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REVIEW ARTICLE

Molecular genetic analysis of organogenesis in vitro with temperature-sensitive mutants

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Abstract About 20 years ago, molecular genetic analysis of tissue culture responses with temperature-sensitive mutants of Arabidopsis was launched in the laboratory of Prof. Atsushi Komamine as a new approach to mechanisms of plant organogenesis in vitro. In this and later studies, many interesting mutants were isolated and characterized, which led to the identification of unexpected genes as important players involved in organogenesis in vitro. The present article provides an overview of the outcomes from these molecular genetic studies derived from Komamine's work.

Keywords Arabidopsis · Dedifferentiation · Organogenesis in vitro · Root formation · Shoot regeneration · Temperature-sensitive mutant

Introduction

Since pioneering work by Skoog and Miller (1957), which demonstrated chemical induction of plant organogenesis by exogenous application of cytokinin and auxin in tissue culture, organogenesis in vitro has fascinated plant physiologists and provided a basis for various manipulations in plant biotechnology. However, the molecular mechanisms underlying organogenesis in vitro were substantially inaccessible for a long time because of the lack of efficient means to study them. In the 1980s, Arabidopsis (specifically *Arabidopsis thaliana*) emerged as a new model plant particularly suitable for molecular genetic studies. In the following decade, various experimental procedures, including tissue culture protocols, were developed for Arabidopsis research. This situation inspired us to begin molecular genetic analysis of in vitro organogenesis with Arabidopsis as one of the main research areas in Prof. Komamine's laboratory. In the present article, I review molecular genetic studies on plant organogenesis in vitro with a focus on our own findings.

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Tissue culture of Arabidopsis

To develop methods of *Agrobacterium tumefaciens*-mediated transformation of Arabidopsis, various factors affecting callus formation and shoot regeneration were examined in tissue culture (Akama et al. 1992; Valvekens et al. 1988). Based on these reports, we further optimized tissue culture conditions and set a standard protocol of the induction of shoot regeneration for genetic analysis, as follows (Tamaki et al. 2009; Yasutani et al. 1994). Hypocotyl or root segments are excised from seedlings and first cultured on callus-inducing medium (CIM), which is a B5 medium that contains 0.5 mg/L 2,4-D and 0.1 mg/L kinetin as phytohormones, under constant dim light for 4–4.5 days. During culture on CIM, cell proliferation is reactivated in the stele to initiate callus formation. The explants are then cultured on shoot-inducing medium (SIM), a B5 medium that contains 0.15 mg/L IAA and 0.5 mg/L 2-isopentenyladenine (2iP) or 0.04 mg/L IAA and 1.5 mg/L 2iP, under constant dim light. After transfer onto SIM, shoot apical meristems (SAMs) of adventitious buds are induced on the callus surface, which eventually regenerate shoots.

Christianson and Warnick (1983) characterized shoot regeneration from leaf explants using *Convolvulus arvensis* in tissue-transfer experiments and divided the process of

shoot organogenesis into three phases, based on the temporal requirement for a specific balance of phytohormones in the control of organogenesis. The first phase corresponds to 'dedifferentiation'. In this phase, explants acquire 'competence', which is defined as the ability to readily respond to phytohormonal signals of induction of organogenesis. The competent explants are destined and determined for specific organ development by inductive signals through the second phase. Then the morphogenesis of organs proceeds independently of exogenously supplied phytohormones during the third phase. This concept can be applied to shoot regeneration of Arabidopsis. In Arabidopsis, pre-culture on CIM is indispensable for effective induction of shoot regeneration, and explants can therefore be assumed to acquire competence for shoot regeneration during the CIM culture. Thus, the two-step procedure of Arabidopsis tissue culture is advantageous in the dissection of organogenesis in vitro because it enables the least ambiguous distinction of the 'competence acquisition/dedifferentiation' phase from the subsequent phases.

When hypocotyl and root explants cultured on CIM are transferred onto root-inducing medium (RIM), a B5 medium that contains 0.5 mg/L indole-3-butyric acid, many roots are regenerated over the entire explants. If hypocotyl segments are directly cultured on RIM without pre-culture on CIM, two to three adventitious roots are induced only in the region close to the basal end. These protocols were employed in tissue culture experiments for studying root organogenesis de novo (Konishi and Sugiyama 2003; Sugiyama 2003). Additionally, we developed a tissue culture system specifically suited for investigation of lateral root formation (Ohtani et al. 2010). In this system, which uses explants from very young (4-day-old) seedlings, lateral roots newly and rapidly form with good synchrony on primary roots upon culture on RIM.

