Genetic analysis of adventitious root formation with a novel series of temperature-sensitive mutants of *Arabidopsis thaliana*

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Summary

When cultured on media containing the plant growth regulator auxin, hypocotyl explants of *Arabidopsis thaliana* generate adventitious roots. As a first step to investigate the genetic basis of adventitious organogenesis in plants, we isolated nine temperature-sensitive mutants defective in various stages in the formation of adventitious roots: five *root initiation defective* (*rid1* to *rid5*) mutants failed to initiate the formation of root primordia; in one *root primordium defective* (*rpd1*) mutant, the development of root primordia was arrested; three *root growth defective* (*rgd1*, *rgd2*, and *rgd3*) mutants were defective in root growth after the establishment of the root apical meristem. The temperature sensitivity of callus formation and lateral root formation revealed further distinctions between the isolated mutants. The *rid1* mutant was specifically defective in the reinitiation of cell proliferation from hypocotyl explants, while the *rid2* mutant was also defective in the reinitiation of cell proliferation from root explants. These two mutants also exhibited abnormalities in the formation of the root apical meristem when lateral roots were induced at the restrictive temperature. The *rgd1* and *rgd2* mutants were deficient in root and callus growth, whereas the *rgd3* mutation specifically affected root growth. The *rid5* mutant required higher auxin concentrations for rooting at the restrictive temperature, implying a deficiency in auxin signaling. The *rid5* phenotype was found to result from a mutation in the *MOR1/GEM1* gene encoding a microtubule-associated protein. These findings about the *rid5* mutant suggest a possible function of the microtubule system in auxin response.

Key words: *Arabidopsis thaliana*, Temperature-sensitive mutant, Adventitious root formation, Cell proliferation, Dedifferentiation, Root primordium, Root apical meristem, Auxin, microtubule, *MOR1*

Introduction

During the post-embryonic development of plants, new axes of growth emerge through lateral or adventitious organogenesis, and the reiteration of this process builds up the complex pattern of a plant body. Regulation of such lateral or adventitious organogenesis provides a flexible way for plants to alter their form and resource allocation in response to environmental changes or after injury. In this context, lateral or adventitious organogenesis plays an essential role in the post-embryonic development and survival of plants.

Among the processes of lateral and adventitious organogeneses, lateral root formation has been extensively studied by various approaches using the model plant, *Arabidopsis thaliana*. Lateral root formation is considered to consist of two distinct phases: lateral root initiation and the establishment of the root apical meristem (Laskowski et al., 1995; Celenza et al., 1995). The histology of both these phases have been described in detail (Malamy and Benfey, 1997). During the first phase, a lateral root primordium originates from a few pericycle cells, called founder cells, that lie in the cell file adjacent to either of the xylem poles. During the second phase, the root apical meristem is established within the primordium. Thereafter, the root apical meristem is activated and becomes responsible for lateral root growth.

Accumulating pieces of physiological and genetic evidence have demonstrated a critical role of the plant growth regulator auxin, which is supplied by shoot tissues through the polar transport system, in the initiation of lateral roots (Reed et al., 1998; Celenza et al., 1995; Hobbie and Estelle, 1995; Ruegger et al., 1998; Fukaki et al., 2002; Xie et al., 2000). Formerly, the cell cycle of pericycle cells was considered to be arrested in G_2 phase and to recommence in response to auxin to initiate lateral root formation (Blakely and Evans, 1979). However, recent studies have demonstrated that the actual situation is more complex. According to the work of Dubrovsky et al. (Dubrovsky et al., 2000), pericycle cells at the xylem poles continue to divide without interruption during their passage through the elongation and differentiation zones, and only some of the dividing pericycle cells are committed in a stochastic manner to the asymmetric formative division that gives rise to lateral root primordia. Beeckman et al. (Beeckman et al., 2001) proposed a slightly different but essentially similar scenario of lateral root initiation, in which pericycle cells at the xylem poles, leaving the root meristematic region, progress via S phase to G_2 phase after a transient arrest in G_1 phase, to become competent to initiate lateral roots.

There seems to be another control point for lateral root initiation apart from the developmental control point discussed above. Auxin applied exogenously to mature roots is thought to act on this later control point to induce pericycle cells

arrested in G_1 phase to recommence the cell cycle. Therefore, different patterns of cell division among pericycle cells, position-dependent and stochastic determination of cell fate, and dual control points for lateral root initiation make the initial scene of lateral root formation highly complicated and difficult to access.

At the onset of adventitious root formation in tissue culture, no cells are specified to form adventitious roots. They first dedifferentiate, i.e., acquire competence for cell proliferation and organ regeneration (Ozawa et al., 1998), then initiate cell proliferation and form adventitious root primordia. All these events can be induced reproducibly under the control of exogenous phytohormones. Therefore, adventitious root formation may provide a useful experimental system with which to study the entire process of root formation, including the pre-morphogenesis stages.

Previously, we characterized temperature-sensitive mutants of *A. thaliana* to dissect the process of shoot and root regeneration via callus formation (Yasutani et al., 1994; Ozawa et al., 1998; Sugiyama, 2003). Here we extend this approach to elucidate the process of adventitious root formation. We report the isolation and phenotypic analysis of a series of novel mutants that are temperature-sensitive in various steps of adventitious root formation.

Materials and methods

Plant materials

In the present study, the Landsberg *erecta* (L*er*) strain of *Arabidopsis thaliana* (L.) Heynh. was used as the wild type. The *srd2* mutant of L*er* origin, which was isolated as temperature-sensitive for shoot redifferentiation (Yasutani et al., 1994) and later also shown to be defective in dedifferentiation and root development (Ozawa et al., 1998), was used together with newly isolated mutants. The *scarecrow-2* (*scr-2*) mutant in the Wassilewskija background (Scheres et al., 1995) and the *microtubule organization 1-1* (*mor1-1*) mutant in the Columbia background (Whittington et al., 2001) were kindly provided by Dr Ben Scheres (Utrecht University, The Netherlands) and Dr Geoffrey O. Wasteneys (The Australian National University, Australia), respectively. These mutants were used for allelism tests.

Plant growth conditions

For the source materials of tissue culture, plants were grown aseptically as described in our previous paper (Ozawa et al., 1998). Seeds were surface-sterilized in a solution of about 1.0% sodium hypochlorite and 0.1% (w/v) Triton X-100 for 10 minutes. The seeds were rinsed several times with sterile water, then sown on germination medium (GMA). Plates were incubated at 22°C on a tilt of 45° under continuous light at a fluence rate of 8-14 μmol/m^2/second. GMA is MS medium (Murashige and Skoog, 1962) supplemented with 10 g/l sucrose, buffered with 0.5 g/l 2-(*N*-morpholino)ethanesulphonic acid (MES) to pH 5.7, and solidified with 1.5% agar.

