development of callus in some explants of L1045 (*srd1*) was associated with the formation of purple spots, whereas no purple spots were observed on calluses of L131 (*srd2*). This result suggests that the *srd1* mutation is leaky for inhibition of progression from stage 2 to stage 3 at the restrictive temperature.

The growth of seedlings was affected remarkably by *srd2* and *srd3* mutations at the early phase of development just

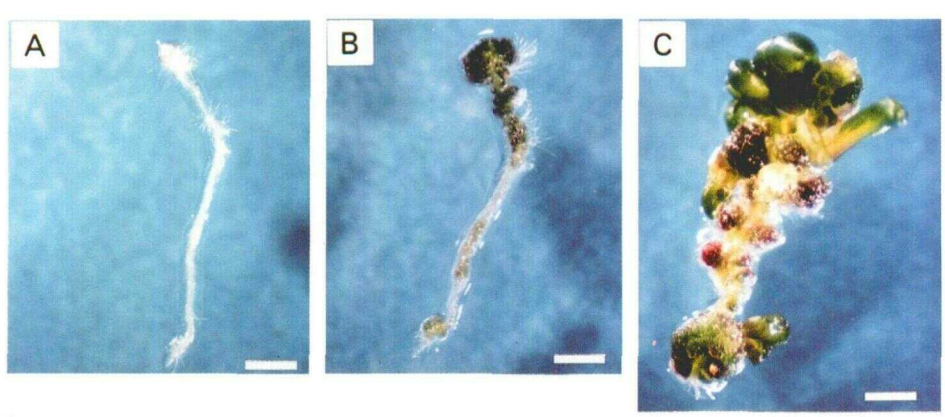

Figure 4. Serial observations of a root explant from a wild-type plant cultured at 22°C. Photographs show the same explant on the 8th d of culture (4 d after transfer onto SIM) (A), on the 12th d (B), and on the 20th d (C). Bars = 1 mm.

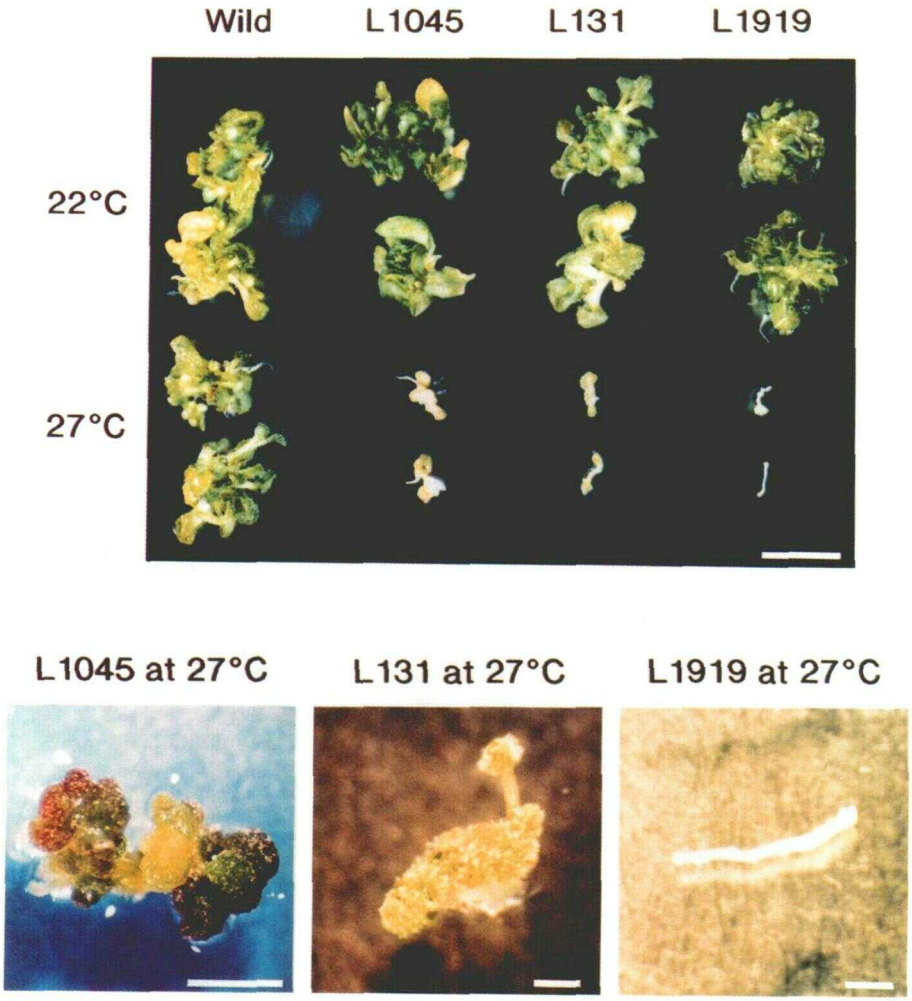

Figure 5. Terminal phenotypes of root explants of *srd* mutants. Root explants were cultured for 4 d on CIM and then for 3 weeks on SIM at 22°C or 27°C. Bars = 2 cm in the upper panel and 2 mm in the lower panels.

