

**Arabidopsis ENHANCER OF SHOOT REGENERATION 2 and PINOID are involved in in vitro shoot regeneration**

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**Abstract**  ENHANCER OF SHOOT REGENERATION 2 (ESR2), like ESR1, plays several critical roles during *in vitro* shoot regeneration. We investigated the genetic interaction between ESR2 and PINOID (PID) during shoot regeneration in this study. Both *esr2* and *pid* mutations markedly decreased the efficiency of shoot regeneration from root segments at comparable levels (27% and 35% of shoot numbers on wild type, respectively); while *esr2-2 pid* double mutants dramatically decreased the efficiency of shoot regeneration (4.7% of shoot numbers on wild type). Our results demonstrated an additive or synergistic effect of these two mutations on shoot regeneration. Expression of ESR2 in *pid* explants during shoot regeneration was abnormal after day 5, although *pid* mutation did not affect ESR2 expression until day 5. In conclusion, PID appears to be required for late development of the shoot apical meristem during shoot regeneration.

**Key words:**  ESR2, PID, shoot regeneration

In many species, *in vitro* organogenesis or somatic embryogenesis is the first step in the development of transgenic plants from single transformed cells. Although organogenesis in tissue culture is directed by the appropriate plant hormones, (i.e., auxins and cytokinins), optimal conditions, including hormone and nutrient concentrations, vary widely among plant species, even within varieties or cultivars of the same species. Despite recent advances in the understanding of the molecular mechanisms underlying the actions of auxins and cytokinins, little is known about the developmental events downstream of these signaling proteins. Therefore, for the purposes of molecular breeding and biotechnology, it is important to elucidate the mechanisms downstream of hormonal action that regulate shoot differentiation in tissue culture.

Recently, Atta et al. (2009) demonstrated that, during *Arabidopsis* tissue culturing, lateral root meristem (LRM)-like primordia were generated from the pericycle cells adjacent to the xylem poles in the plant roots or from hypocotyls by incubation on callus-inducing medium (CIM) containing a high concentration of 2,4-dichlorophenoxyacetic acid (2,4-D). In addition, subsequent incubation of the LRM-like primordia on shoot-inducing medium (SIM) containing cytokinin converted the primordia to shoot meristems (SAMs). Thus, shoots were regenerated from the SAMs originated from pericycle cells via LRM-like primordia. The LRM-like structures have also been shown to develop from the aerial parts of plants upon incubation on CIM, and the cytokinin signal subsequently converts the LRM-like structures to SAMs (Sugimoto et al. 2010). Therefore, transdifferentiation of the early LRM-like primordia into SAMs appears to be a general mechanism of *in vitro* shoot regeneration.

**ENHANCER OF SHOOT REGENERATION 1 (ESR1),** [also known as DORNRÖSCHEN or DRN (Kirch et al. 2003)] and ESR2 (Ikeda et al. 2006) [also known as DRNL (Kirch et al. 2003), SOB2 (Ward et al. 2006), and BOLITA (Marsh-Martinez et al. 2006)] are thought to play critical roles during *in vitro* shoot regeneration, and they encode similar transcription factors belonging to the ethylene-responsive factor (ERF) family (Mase et al. 2007). Recently, we demonstrated that ESR1 expression was initiated in a small number of cells in the LRM-like structures, which had been induced by incubating root explants on SIM after pre-incubation on CIM (Matsuo et al. 2011). Subsequently, ESR1-expressing cells proliferated to form SAM-like structures, suggesting that ESR1 may direct conversion of LRM into SAM in tissue culture. By contrast, ESR2 expression was initiated in small regions of SAM-like structures and continued...
through shoot formation on SAM-like structures. ESR2 appeared to function in SAM-like structures after conversion of LRM into SAM.

Proper auxin distribution is important for correct cell specification in the early embryo (Møller and Weijers 2009). PIN-FORMED 1 (PIN1) and other members of its protein family are auxin efflux carriers that transport auxin across cellular membranes, and PIN-dependent auxin transport is crucial for auxin distribution in SAM (reviewed in Vernoux et al. 2010). Auxin gradients and maxima in SAM are created by polar auxin transport, and PIN proteins determine the extent of polar auxin transport through their asymmetric subcellular localization. The pin1-4 mutation showed decreased numbers of shoot on callus explants (Gordon et al. 2007), suggesting that PIN function is also required for efficient in vitro shoot regeneration.

