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Original Paper

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 apical 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* (Marsch-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

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; 2-ip, N^6-Δ^2-isopentenyladenine; CIM, callus-inducing medium; MS salts, Murashige and Skoog salts; SIM, shoot-inducing medium; PCR, polymerase chain reaction; qRT-PCR, quantitative reverse-transcription PCR; YFP, Yellow Fluorescent Protein.

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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 (Moller 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), 1% sucrose, and 0.25% gellan gum (Wako Pure Chemical Industries, Ltd.). The CIM contained Gamborg's B5 salts (Sigma-Aldrich), 2% glucose, Gamborg's B5 vitamins, 2 μM 2,4-D, and 0.25% gellan gum. The SIM contained MS salts, Gamborg's B5 vitamins, 1% sucrose, 12.5 μM N^6-Δ^2-isopentenyladenine (2iP), 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. The *esr2-2* mutant allele has been described previously (Matsuo et al. 2011), and the *pid* mutant allele (Salk_049736) was obtained from the *Arabidopsis* Biological Resource Center (ABRC; Columbus, OH, USA). These mutants were genotyped by PCR with the following primers (forward and reverse, respectively): *ESR2*F4 (5′-TTT CGC TTA CCC GCC TTG TAA TC-3′), *ESR2*F4mut (5′-TTT CGC TTA CCC GCC TTG TAA TAT -3′), and *ESR2*R (5′-ATT CCA CCA TTT CCG TTC TGC TGC A-3′) for *ESR2*; and *PID*-F (5′-ATT TTG CGA TGA AAG TTG TGG -3′), *PID*-R (5′-CAG TCG GGA AAC TCA ACT GTC -3′), and 35S-MF (5′-CTC TAT ATA AGG AAG TTC ATT TCA TTT GG-3′) for *PID*. These mutants were backcrossed three times with wild-type plants to eliminate undesired mutations.

Transgenic plant lines carrying *PID*::GUS (Benjamins et al. 2001) were obtained from the ABRC.

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 CGC CG-3′ and PID-RTR 5′-CCT CTC 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 have cotyledons whose abnormalities 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

Table 1. Frequencies of cotyledon phenotypes in mutant seedlings.

Parent genotype	Frequency of cotyledon number (%)							Total number of seedlings
	No cotyledons	One cotyledons	Two cotyledons	Three cotyledons	Four cotyledons	Two fused cotyledons	Others*	
Col-0	0.00	0.00	100	0.00	0.00	0.00	0.00	156
esr2-2/esr2-2	0.00	1.83	97.25	0.00	0.00	0.92	0.00	427
pid/PID	0.00	0.00	88.51	10.34	1.15	0.00	0.00	174
esr2-2/ESR2 pid/PID	2.41	1.20	84.94	7.83	0.00	0.00	3.61	166

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.

Figure 1. Phenotypes of *esr2-2* and *pid* seedlings 5 days after sowing. (A) Wild-type (Col-0); (B) *pid*; (C) *esr2-2*; (D–F) *esr2-2 pid* double mutants.

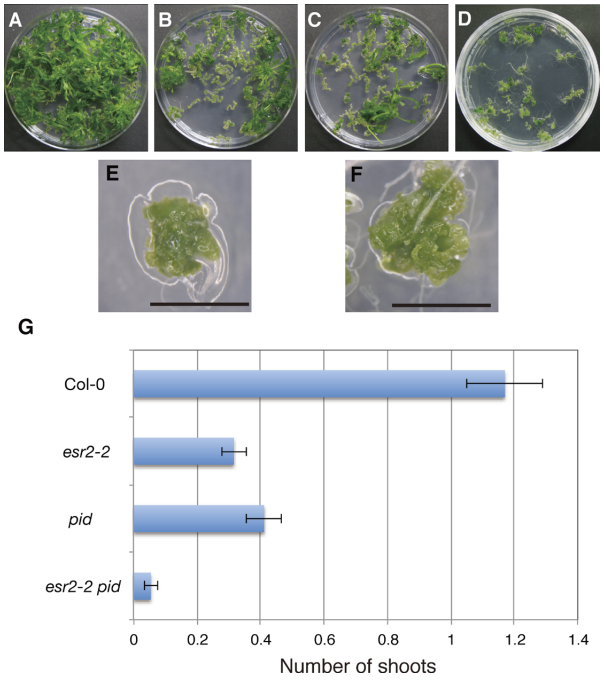

Figure 2. *In vitro* shoot regeneration in *esr2-2* and *pid* mutant plants. After 4-day pre-incubation period on CIM, each root explant (approximately 5 mm in length) was transferred onto SIM and incubated for 4 weeks. (A–F) Photographs of regenerated shoots from root explants; (A) Col-0; (B) *esr2-2*; (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.