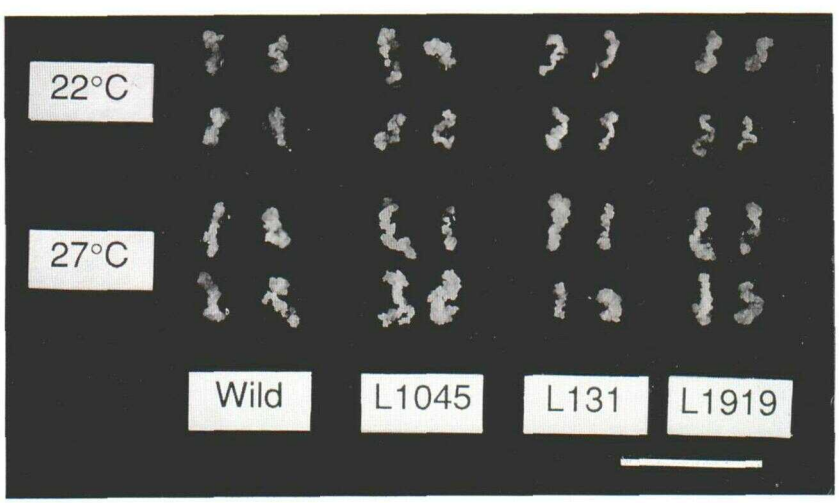

Figure 6. Effects of temperature on the formation of callus from root segments of *srd* mutants. Root explants were cultured for 3 weeks on CIM at 22°C or at 27°C. Bar = 2 cm.

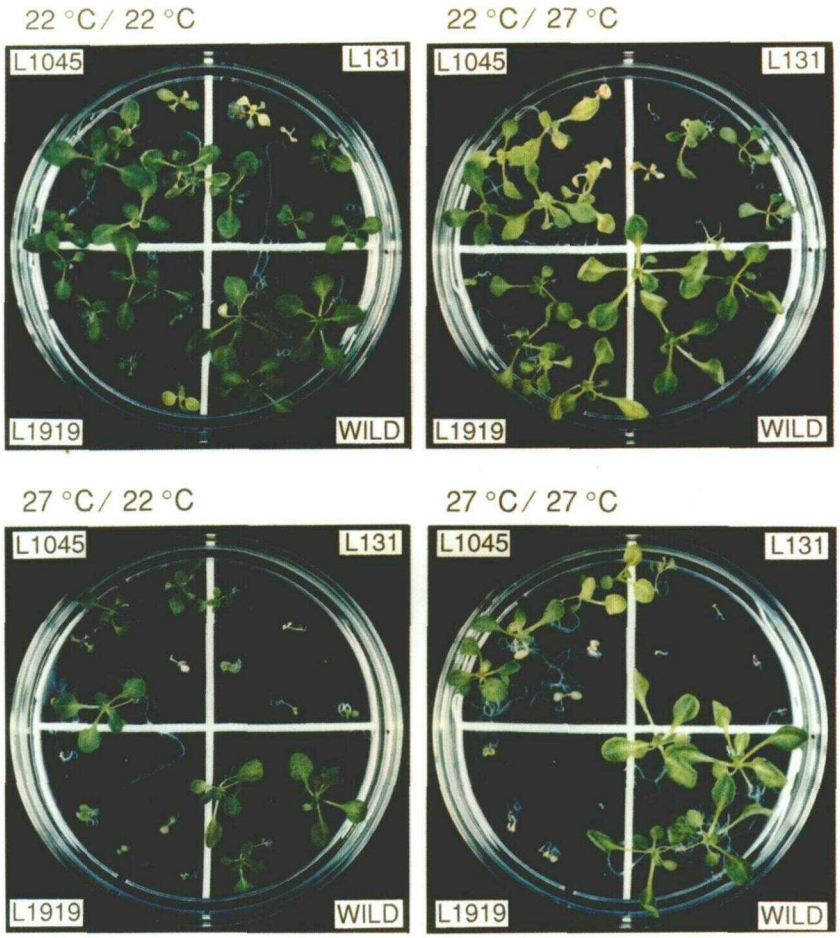

Figure 7. Effects of temperature on the growth of seedlings of *srd* mutants. Seeds of the wild type, L1045, L131, and L1919 were sowed on GM. Incubation was performed at 22°C for 21 d (22°C/22°C), at 27°C for 21 d (27°C/27°C), at 22°C for 7 d and then at 27°C for 14 d (22°C/27°C), or at 27°C for 7 d and then at 22°C for 14 d (27°C/22°C).

after germination. A possibility has arisen from this result that development of seedlings and organogenesis in tissue cultures share a process that involves *SRD2* and *SRD3*. Further characterization of *srd* mutants is expected to provide various information useful for understanding organogenesis in vivo as well as in vitro.