Tissue culture

Tissue culture experiments for the phenotypic characterization of mutants were performed in a similar way to that described by Ozawa et al. (Ozawa et al., 1998). Hypocotyl or root segments of 5 mm in length were excised from 12- to 14-day-old seedlings and cultured under continuous light (15-25 μmol/m^2/second) either at 22°C or at 28°C. As a root segment, we used a 5 mm section of primary root that was 5 mm away from the root apex. For the induction of adventitious roots and lateral roots, hypocotyl and root explants were cultured on root-inducing medium [RIM: B5 medium (Gamborg et al., 1968) supplemented with 20 g/l glucose and 0.5 mg/l indole-3-butyric acid (IBA)]. To induce callus formation, hypocotyl and root explants were cultured on callus-inducing medium [CIM: B5 medium supplemented with 20 g/l glucose, 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.1 mg/l kinetin]. All tissue culture media were buffered to pH 5.7 with 0.5 g/l MES, and solidified with 2.5 g/l gellan gum.

Isolation of mutants

L*er* seeds were mutagenized by treatment with 0.3% ethyl methanesulfonate solution for 20 hours at room temperature. In the primary screening, about 8,000 M_2 seedlings were tested for their ability to form adventitious roots at the restrictive (28°C) and permissive (22°C) temperatures. Adventitious root formation was induced from hypocotyl segments of M_2 seedlings by culture on RIM at 28°C, and at the same time from the remaining shoots (the top part of hypocotyl plus shoot tip including cotyledons) by culture on RIM at 22°C. M_2 plants that showed some defects in adventitious root formation from hypocotyl explants at 28°C but could form almost normal roots at 22°C from shoots were selected as candidate temperature-sensitive mutants. An M_3 line was constructed from the rooted shoot of each M_2 candidate. Secondary screening was carried out using 4-24 hypocotyl segments of every M_3 line. One half of the segments were cultured on RIM at 22°C and the other half at 28°C. M_3 lines that exhibited temperature-dependent defects in adventitious root formation from hypocotyl explants during secondary screening were finally isolated as mutant lines. Before phenotypic analysis, they were genetically purified with three rounds of backcrosses followed by two rounds of self-reproduction.

Complementation tests

Mutant lines were categorized into three classes on the basis of their phenotypic similarities (see Results). Complementation tests were carried out for any combinations of two mutant lines belonging to the same class, by examining the temperature sensitivity of adventitious root formation from hypocotyl explants of F_1 seedlings derived from crosses between them. To test the allelism of *rid5* with *mor1*, F_1 progenies produced by crossing *rid5* with *mor1-1* were tested for adventitious rooting in tissue culture and seedling morphology at 28°C. Allelism between *rgd3* and *scr* was assessed by monitoring adventitious root growth at 28°C of F_1 progenies derived from a cross between *rgd3* and *scr-2*.

Chromosome mapping

Mutants were crossed to the Columbia strain and the resultant F_2 populations were used for chromosome mapping. F_2 plants were examined for the temperature sensitivity of adventitious root formation in hypocotyl explants. Temperature-sensitive F_2 plants were judged to be homozygous for the mutant allele responsible for the defect in adventitious root formation. DNA was extracted from the cotyledons of these F_2 seedlings by grinding them in 200 μl of extraction buffer [200 mM Tris-HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA, 0.5% SDS]. DNA was recovered by ethanol-precipitation and subjected to simple sequence length polymorphism (SSLP) and cleaved amplified polymorphic sequence (CAPS) analyses (http://www.arabidopsis.org/aboutcaps.html).

Whole-mount preparations

Hypocotyl explants were fixed overnight at 4°C in a 9:1 mixture of ethanol and acetic acid, hydrated through a graded series of ethanol, and mounted with a drop of clearing solution (a mixture of 8 g chloral hydrate, 2 ml water, and 1 ml glycerol). Root explants were cleared after fixation in 25 mM sodium phosphate buffer (pH 7.0) that contained 2% formaldehyde and 1% glutaraldehyde, or without fixation in some cases. Cleared samples were observed under a light microscope equipped with Nomarski optics (BX50-DIC; Olympus).

Table 1. Chromosome mapping of *RID*, *RPD* and *RGD* loci

Locus	Chromosome	Flanking markers	Recombinant chromosomes/ examined*
RID1	I	g2395	1/24
		UFO	4/24
RID2	V	ILL2	2/328
		PLC1	3/328
RID3	III	F1P2-TGF	6/148
		ciw4	4/148
RID4	II	nga361	3/476
		m323	7/476
RID5	II	nga361	7/324
		ve017	4/324
RPD1	IV	g8300	2/42
		CAT2	1/42
RGD1	I	ciw1	2/106
		g4026	1/106
RGD2	III	nga162	5/42
		ArLIM15	1/42
RGD3	III	ciw4	2/42
		nga6	3/42

*Number of recombinant chromosomes/total number examined.

Results

Isolation of temperature-sensitive mutants defective in adventitious root formation

We screened mutagenized populations of *A. thaliana* for their capacity to form adventitious roots from hypocotyl explants and isolated 10 temperature-sensitive mutant lines. Temperature sensitivity was a recessive trait in all these mutants. Complementation tests (data not shown) and chromosome mapping (Table 1) indicated that the 10 mutant alleles represented nine loci.

Comparison of map positions and root phenotypes of our temperature-sensitive mutants with those of mutants known to be deficient in root development suggested possible allelism between *rgd3* and *scr* (Scheres et al., 1995; Di Laurenzio et al., 1996). These two mutants appeared to share a part of characters such as defects in the maintenance of root meristematic activity (Sabatini et al., 2003) (see below for the *rgd3* phenotype). However, adventitious root formation and root growth of the F_1 progenies derived from a cross between *rgd3* and *scr-2* were normal at 28°C, and sequence analysis detected no mutations in the *SCR* gene, including its promoter region, of the *rgd3* mutant (data not shown). Accordingly, we concluded that *rgd3* is not allelic to *scr*.