Isolation of temperature-sensitive mutants

In our project, we aimed to harness the power of forward genetics to identify genes with important functions for elementary developmental processes involved in organogenesis in vitro. As a first step to achieving this purpose, we attempted to isolate mutants that are conditionally defective for organogenesis in vitro only at high temperatures; these conditional mutants are useful in genetic dissection of organogenesis. They were expected to be very informative about the critical timing of functions of the mutated genes during organogenesis and pleiotropic functions shared by different physiological phenomena. In addition, conditional mutations allow us to study essential genes whose total inactivation is lethal.

For isolation of temperature-sensitive mutants, we screened an ethylmethane sulfonate-mutagenized population of Arabidopsis with shoot regeneration, root regeneration, or adventitious root formation as an index phenotype (Konishi and Sugiyama 2003; Sugiyama 2003; Yasutani et al. 1994). For the screenings, explants were prepared from donor plants that had been grown at 22 °C (permissive temperature), and tissue culture of the explants was performed at 22 and 27–28 °C (restrictive temperature) to compare organogenic responses between temperatures. Mutant candidates that showed temperature-dependent defects were selected and genetically purified by repeated back-crosses with the wild-type. Finally, we established more than 10 lines of temperature-sensitive mutants (Table 1).

Control of cell proliferation competence

Taking advantage of the temperature-sensitive nature of the isolated mutants, we characterized them by examining effects of temporal exposure to the restrictive temperature on tissue culture responses. As a first example here, I introduce the case of the *srd2* mutant. The phenotypic analysis of *srd2* divided the dedifferentiation phase into two subphases, acquisition of competence for cell proliferation and acquisition of competence for shoot organogenesis, and later led to molecular insights into the control of cell proliferation competence.

In the *srd2* mutant, shoot regeneration from root explants was severely inhibited by a restrictive temperature given only after transfer onto SIM; shoot regeneration from hypocotyl explants, however, was highly sensitive to a restrictive temperature given during either CIM culture or SIM culture (Ozawa et al. 1998; Fig. 1a). Although CIM culture of root explants of *srd2* at the restrictive temperature induced cell proliferation in the stele, which developed into calluses, such proliferation was not detected in hypocotyl explants of this mutant cultured under the same condition (Ozawa et al. 1998; Fig. 1b). These phenotypes indicate that hypocotyl and root explants differ in the requirement of reactivation of cell division for *SRD2* function in the dedifferentiation phase. In other words, hypocotyl dedifferentiation involves a hypocotyl-specific, *SRD2*-dependent subphase prior to the reactivation of cell proliferation, in addition to the *SRD2*-independent subphase common to both hypocotyl and root explants during which cells become competent for shoot organogenesis. This difference between tissues can be further explained by assuming that hypocotyl stele cells, which are incompetent for cell proliferation, acquire proliferation competence depending on *SRD2* function during CIM culture. Root stele cells, however, maintain a high proliferation competence and do not need the *SRD2* function prior to re-entry into the cell cycle (Ozawa et al. 1998).

Table 1 List of Arabidopsis mutants isolated as being temperature-sensitive for organogenesis in vitro