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LITERATURE CITED

Agache S, Bachelier B, de Buyser J, Henry Y, Snape J (1989) Genetic analysis of anther culture response in wheat using aneuploid, chromosome substitution and translocation lines. Theor Appl Genet **77:** 7–11

Breton AM, Sung ZR (1982) Temperature-sensitive carrot variants impaired in somatic embryogenesis. Dev Biol **90:** 58–66

Estelle MA, Somerville C (1987) Auxin-resistant mutants of *Arabidopsis thaliana* with an altered morphology. Mol Gen Genet **206:** 200–206

Frankenberger EA, Hasegawa PM, Tigchelaar EC (1981) Diallel analysis of shoot-forming capacity among selected tomato genotypes. Z Pflanzenphysiol **102:** 233–242

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res **50:** 151–158

Giuliano G, Lo Schiavo F, Terzi M (1984) Isolation and developmental characterization of temperature-sensitive carrot cell variants. Theor Appl Genet **67:** 179–183

Hauge BH, Hanley SM, Cartinhour S, Cherry JM, Goodman M, Koornneef M, Stam P, Chang C, Kempin S, Medrano L, Meyerowitz EM (1993) An integrated genetic/RFLP map of the *Arabidopsis thaliana* genome. Plant J **3:** 745–754

Koornneef M, Bade J, Hanhart C, Horsman K, Schel J, Soppe W, Verkerk R, Zabel P (1993) Characterization and mapping of a gene controlling shoot regeneration in tomato. Plant J **3:** 131–141

Koornneef M, Hanhart CJ, Martinelli L (1987) A genetic analysis of cell culture traits in tomato. Theor Appl Genet **74:** 633–641

Langridge P, Lazzeri P, Lorz H (1991) A segment of rye chromosome 1 enhances growth and embryogenesis of calli derived from immature embryos of wheat. Plant Cell Rep **10:** 148–151

Leyser HMO, Lincoln A, Timpte C, Lammer D, Turner J, Estelle M (1993) *Arabidopsis* auxin-resistance gene *AXR1* encodes a protein related to ubiquitin-activating enzyme E1. Nature **364:** 161–164

Lo Schiavo F, Giuliano G, de Vries SC, Genga A, Bollini R, Pitto L, Cozzani F, Nuti-Ronchi V, Terzi M (1990) A carrot cell variant temperature sensitive for somatic embryogenesis reveals a defect in the glycosylation of extracellular proteins. Mol Gen Genet **223:** 385–393

Lo Schiavo F, Giuliano G, Sung ZR (1988) Characterization of a temperature-sensitive carrot cell mutant impaired in somatic embryogenesis. Plant Sci **54:** 157–164

Mathias RJ, Fukui K, Law CN (1986) Cytoplasmic effects on the tissue culture response of wheat (*Triticum aestivum*) callus. Theor Appl Genet **72:** 70–75

Meyerowitz EM (1989) *Arabidopsis*, a useful weed. Cell **56:** 263–269

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant **15:** 473–497

Nadolska-Orczyk A, Malepszy S (1989) In vitro culture of *Cucumis sativus* L. 7. Genes controlling plant regeneration. Theor Appl Genet **78:** 836–840

Ohki S, Bigot C, Mousseau J (1978) Analysis of shoot-forming capacity in vitro in two lines of tomato (*Lycopersicon esculentum* Mill.). Plant Cell Physiol **19:** 27–42

Reich B, Bingham ET (1980) The genetic control of bud formation from callus cultures of diploid alfalfa. Plant Sci Lett **20:** 71–77

Schnall JA, Cooke TJ, Cress DE (1988) Genetic analysis of somatic embryogenesis in carrot cell culture: initial characterization of six classes of temperature-sensitive variants. Dev Genet **9:** 49–67

Tomes DT, Smith OS (1985) The effect of parental genotype on initiation of embryogenic callus from elite maize (*Zea mays* L.) germplasm. Theor Appl Genet **70:** 505–509

Valvekens D, van Montagu M, van Lijsebettens M (1988) *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants by using kanamycin selection. Proc Natl Acad Sci USA **85:** 5536–5540

Yu K, Pauls KP (1993) Identification of a RAPD marker associated with somatic embryogenesis in alfalfa. Plant Mol Biol **22:** 269–277