The isolated mutants showed various genetic lesions in adventitious root formation at the restrictive temperature (Figs 1, 2). They could be roughly categorized into three types: (1) defects in the initial stage and/or pre-morphogenic stage of root formation, (2) defects in the development of primordia, and (3)

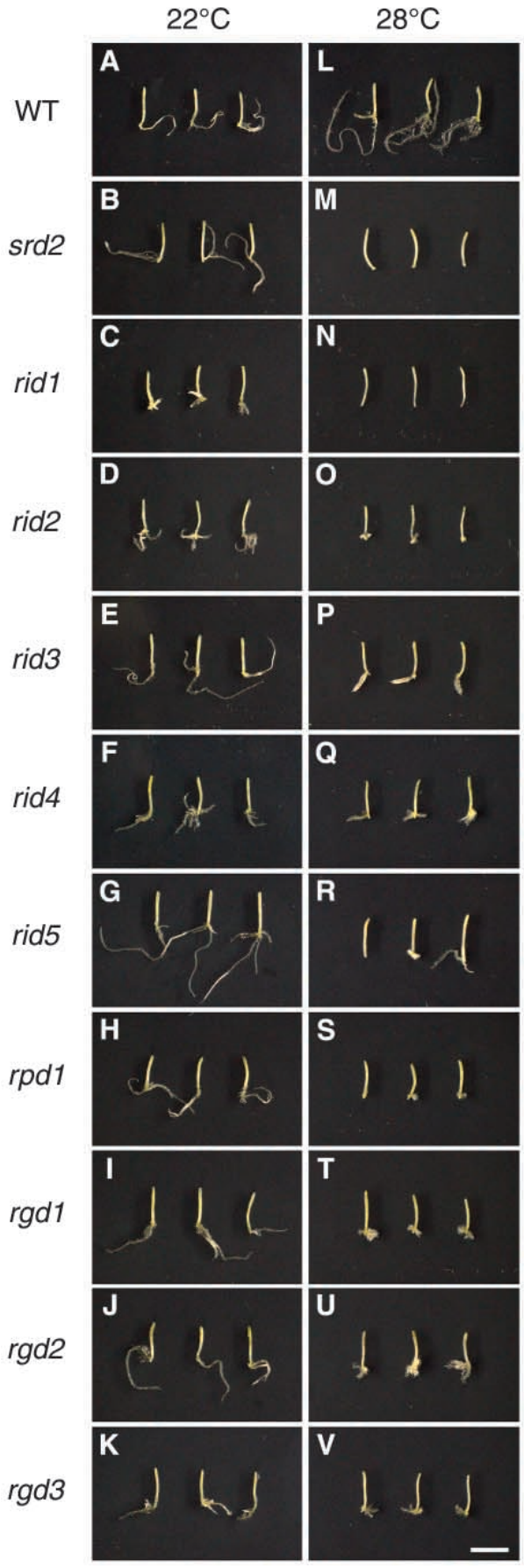


Fig. 1. Adventitious root induction from hypocotyl explants cultured on RIM for 16 days at 22°C (A-K) or at 28°C (L-V). (A,L) Wild type, (B,M) *srd2*, (C,N) *rid1*, (D,O) *rid2*, (E,P) *rid3*, (F,Q) *rid4*, (G,R) *rid5*, (H,S) *rpd1*, (I,T) *rgd1*, (J,U) *rgd2*, (K,V) *rgd3*. Scale bar: 5 mm.

defects in the growth of roots after the establishment of the root apical meristem. According to this classification, the mutants were designated *rid1* to *5* (*root initiation defective 1* to *5*), *rpd1* (*root primordium defective 1*), and *rgd1* to *3* (*root growth defective 1* to *3*). Of these, only *rpd1* had two alleles (*rpd1-1* and *rpd1-2*). *rpd1-1* showed more pronounced aberrancies than *rpd1-2*, and was used for subsequent analyses.

On closer observation, the rooting phenotypes of the mutants were as follows. The *rid1* hypocotyl did not show any signs of cell proliferation at 28°C. At 22°C, the *rid1* mutation was still effective and delayed root initiation by up to 10 days relative to the wild type (Fig. 2). The *rid2*, *rid3*, *rid4* and *rpd1* mutants, when observed at 6 days on RIM, had formed only young root primordia at 28°C, whereas at 22°C, they had completed adventitious root formation at this time (Fig. 2). After a longer period (16 days) of culture on RIM at 28°C, the *rid2*, *rid3* and *rid4* mutants eventually established adventitious roots although they were deformed to some extent (Fig. 1). Therefore, the main defects of *rid2*, *rid3* and *rid4* were considered to lie in the initial stages prior to the development of root primordia, and to specifically delay the timing of root primordium formation, with limited influence on the later stages. In contrast to the *rid* mutants, the majority of *rpd1* root primordia remained undeveloped after 16 days incubation at 28°C. This suggests that the *rpd1* mutation primarily affected the development of the root primordia.

The rooting phenotype of the *rid5* mutant at the restrictive temperature was highly variable among explants (Fig. 1). Some explants cultured at 28°C could form adventitious roots that were almost normal in morphology and growth, whereas others gave no indication of cell division that could lead to the formation of root primordia. Thus, the *rid5* mutation reduced