Mutant	Organogenesis system used for screening	Notable phenotypic feature in tissue culture	Responsible gene	Gene function	References
shoot redifferentiation defective 1 (*srd1*)	Shoot regeneration from root explants (culture on CIM and then on SIM)	Defective in shoot bud development	–[a]	–	Yasutani et al. (1994), Ozawa et al. (1998)
shoot redifferentiation defective 2 (*srd2*)	Shoot regeneration from root explants (culture on CIM and then on SIM)	Defective in callus initiation from hypocotyl explants, shoot bud development, and root morphogenesis but not much affected in callus initiation from root explants and callus growth	At1g28560	Transcription of snRNAs	Yasutani et al. (1994), Ozawa et al. (1998), Huang et al. (2003), Ohtani and Sugiyama (2005), Ohtani et al. (2008, 2010)
shoot redifferentiation defective 3 (*srd3*)[b]	Shoot regeneration from root explants (culture on CIM and then on SIM)	Defective in the acquisition of competence for shoot organogenesis	–	–	Yasutani et al. (1994), Ozawa et al. (1998)
root initiation defective 1 (*rid1*)	Root formation from hypocotyl explants (culture on RIM)	Defective in callus initiation from hypocotyl explants, shoot bud development, and root morphogenesis but not much affected in callus initiation from root explants and callus growth	At1g26370	Pre-mRNA splicing	Konishi and Sugiyama (2003), Ohtani et al. (2013)
root initiation defective 2 (*rid2*)	Root formation from hypocotyl explants (culture on RIM)	Defective in callus initiation from hypocotyl explants but not much affected in callus initiation from root explants	At5g57280	Pre-rRNA processing	Konishi and Sugiyama (2003), Ohbayashi et al. (2011)
root initiation defective 3 (*rid3*)	Root formation from hypocotyl explants (culture on RIM)	Delayed in root initiation and defective in shoot bud development	At3g49180	–	Konishi and Sugiyama (2003), Tamaki et al. (2009)
root initiation defective 4 (*rid4*)	Root formation from hypocotyl explants (culture on RIM)	Defective in the size control of lateral root primordia, leading to root fasciation	–	–	Konishi and Sugiyama (2003), Otsuka and Sugiyama (2012)
root initiation defective 5 (*rid5*)	Root formation from hypocotyl explants (culture on RIM)	Reduced in the ability to initiate root formation in response to auxin	At2g35630	Microtubule organization	Konishi and Sugiyama (2003)
root primordium defective 1 (*rpd1*)	Root formation from hypocotyl explants (culture on RIM)	Defective in root primordium development and callus development	At4g33495	–	Konishi and Sugiyama (2003, 2006)
root growth defective 1 (*rgd1*)	Root formation from hypocotyl explants (culture on RIM)	Defective in root growth	–	–	Konishi and Sugiyama (2003)
root growth defective 2 (*rgd2*)	Root formation from hypocotyl explants (culture on RIM)	Defective in root growth	–	–	Konishi and Sugiyama (2003)
root growth defective 3 (*rgd3*)	Root formation from hypocotyl explants (culture on RIM)	Defective in root growth and shoot bud development but not much affected in callus growth	At3g54280	–	Konishi and Sugiyama (2003), Tamaki et al. (2009)

Table 1 continued

Mutant	Organogenesis system used for screening	Notable phenotypic feature in tissue culture	Responsible gene	Gene function	References
lignescens (*lig*)	Root formation from hypocotyl explants (culture on RIM)	Ectopically lignified	At5g15770	Acetylation of glucosamine-6-phosphate	Nozaki et al. (2012)
root redifferentiation defective 1 (*rrd1*)	Root formation from hypocotyl explants (culture on CIM and then on RIM)	Defective in the size control of lateral root primordia, leading to root fasciation	–	–	Sugiyama (2003), Otsuka and Sugiyama (2012)
root redifferentiation defective 2 (*rrd2*)	Root formation from hypocotyl explants (culture on CIM and then on RIM)	Defective in the size control of lateral root primordia, leading to root fasciation	–	–	Sugiyama (2003), Otsuka and Sugiyama (2012)
root redifferentiation defective 3 (*rrd3*)	Root formation from hypocotyl explants (culture on CIM and then on RIM)	Reduced in the ability to form roots[c]	–	–	Sugiyama (2003)
root redifferentiation defective 4 (*rrd4*)	Root formation from hypocotyl explants (culture on CIM and then on RIM)	Defective in callus formation from hypocotyl explants but not much affected in adventitious rooting of hypocotyl explants and callus formation from root explants	–	–	Sugiyama (2003)

[a] – indicates unidentifed, unknown, or unpublished genes and functions

[b] This mutant line was extinguished by the complete loss of germination ability during seed storage

[c] The temperature dependency of the defects was unclear in the established line after genetic purification

The tendency for the temperature sensitivity of callus initiation to be much higher in hypocotyl explants than in root explants was also observed in *rid1* and *rid2* as well as in *srd2* (Konishi and Sugiyama 2003; Ohbayashi et al. 2011). These findings suggested that cell proliferation competence is controlled by a particular set of genes including *SRD2*, *RID1*, and *RID2*. These genes were identified by positional cloning and tested for the functions presumed from their sequences, which demonstrated that the *SRD2*, *RID1*, and *RID2* genes participate in snRNA transcription, pre-mRNA splicing, and pre-rRNA processing, respectively (Ohbayashi et al. 2011; Ohtani and Sugiyama 2005; Ohtani et al. 2013). Because transcription of spliceosomal snRNAs, essential components of the spliceosome, requires SRD2 activity, *SRD2* is indirectly involved in pre-mRNA splicing, and, in this respect, the functions of *SRD2* and *RID1* overlap. Expression analysis of *SRD2*, *RID1*, and *RID2* showed that they are partly similar in expression patterns. All these genes are expressed more strongly in the root stele than in the hypocotyl stele, and expression in the hypocotyl stele is elevated rapidly upon CIM culture. The spatial and temporal changes in the expression of these genes are in good agreement with their hypothesized roles in the control of cell proliferation competence. Taken together with the molecular functions of these genes in pre-mRNA splicing and pre-rRNA processing, we can reasonably speculate that the abundance of spliceosomal and ribosomal factors serves to limit cell proliferation competence.