The PINOID (PID) serine/threonine kinase regulates subcellular localization of PIN protein by phosphorylation of PIN proteins. Recently, Chandler et al. (2011a) reported a genetic interaction between DRN (ESR1) and DRNL (ESR2) with PIN1 or PID during cotyledon development. The investigators demonstrated that the function of ESR1 overlaps with that of PID, and the function of ESR2 overlaps with that of PIN1 in cotyledon organogenesis.

In this study, we investigated the genetic interaction between ESR2 and PID by using esr2-2 pid double mutants, focusing on in vitro shoot regeneration. Our results demonstrated that both ESR2 and PID were required for efficient shoot regeneration.

Materials and methods

Plant materials and growth conditions

Seeds were surface-sterilized and sown on Murashige and Skoog (MS) medium supplemented with MS salts (Wako Pure Chemical Industries, Ltd.; Osaka, Japan), Gamborg’s B5 vitamins (Sigma-Aldrich; St. Louis, MO, USA), 2% glucose, Gamborg’s B5 vitamins, 2 µM 2,4-D, and 0.25% gellan gum. Plants were grown at 22°C under continuous light for 2 weeks, and then subjected to preparation of root explants (ABRC; Columbus, OH, USA). These mutants were genotyped for effi cient subcellular localization of PIN protein by phosphorylation of PIN proteins. Recently, Chandler et al. (2011a) reported a genetic interaction between DRN (ESR1) and DRNL (ESR2) with PIN1 or PID during cotyledon development. The investigators demonstrated that the function of ESR1 overlaps with that of PID, and the function of ESR2 overlaps with that of PIN1 in cotyledon organogenesis.

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Histochemical analysis of GUS activity

Root samples were incubated in X-Gluc solution [1 mg ml⁻¹ of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt, 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide, 100 mM sodium phosphate (pH 7.4), 10 mM EDTA, and 0.1% Triton X-100] overnight at 37°C. After staining, the solutions were removed from the root samples, and the chlorophyll was bleached with ethanol.

RNA isolation and cDNA synthesis

Total RNA isolation and cDNA synthesis were performed as previously described (Mase et al. 2007).

qRT-PCR analysis

Real-time qRT-PCR was performed as previously described (Matsuo et al. 2009). The primers used were as follows: PID (PID-RTF 5’-AGG AGG AAA CCA CCA GCC CGG-3’ and PID-RTR 5’-CTT CAC GTA CTG GTT GTC GTT A-3’).

Results

Genetic interaction between ESR2 and PID in in vitro shoot regeneration

Arabidopsis possesses eight PIN protein members (Paponov et al. 2005) and a PID that is capable of regulating the subcellular localization of multiple PIN proteins (Huang et al. 2010; Kleine-Vehn et al. 2009; Michniewicz et al. 2007). To investigate the relationship between ESR2 and auxin localization during in vitro shoot regeneration, an esr2-2 pid double mutant line was generated using a pid allele reported by Cheng et al. 2008. We were unable to generate esr1-1 pid double mutants, possibly because of their lethality.

Figure 1 illustrates the cotyledon phenotypes of pid, esr2-2, and esr2-2 pid double mutants. As reported by Michniewicz et al. (2007), pid seedlings have three cotyledons in many instances (Figure 1B, Table 1). The esr2 single mutation caused the generation of abnormal cotyledons at low frequencies (Figure 1C), as reported previously (Chandler et al. 2007; Matsuo et al. 2011). Genotyping revealed that the majority of esr2-2 pid seedlings with abnormal cotyledons were enhanced compared with the cotyledons of esr2-2 or pid seedlings (Figure 1D, E, F, Table 1). Frequencies of abnormal cotyledons (one cotyledon or two fused cotyledons in Table 1) emerging on progenies from the parental genotype esr2-2 (2.75%) were higher than frequencies on progenies from the parental genotype
drnl-1 (0.71%; Chandler et al. 2011a), suggesting that esr2-2 is a stronger allele than drnl-1. Our pid plants were sterile, whereas pid-2 plants appeared to be partially fertile (Chandler et al. 2011b), suggesting that pid is a stronger allele than pid-2. Our double mutants had more severe phenotypes than the drnl-1 pid-2 double mutants did, probably because we used stronger alleles of both mutations than drnl-1 and pid-2 generated by Chandler and colleagues (2011a, b). In contrast to abnormal cotyledons, esr2-2 pid mutants grew normally until their transition to the reproductive phase, and they produced pin-like inflorescence stems as well as pid single mutants (data not shown).