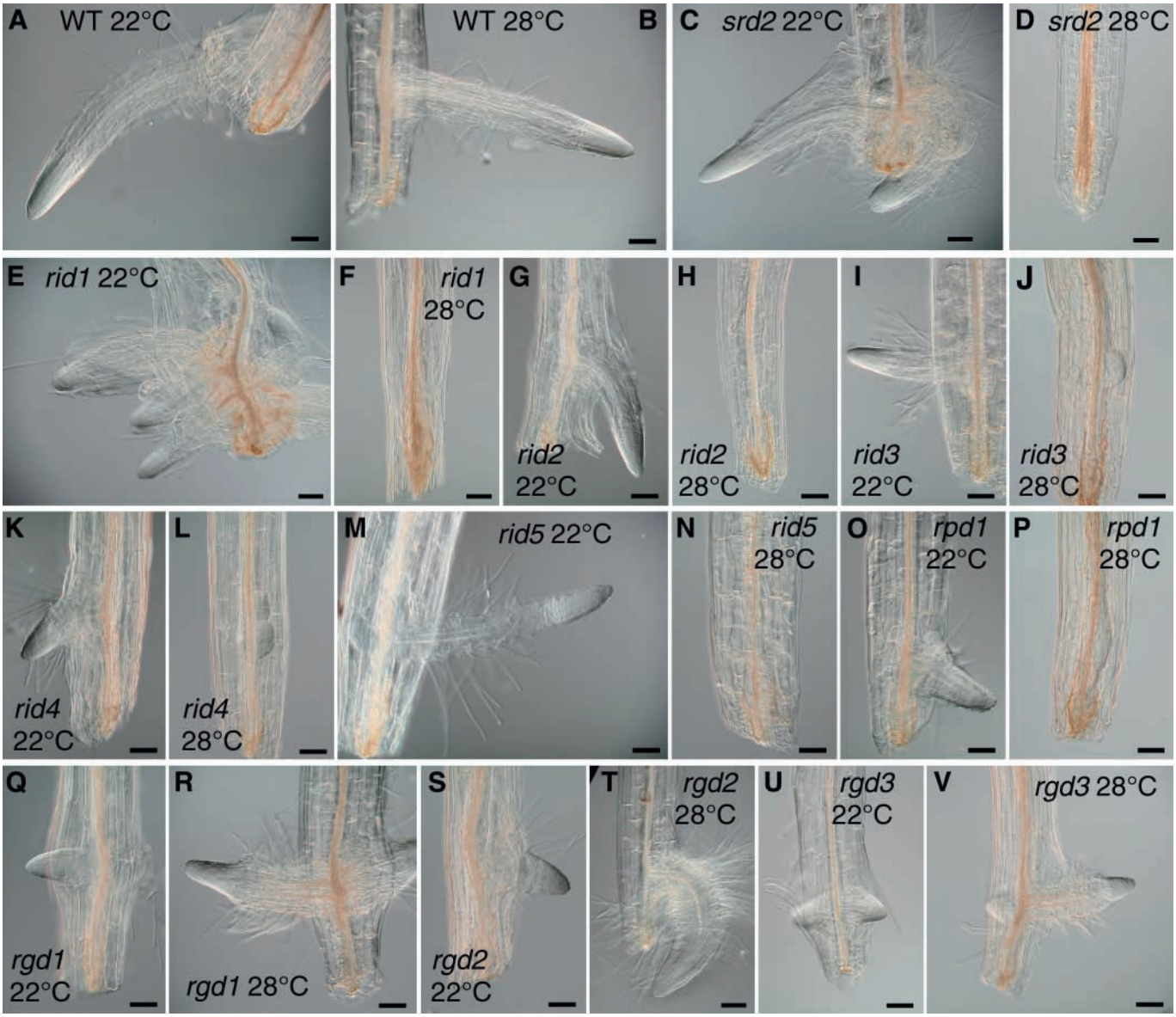


Fig. 2. Early stage of adventitious root formation. Hypocotyl explants were cultured on RIM at 22°C (A,C,E,G,I,K,M,O,Q,S,U) or at 28°C (B,D,F,H,J,L,N,P,R,T,V). (A,B) Wild type at day 6, (C,D) *srd2* at day 11, (E,F) *rid1* at day 16, (G,H) *rid2* at day 6, (I,J) *rid3* at day 6, (K,L) *rid4* at day 6, (M,N) *rid5* at day 6, (O,P) *rpd1* at day 6, (Q,R) *rgd1* at day 6, (S,T) *rgd2* at day 6, (U,V) *rgd3* at day 6. Scale bar: 100 μm.

the frequency of root initiation at 28°C without affecting the later stages of root formation.

The *rgd* mutants showed neither an apparent delay nor a reduction in frequency in adventitious root formation even at 28°C (Fig. 2). In these mutants, however, the subsequent growth of adventitious roots was strongly inhibited at 28°C (Fig. 1).

The *srd2* mutant, which was originally isolated as temperature-sensitive for shoot redifferentiation (Yasutani et al., 1994), exhibited a very similar phenotype to that of *rid1* with respect to adventitious root formation (Figs 1, 2). Lack of cell proliferation at 28°C in *srd2* is consistent with its previously reported defects in the process of cell proliferation during the dedifferentiation of hypocotyl explants (Ozawa et al., 1998).

Auxin dependency of adventitious root formation in the *rid5* mutant

As described in the previous paragraph, the *rid5* mutation specifically influenced the initiation of adventitious roots at 28°C. In tissue culture, the rate of adventitious rooting generally depends on the concentration of exogenous auxin, which is required for dedifferentiation and subsequent root initiation. Taking this into consideration, we examined the effects of IBA on the rooting rate of wild type and *rid5* (Fig. 3). In our standard RIM, the IBA concentration is set to 0.5 mg/l. The rooting rate of the wild type was gradually reduced as IBA concentrations were lowered. In the wild type, the IBA concentration required for maximal rooting was a little higher at 22°C than at 28°C. Compared with the wild type, the *rid5* mutant showed clear temperature sensitivity in auxin-dependent rooting. The IBA-response curve for the rooting rate of *rid5* was similar to that of the wild type at 22°C, but was shifted greatly at 28°C. These results imply that the *rid5* mutation is detrimental to auxin signaling that induces root formation. Similar experiments using the other mutants did not detect such a shift in the auxin-response curve with an increase in temperature (data not shown).

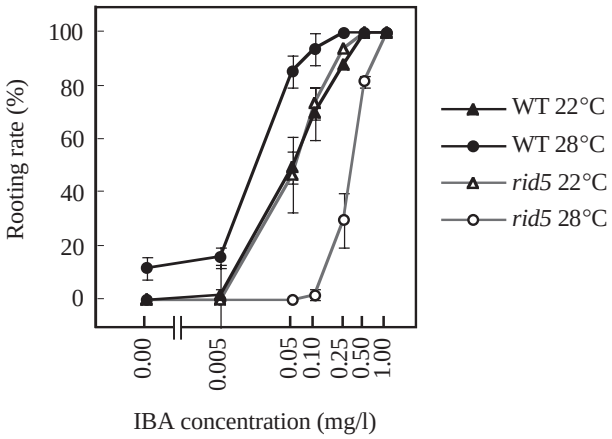


Fig. 3. Effect of auxin concentration on the rooting rate of the *rid5* mutant. Hypocotyl explants of the wild type and *rid5* were cultured on RIM containing various concentrations of IBA at either 22°C or 28°C. Each symbol represents an average value of data obtained from two independent experiments, in which 25 hypocotyls were used for every point. Vertical lines indicate standard deviation.

Effects of the mutations on lateral root formation

Root explants of the mutants were cultured on RIM at 22°C and 28°C to induce lateral root formation, and were observed after 16 days in culture. *srd2*, *rid1*, *rid2* and *rid4* exhibited aberrant lateral roots morphology under restrictive conditions (Fig. 4). The *rid1* mutant showed the most severe phenotype, with lateral root primordia developing into massive structures. In the root explants of the *rid4* mutant, RIM culture at 28°C induced fasciated lateral roots at a high frequency (data not shown).

Effects of the mutations on cell proliferation

To examine the effects of these mutations on cell proliferation independently of tissue organization, we tested the temperature sensitivity of callus formation in these mutants by culturing hypocotyl and root explants on CIM for 24 days at either 22°C or 28°C.