SAM assembly during shoot regeneration

In explants cultured on CIM, cells divide actively throughout developing calluses. When the explants are transferred from CIM to SIM for the induction of shoot regeneration, cell division soon ceases and is then reactivated locally in the callus surface to produce mounds of dense cells, in which adventitious bud SAMs are constructed. In relation to these morphological changes during shoot regeneration, expression patterns of known SAM genes such as *WUS*, *CUC1*, *CUC2*, and *STM* have been analyzed (Cary et al. 2002; Gordon et al. 2007), with results indicating that high expression of *CUC*s marks sites of formation of pre-meristematic cell mounds.

For detailed investigation of shoot regeneration and SAM gene expression, we chose *rid3* and *rgd3* from among the isolated temperature-sensitive mutants because their

shoot regeneration exhibited distinct sensitivity to a restrictive temperature introduced after transfer onto SIM (Tamaki et al. 2009). Microscopic observation of hypocotyl explants of these mutants cultured on SIM at 28 °C after CIM culture at 22 °C showed a very different morphology from wild-type explants cultured under the same conditions, and morphology was also quite different between the mutants. In the *rid3* explants, calluses were covered with irregularly large cell mounds, which did not give rise to adventitious buds. In contrast, the callus surface of the *rgd3* explants was smooth with no visible cell mounds. In association with these morphological defects, *CUC1* expression was enhanced and expanded in the *rid3* explants while it was suppressed to a very low level in the *rgd3* explants.

From the viewpoint of spatial organization of cell proliferation, the role of *RID3* is of particular interest. We found that expression of the *RID3* gene, which is uniformly high in calluses during CIM culture, is unevenly decreased from the callus surface after transfer onto SIM and that pre-meristematic cell mounds appear at sites where *RID3* expression is relatively low (Tamaki et al. 2009). Comparison of this expression pattern and the *CUC1* expression pattern, together with the effects of the *rid3* mutation on *CUC1* expression, strongly suggested that *RID3* plays a critical part in the control of *CUC1* expression by confining it to small spots, which then reactivates cell proliferation locally, resulting in the formation of properly organized, pre-meristematic cell mounds (Fig. 2). Further analysis of the molecular function of *RID3* would provide a key to approaching de novo patterning systems operating in shoot regeneration.

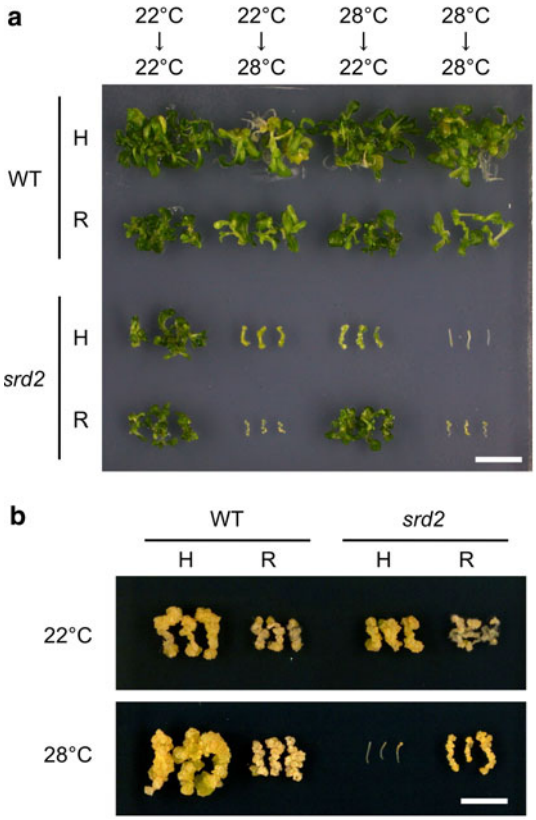

Fig. 1 Temperature sensitivity of tissue culture responses of *srd2*. Hypocotyl (*H*) and root (*R*) explants of the wild-type (*WT*) and *srd2* were cultured under various temperature regimes. **a** Explants were cultured on CIM for 4 days at 22 or 28 °C and then cultured on SIM for 24 more days at 22 or 28 °C. **b** Explants were cultured on CIM for 30 days at 22 or 28 °C. *Scale bars* 1 cm. Photographs provided by Dr. Misato Ohtani (RIKEN)