We investigated the effects of these mutations on in vitro shoot regeneration. After a 4-day pre-incubation period on CIM, each root explant (approximately 5 mm in length) was transferred onto SIM and incubated for 4 weeks. (A–F) Photographs of regenerated shoots from root explants; (A) Col-0; (B) esr2-2; (C) pid; (D) esr2-2 pid; (E, F) esr2-2 pid (enlarged view). (G) The number of regenerated shoots per root segment. We used 110–392 root segments for each experiment. Error bars indicate standard errors. Scale bars = 5 mm.

Table 1. Frequencies of cotyledon phenotypes in mutant seedlings.

<table>
<thead>
<tr>
<th>Parent genotype</th>
<th>Frequency of cotyledon number (%)</th>
<th>Total number of seedlings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No cotyledons</td>
<td>One cotyledons</td>
</tr>
<tr>
<td>Col-0</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>esr2-2/esr2-2</td>
<td>0.00</td>
<td>1.83</td>
</tr>
<tr>
<td>pid/PID</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>esr2-2/ESR2 pid</td>
<td>2.41</td>
<td>1.20</td>
</tr>
</tbody>
</table>

Plants of parental genotype were self-propagated, and their progeny were observed. *Two or three cotyledons are presented, with one cotyledon among them being smaller than the remaining cotyledons.

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**PID expression during shoot regeneration**

We performed qRT-PCR to investigate time courses of PID expression during shoot regeneration (Figure 3). After a 4-day pre-incubation period on CIM, root segments from wild-type or esr2-2 mutants were transferred onto SIM, followed by periodic total RNA preparation for cDNA synthesis. The PID transcripts in wild-type explants increased, with their level reaching approximately thrice the level on day 7 as that on day 0, followed by a subsequent reduction on day 10. These results suggest that PID may function during shoot regeneration. The PID expression patterns in esr2-2 explants during shoot regeneration were similar to those observed in the wild-type explants, indicating that esr2-2 mutation did not affect PID expression levels until day 7. The PID transcript levels in esr2-2 explants significantly decreased to 74% of those observed in wild-type explants on day 10.

We also investigated PID expression sites in root explants during shoot regeneration by using the PID promoter::GUS fusion gene (Figure 4). After a 4-day pre-incubation period on CIM, root segments from transgenic Arabidopsis carrying the PID::GUS were incubated on SIM. Although GUS expression was not detected in root explants on day 0 after excision from mature wild-type or esr2-2 mutants, GUS expression was detected in the vascular tissues of both root explants after a 4-day incubation period on CIM (SIM 0). The GUS expression levels increased in LRM-like structures close to the vascular bundles of both root explants by incubation on SIM. Many SAM-like structures were formed on explants generated from wild-type explants, although very few of these structures were formed on esr2-2 explants on day 7 after incubation on SIM. The SAM-like structures were distinguishable from LRM-like structures by the presence or absence of leaf primordia. Strong GUS expression was detected in the SAM-like structures of the wild-type explants. The decrease in the PID transcript levels in esr2-2 explants, as determined by qRT-PCR on day 10 (Figure 3), may reflect fewer SAM-like structures, since esr2 mutation decreased shoot regeneration efficiency compared with wild type (Figure 2). These results suggest that PID expression is found in LRM- and SAM-like structures, and may function through the formation of LRM and their conversion into SAMs.