Callus formation was completely blocked in the hypocotyls of the *srd2* and *rid1* mutants at 28°C (Fig. 5). Root explants of

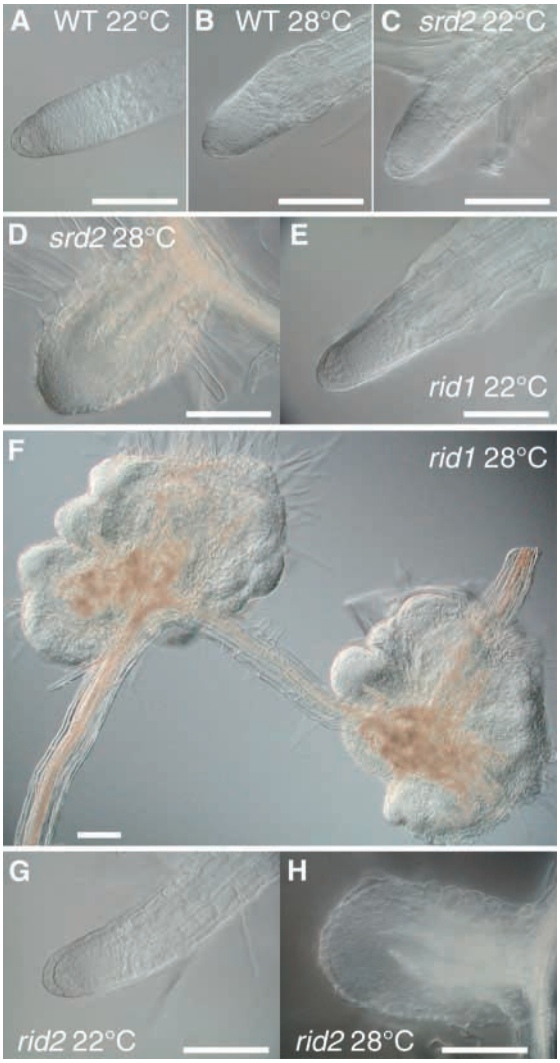
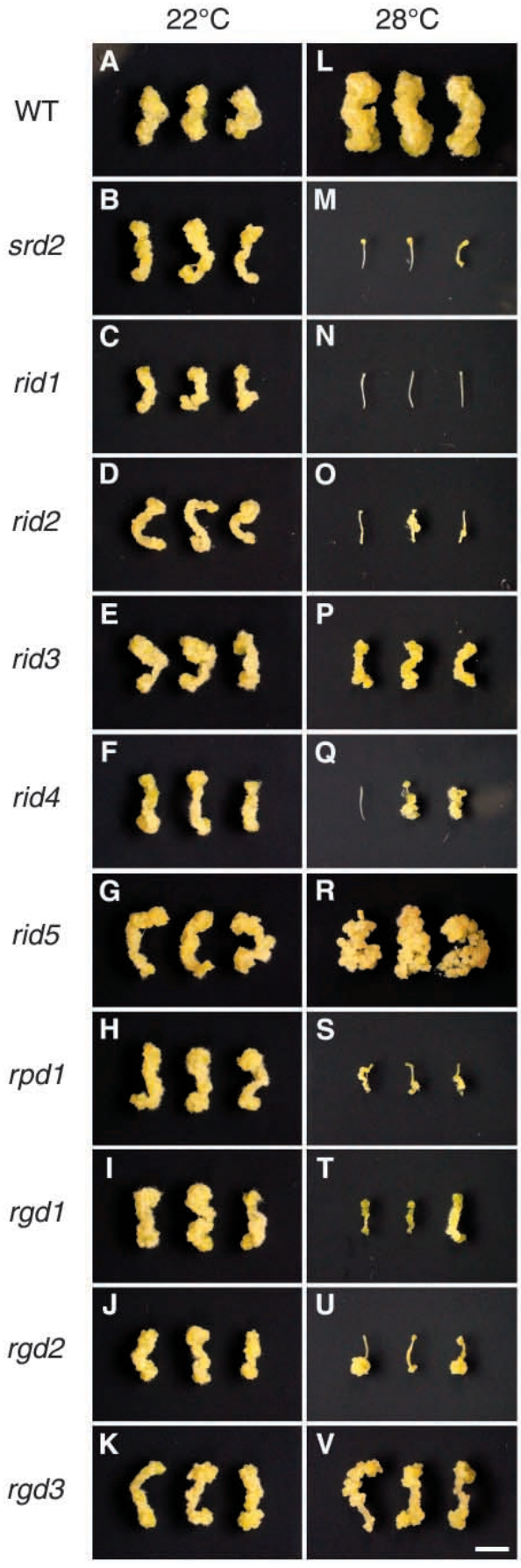


Fig. 4. Lateral root formation. Primary root explants were cultured on RIM for 16 days at 22°C (A,C,E,G) or 28°C (B,D,F,H). (A, B) Wild type, (C,D) *srd2*, (E,F) *rid1*, (G,H) *rid2*. Scale bar: 100 μm.



both mutants, however, formed calli at 28°C that were similar to those formed at 22°C (Fig. 6). The *srd2* and *rid1* mutations thus interfere with cell proliferation from hypocotyl explants, but not with cell proliferation from root explants.

Callus formation from hypocotyl explants of the *rid2* mutant was highly temperature-sensitive and severely inhibited at 28°C (Fig. 5). Root explants of *rid2* could form calli at 28°C, but their appearance was unusual (Fig. 6Z). In this case, calli formed at intervals where no cell proliferation was observed. This is quite different from normal callus formation, which takes place all over the explant. The discontinuous callusing phenotype of the *rid2* mutant may be attributable to a deficiency in the initiation of cell proliferation from root pericycle cells that are competent but quiescent (see Discussion for details).

Callus formation by the *rid3* mutant appeared to be temperature-insensitive both in hypocotyl explants and in root explants after 24 days in culture on CIM (Figs 5, 6). When observed on day 4 in culture, however, callus formed at 28°C from hypocotyl explants was markedly smaller than that formed at 22°C (data not shown), suggesting leaky defects of the *rid3* mutation in the initial stage of cell proliferation or in the dedifferentiation stage that precedes cell proliferation.

Callus formation in both hypocotyl and root explants of the *rid4* mutant showed temperature sensitivity (Figs 5, 6). At 28°C, variable suppression of cell proliferation was observed among explants. In some explants, cell proliferation was strongly inhibited, whereas it was only partially inhibited in others. Such phenotypes could be explained by assuming that the defect in the *rid4* mutant during callus formation is restricted to the initial step of cell proliferation and that the *rid4* explants are able to develop calli once they pass through this step by chance.

In the cases of the *rpd1*, *rgd1* and *rgd2* mutants, both hypocotyl and root explants could initiate callus formation at 28°C but there were defects in callus development or growth (Figs 5, 6). The *rid5* and *rgd3* mutants did not show apparent temperature sensitivity in callus formation from hypocotyl and root explants (Figs 5, 6).