Root primordium development

Several of the isolated mutants displayed peculiar abnormalities in root morphogenesis at a restrictive temperature. These mutants were closely investigated for root primordium development by using the tissue culture system to induce lateral root formation semi-synchronously. In this system, exogenous auxin triggers the initiation of development of lateral root primordia, and root primordium development then proceeds as in planta (Ohtani et al. 2010). At the beginning, a few rounds of asymmetric, anticlinal cell division, called formative cell division, occur in the pericycle, which forms the base of a root primordium. Then, periclinal cell divisions start to increase cell layers of the primordium, and ordered cell divisions build it up into a cone shape. In the subsequent stage, cell proliferation ceases, and the primordium grows only by cell expansion for a short time. Finally, cell division resumes in the apical region, thereby establishing the structure of the root apical meristem (RAM).

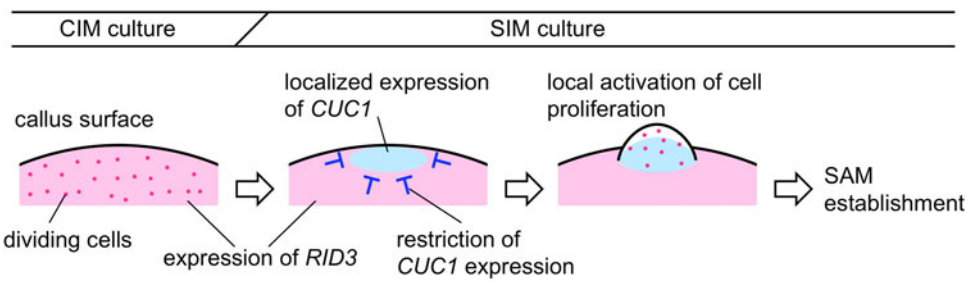

Fig. 2 Hypothetical scheme for the function of *RID3* in the restriction of *CUC1* expression and cell proliferation during shoot regeneration

When explants of *srd2* were cultured in the semi-synchronous lateral root induction system at the restrictive temperature, they produced hemispherical knobs instead of lateral roots (Ohtani et al. 2010). Detailed inspection of these knobs revealed that the *srd2* mutation had altered cell organization of the root primordium and maintained primordial cell division for a long period, resulting in the formation of abnormal hemispherical laterals lacking the RAM structure. In the early stages of development of root primordia, the *srd2* mutation reduced the amount of PIN auxin efflux facilitators and, probably by this means, interfered with the generation of an auxin gradient and the establishment of the apical–basal axis. In accordance with the function of *SRD2* in snRNA transcription, spliceosomal snRNAs were markedly decreased in developing root primordia of *srd2* even before deformation became evident. Notably, the *rid1* mutant, which is impaired in pre-mRNA splicing, also produced severely deformed lateral roots at the restrictive temperature, possibly resulting from uncontrolled and excess primordial cell divisions. These findings collectively suggested that splicing-related factors are particularly important for expression of PINs in polarized pattern generation during the development of lateral root primordia.

In the last part of this article, I introduce just briefly three other interesting mutants, *rrd1*, *rrd2*, and *rid4*, which were characterized by frequent formation of fasciated lateral roots at the restrictive temperature (Konishi and Sugiyama 2003; Sugiyama 2003). Results of temperature-shift experiments using the semi-synchronous lateral root induction system implied that these mutations have a specific effect on formative cell division at the very early stage of root primordium development to broaden the basal width of each primordium (Otsuka and Sugiyama 2012). Therefore, these mutants are expected to be very useful for investigation of molecular mechanisms controlling formative cell division.

Perspectives

As described above, molecular genetic analysis of plant organogenesis in vitro, which originated in Prof. Komamine's laboratory, has successfully provided novel and unique clues to understanding the mechanisms underlying organogenesis. In my view, the methodologically important choice in these studies was to use various Arabidopsis mutants that are temperature-sensitive for tissue culture responses. Judging from the scale of screening carried out so far, the current collection of temperature-sensitive mutants is far from saturation. Isolation of more temperature-sensitive mutants and extending molecular genetic analysis with them would be a promising way to elucidate the molecular basis of organogenesis in vitro.

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