**ESR2 expression in pid explants during shoot regeneration**

The esr2-2 mutation appeared not to affect PID expression at early stages prior to SAM formation. We also investigated the effects of pid mutation on ESR2 expression during shoot regeneration (Figure 5). After a 4-day pre-incubation period on CIM, root segments from wild-type or pid plants were transferred onto SIM. Total RNAs were periodically prepared from root explants and qRT-PCR experiments were conducted. The ESR2 transcript levels gradually increased after day 2 and continued to increase until day 5, after which time they slightly decreased, as previously reported (Matsuo et al. 2009). The decrease in ESR2 expression can be attributed
to its restricted expression in the leaf primordia tips during the late stage of SAM formation (Matsuo et al. 2011). In contrast to the wild-type explants, ESR2 expression levels in pid explants continued to increase after day 5. Defects in SAM development may direct persistent ESR2 expression.

Discussion

A proper auxin gradient is important for SAM formation (Moller and Weijers 2009). The PID serine/threonine kinase plays a crucial part by regulating subcellular localization of PIN protein via phosphorylation. In this study, we investigated genetic interaction between ESR2 and PID during shoot regeneration. Both esr2-2 and pid mutations decreased the efficiency of shoot regeneration from the root segments at comparable levels (27% and 35% of the number of shoots on wild-type, respectively), while esr2-2 pid mutants dramatically decreased the efficiency of shoot regeneration (4.7% of the number of shoots on wild-type) (Figure 2). These results suggest that both ESR2 and PID are required for efficient shoot regeneration.

We also investigated PID expression during shoot regeneration. Expression of PID was induced in the vascular tissues of root explants by incubation on CIM (Figure 4), suggesting that PID expression was induced by 2,4-D (CIM is the basal medium containing only 2,4-D as a plant hormone), although it is possible that cutting-induced stress may have influenced this process. Exogenous auxin appeared to stimulate PID expression in a portion of vascular tissues from root explants.

Subsequent incubation on SIM induced PID expression in LRM-like structures. As PID was also expressed in SAM-like structures after the conversion of LRM into SAM, auxin distribution may be important for the growth of SAMs. This result is consistent with the fact that PID is expressed in the SAMs of wild-type seedlings (Benjamin et al. 2001). The esr2-2 mutation did not affect PID expression until day 7 after transfer onto SIM during shoot regeneration, although PID expression in esr2-2 explants decreased by 26% compared with that in the wild-type explants on day 10 (Figure 3). The decrease may reflect the reduced numbers of regenerated shoots compared with those of the wild-type, since PID was expressed strongly in SAM-like structures (Figure 4).

During shoot regeneration, ESR2 expression in wild-type explants was initiated on day 3 after transfer onto SIM and continued to increase until day 5 (Figure 5). Subsequently, ESR2 expression decreased, probably because it is restricted to the leaf primordia tips during the late stages of SAM formation, whereas ESR1 is expressed in the whole SAM structures during early stages (Matsuo et al. 2011). On the other hand, ESR2 expression in pid explants continued to increase even after day 5. Taken together with the fact that the numbers of mature SAM-like structures were reduced on pid explants (Figure 2), persistent ESR2 expression was probably caused by the defects in SAM development. However, ESR2 expression during early stages of SAM formation was normal, suggesting that PID function is not required for the conversion of LRMs into SAMs, since ESR2 was expressed normally in pid explants until day 5, and ESR2 expression initiated after the conversion which had occurred within a few days after transfer onto SIM (Matsuo et al. 2011).

Chandler et al. (2007) reported that approximately a quarter of the progeny of drn-1 drnL-2/DRNL plants had pin-like embryos, with a complete absence of cotyledons. We also observed that esr1 esr2 double mutants formed pin-like inflorescence stems at a low frequency (unpublished data). Proper auxin distribution may be disturbed in esr1 esr2 double mutants. Although we examined auxin distribution in explants during shoot regeneration by using the auxin reporter system DR5::YFP, visible differences in YFP distribution between the wild-type and esr2-2 explants were not observed. An imperceptible auxin gradient may affect SAM development. Our results demonstrated that PID regulated in vitro shoot regeneration as well as ESR2 did, although their genetic interactions were not clear.

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References