Seedling phenotypes of the mutants

To assess the effect of the mutations on other developmental processes, the whole-plant phenotypes of seedlings grown on vertically placed GMA at 22°C or 28°C for 12 days were observed (Fig. 7). At 22°C, the mutant seedlings looked almost normal except *rid1* and *rid4*. The primary roots of these two mutants were short relative to the wild type and the other mutants. At 28°C, the primary roots of all the mutants were much shorter than those formed at 22°C (Fig. 7A,B). The shoot phenotypes of the mutants at 28°C varied somewhat between repeated experiments. Formation of linear true leaves, lacking leaf lamina in severe cases, was reproducibly and frequently observed in the *rid3* seedlings (Fig. 7L,M).

rid5 mutant seedlings exhibited peculiar temperature-dependent abnormalities. Their primary root grew leftward at

Fig. 5. Callus formation from hypocotyl explants. Hypocotyl explants were cultured on CIM for 24 days at 22°C (A-K) or at 28°C (L-V). (A,L) Wild type, (B,M) *srd2*, (C,N) *rid1*, (D,O) *rid2*, (E,P) *rid3*, (F,Q) *rid4*, (G,R) *rid5*, (H,S) *rpd1*, (I,T) *rgd1*, (J,U) *rgd2*, (K,V) *rgd3*. Scale bar: 5 mm.

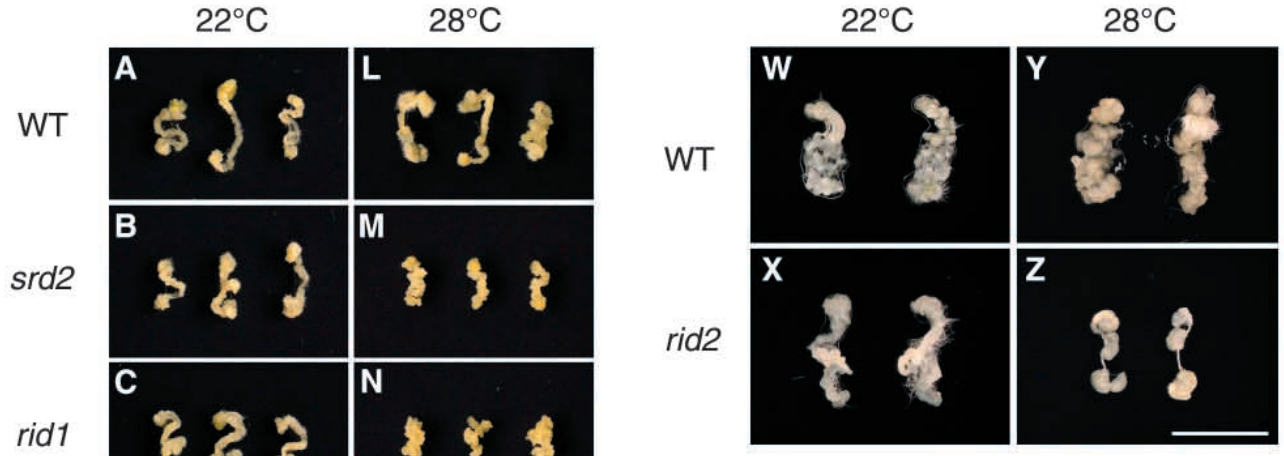


Fig. 6. Callus formation from root explants. Root explants were cultured on CIM for 24 days at 22°C (A–K,W,X) or at 28°C (L-V,Y,Z). (A,L) Wild type, (B,M) *srd2*, (C,N) *rid1*, (D,O) *rid2*, (E,P) *rid3*, (F,Q) *rid4*, (G,R) *rid5*, (H,S) *rpd1*, (I,T) *rgd1*, (J,U) *rgd2*, (K,V) *rgd3*. (W-Z) Higher magnification of the wild type (W,Y) and *rid2* (X,Z). Scale bar: 5 mm.

28°C (Fig. 7B, inset). Left-handed helical growth was induced at 28°C in epidermal cell files of the hypocotyls, as well as the primary roots (Fig. 7F,J). Moreover, the root hairs of *rid5* often branched and the branching was more pronounced at the higher temperature (Fig. 7J).

Allelism between *rid5* and *mor1*

Helical growth similar to those observed in the *rid5* seedling at 28°C have been reported for several microtubule-related mutants and wild-type plants treated with microtubule-depolymerizing or -stabilizing drugs (Rutherford and Masson, 1996; Marinelli et al., 1997; Furutani et al., 2000; Whittington et al., 2001; Thitamadee et al., 2002; Sedbrook et al., 2002). This similarity suggested a possible relationship between *rid5* and the microtubule system. Since *rid5* was mapped near the *MOR1/GEM1* gene encoding a microtubule-associated protein (Whittington et al., 2001; Twell et al., 2002), we tested whether *rid5* is an allele of this gene. F_1 seedlings produced by crossing *rid5* and *mor1-1* all exhibited left-handed helical growth at 28°C (data not shown). Adventitious root formation from the F_1 hypocotyls on RIM containing 0.25 mg/l IBA at 28°C was very poor as compared to the wild-type explants (data not shown). These results confirmed the allelic relationship of *rid5* to *mor1-1*. Sequence analysis of the *MOR1*/*GEM1* gene of *rid5* identified a mutation of TGC to TAC, which causes an amino acid substitution of Tyr for Cys at residue 96 in the gene product.

Discussion

We isolated nine novel temperature-sensitive mutants of *A. thaliana* that are defective in adventitious root formation. On the basis of their phenotypes at the restrictive temperature, we roughly classified these mutants into three groups: (1) the *rid* mutants, defective in the initial stage or the pre-morphogenic stage of adventitious root formation; (2) the *rpd1* mutant, arrests during the development of root primordia, and (3) the

rgd mutants, which can establish adventitious roots but fail to maintain their growth. We note that the mutations, except for *rgd3*, seem to interfere not with tissue organization per se but with mechanisms involved in cell proliferation-related events during adventitious root formation.

Reinitiation of cell proliferation

Adventitious root formation from hypocotyl segments starts with the recommencement of cell division in quiescent hypocotyls. Earlier observations on the *srd2* mutant revealed that, at the restrictive temperature, hypocotyl explants did not reinitiate cell proliferation, whereas root explants could reinitiate cell proliferation and form calli (Ozawa et al., 1998). This suggested that 'reinitiation of cell proliferation' from hypocotyl explants entails two distinct stages: the acquisition of competence for cell proliferation and the resumption of the cell cycle. In our view, the non-dividing cells of root explants are equivalent to the cells of hypocotyl explants that have acquired the competence for cell proliferation. Among the novel series of mutants reported here, the *rid1* phenotype was very similar to that of *srd2*. Hypocotyl explants of *rid1* could not reinitiate cell proliferation at the restrictive temperature but their root explants could (Figs 5, 6). This feature suggests that both the *SRD2* and *RID1* genes are involved in the same process of the acquisition of competence for cell proliferation during dedifferentiation of hypocotyl cells, and that their functions are not required for the resumption and subsequent progression of the cell cycle.

The *RID2* gene may play some essential roles in the second stage of the reinitiation of cell proliferation, i.e., the resumption of the cell cycle in competent but quiescent cells. The root explants of *rid2* formed calli at intervals under the restrictive conditions (Fig. 6Z). One possible explanation for this phenotype is that the *rid2* mutation specifically inhibits the re-entry of non-dividing cells into the cell cycle but does not affect callus formation by dividing cells, such as those of the lateral root primordia present at intervals in the root segments. This hypothesis can also account for the temperature-sensitive

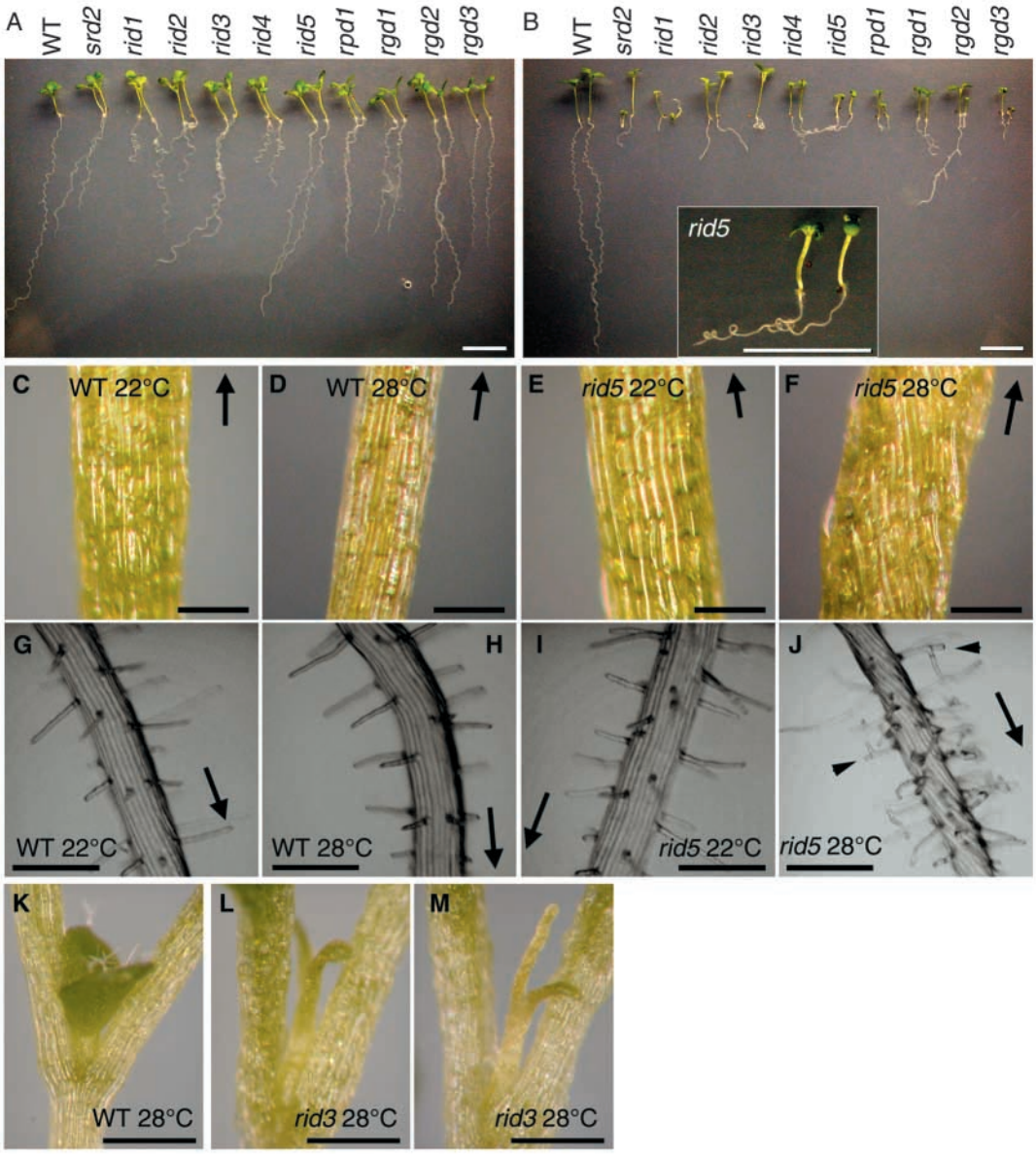


Fig. 7. Phenotypes of seedlings that were grown for 12 days on vertical GMA plates. (A) Seedlings grown at 22°C. (B) Seedlings grown at 28°C. (C-J) Epidermis of the hypocotyl (C-F) and primary root (G-J) of the wild type and *rid5*. (C,G) Wild type at 22°C, and (D,H) at 28°C. (E,I) *rid5* at 22°C. (F,J) *rid5* at 28°C. Arrows indicate the direction of growth. Epidermal cell files of the *rid5* seedling showed left-handed helical growth at 28°C. Arrowheads in (J) indicate branched root hairs. (K-M) True leaves of the wild type (K) and *rid3* (L,M) at 28°C. Bars in (A,B), 1 cm. Scale bars: (C-J) 200 μm; (K,L,M), 500 μm.

initiation of callus and adventitious roots from hypocotyl explants of the *rid2* mutant. Further characterization of the *rid2* mutant is in progress to test this hypothesis.

Auxin response and microtubules

The threshold level of auxin required for adventitious root initiation became higher in the *rid5* mutant at the restrictive temperature (Fig. 3). This finding suggests that the *RID5* gene functions somewhere, directly or indirectly, in the auxin signaling pathway that stimulates cells to proliferate to form adventitious roots. The auxin signaling machinery may require *RID5* function, either for the acquisition of competence or for resumption of the cell cycle, or for both.

In the *rid5* seedlings cultured at the restrictive temperature, the primary roots grew leftward, and the epidermal cell files of the hypocotyls and primary roots formed left-handed helices (Fig. 7). Similar phenotypes have been reported for several microtubule-related mutants (Furutani et al., 2000; Whittington et al., 2001; Thitamadee et al., 2002). Cortical microtubule arrays of *spr* mutants, which display right-handed helical growth (their primary roots grow rightward), are skewed into left-handed obliques (Furutani et al., 2000). Recently, mutants with left-handed helical growth, *lefty1* and *lefty2*, were found to have mutations in α-tubulin genes (Thitamadee et al., 2002). The *lefty* mutant tubulins were incorporated into microtubule polymers, producing right-handed cortical arrays. These studies indicate that the orientation of the cortical microtubule arrays are critical in the handedness of helical growth. In light of these findings, the temperature-sensitive helical growth of the *rid5* mutant very probably results from some alterations in cortical microtubules. Root-hair branching of the *rid5* mutant may also be related to cortical microtubules.

Temperature-sensitive mutants, *mor1-1* and *mor1-2*, of a MAP215 family microtubule-associated protein, MOR1/GEM1, have been described in a recent report (Whittington et al., 2001). They exhibited aberrant cortical microtubule organization and left-handed helical growth at the restrictive temperature. A complementation test and sequence analysis showed that *rid5* is a new mutant allele of the *MOR1/GEM1* gene.

So far, four mutant alleles have been reported for the *MOR1/GEM1* locus: *mor1-1*, *mor1-2*, *gem1-1* and *gem1-2*. The *mor1-1* and *mor1-2* mutations, both of which result in a single amino acid substitutions of the amino-terminal HEAT repeat of the MOR1/GEM1 protein, affect cortical microtubule arrays only in interphase (Whittington et al., 2001). The *gem1-1* and *gem1-2* mutations, both of which result in a carboxy-terminal truncation, cause a severe defect in cytokinesis and consequently lethality (Twell et al., 2002).

Our new mutant allele, *rid5*, results in an amino acid substitution at the amino-terminal region outside of and adjacent to the HEAT repeat. Hence it is possible that the *rid5* mutation specifically interferes with the interphase organization of cortical microtubules like the *mor1* mutations and unlike the *gem1* mutations. This view is consistent with the *rid5* phenotypes, particularly in the left-handed helical growth and the negligible effect on cell proliferation at the restrictive temperature.

Phytohormones, including auxin, function in modulating cyclic changes in the orientations of microtubule arrays between transverse and longitudinal via an oblique orientation (Shibaoka, 1994; Takesue and Shibaoka, 1999). Interestingly, Marinelli et al. (Marinelli et al., 1997) reported a digenic mutant that showed right-handed helical growth and enhanced sensitivity to auxin with respect to its inhibitory effect on primary root elongation. Taken together with the reduced responsiveness to auxin and presumed alterations in the cortical microtubules of the *rid5* mutant, we surmise that the auxin signaling pathway in adventitious root formation is mediated by modulation of the orientation of the microtubule arrays, which involves the *MOR1*/*GEM1* function.

Root primordium development

In the course of adventitious root formation, after reinitiation of cell proliferation cells undergo several rounds of division to develop the root primordia in which the apical meristem subsequently arises. At the restrictive temperature, adventitious root primordia of the *rpd1* mutant were arrested during development, prior to the formation of recognizable apical meristems (Fig. 2). This phenotype suggests the involvement of the *RPD1* gene in the construction of the root apical meristem. However, the *rpd1* mutation also inhibits callus development at early stages. Thus, the *RPD1* gene may participate in the maintenance of the cell proliferation required for both root primordium and callus development.

Establishment of the root apical meristem

When lateral roots were induced from root explants at the restrictive temperature, the *srd2*, *rid1* and *rid2* mutants generated deformed laterals, indicating defects in establishing the root apical meristem (Fig. 4). The acquisition of competence for cell proliferation, which was affected by the *srd2* and *rid1* mutations during adventitious root formation and callus formation from hypocotyl explants, is not required for lateral root formation from root explants that are initially competent. Therefore, the failure to establish apical meristems in lateral root primordia in the *srd2* and *rid1* mutants may suggest a direct involvement of the *SRD2* and *RID1* functions. In the case of the *rid2* mutant, misorganization of the root apical meristems might be a secondary consequence of its leaky deficiency in reinitiation of cell proliferation from the root pericycle.

Root growth

Both adventitious root growth and callus growth were inhibited in the *rgd1* and *rgd2* mutants at the restrictive temperature. The *rgd3* mutant, however, was defective in adventitious root growth but not in callus growth (Figs 1, 5, 6). These results may indicate that *RGD1* and *RGD2* function in general aspects of root and callus growth, and the *RGD3* function is required for a mechanism specific to root growth, e.g., the maintenance of the root apical meristem. Alternatively, the *rgd3* mutant allele may be weak and therefore not showing the full gene function.

Concluding remarks

We report on a series of temperature-sensitive mutants that affect adventitious root formation at different stages (Fig. 8).

Hypocotyl explants of *Arabidopsis thaliana* are initially incompetent for cell proliferation. Upon culture on RIM, they become competent. The *srd2* and *rid1* mutants are defective at this stage. Hypocotyl explants that have acquired competence,

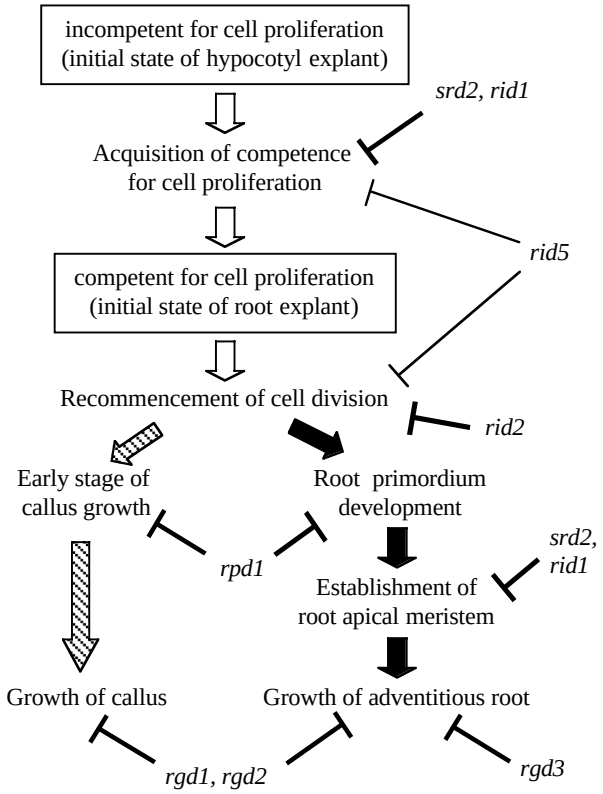


Fig. 8. A hypothetical scheme for adventitious root formation.

which are comparable to root explants, recommence cell division. This step is defective in *rid2*. The *rid5* mutation affects adventitious root formation somewhere in the signaling pathway from auxin to the reinitiation of cell proliferation probably via alterations of cortical microtubule arrays. Proliferating cells undergo cycles of cell division to form the root primordia. The *rpd1* mutant is unable to maintain cell proliferation of the root primordia. The root apical meristem is subsequently established. At this stage, the *srd2* and *rid1* mutants are again affected. The growth of adventitious roots requires the functions of at least three genes, *RGD1*, *RGD2* and *RGD3*. We include the process of callus formation in this scheme to address the effects of mutations in the *RPD1*, *RGD1* and *RGD2* genes in callus formation.

Owing to the temperature-sensitive nature of our series of mutants, they are not likely to represent full loss-of-function alleles. Therefore we cannot assess whether the corresponding genes are required only at the steps affected in the mutants, or whether they serve a more general role. Further analysis of these mutants should nevertheless identify elementary components of the mechanisms underlying adventitious root formation.

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