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, root segments were incubated on SIM, and the numbers of shoots were counted after 4 weeks. The *esr2-2* mutation caused dramatic decreases in the efficiency of shoot formation (Figure 2B), as previously reported (Matsuo et al. 2011). A single mutation of *esr2-2* or *pid* reduced the number of shoots formed on root explants to 27% and 35% of the number on wild-type, respectively (Figure 2B, C, G). The number of shoots formed on root explants from *esr2-2 pid* dramatically decreased to 4.3% of the number on wild-type plants (Figure 2D, G). These results suggest that *PID* regulates shoot regeneration to a comparable degree as *ESR2*. Moreover, the double mutation of *esr2* and *pid* appeared to have an additive or synergistic effect on shoot regeneration. In some instances, dark green calli formed on *esr2-2 pid* root explants (Figure 2E, F) similar to those formed on *ESR1*- or *ESR2*-overexpressing explants on SIM (Banno et al. 2001; Ikeda et al. 2006). The reason for the formation of these calli is currently unclear.

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 LRMs 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

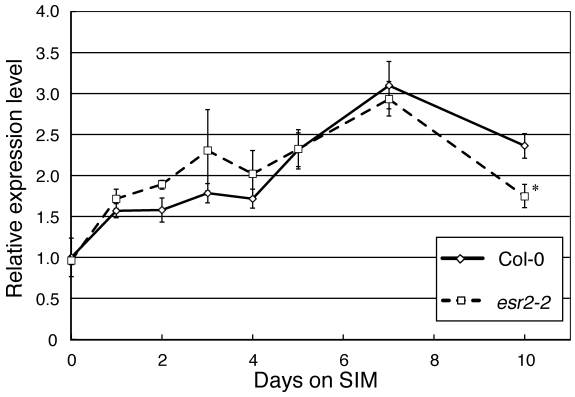

Figure 3. *PID* expression patterns during *in vitro* shoot regeneration. Time courses of *PID* expression during *in vitro* shoot regeneration in wild-type (Col-0) and *esr2-2* explants are displayed. Root explants were incubated on CIM for 4 days, and then transferred onto SIM. Expression levels were measured with qRT-PCR. Each value on the vertical axis indicates a relative level to Col-0 on day 0 calculated by reference to the ubiquitin (*UBQ5*) transcript levels. Numbers on the horizontal axis indicates days after transfer onto SIM. Data represent the average of three independent PCR reactions, and error bars indicate standard deviations. Significant differences from Col-0 are indicated with asterisk (Student's *t* test; $p<0.1$).

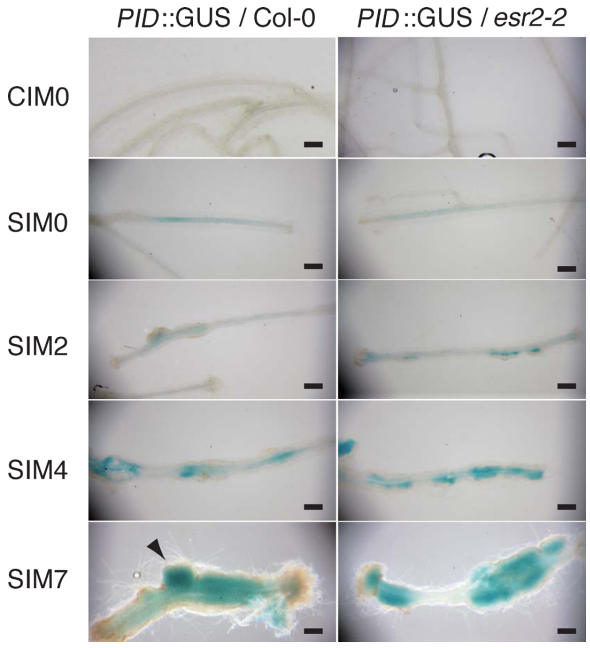

Figure 4. *PID*::GUS expression during *in vitro* shoot regeneration in wild-type (Col-0) and *esr2-2* root explants. After a 4-day pre-incubation period on CIM, *PID*::GUS root explants were incubated on SIM. Then, explants were collected on days 0, 2, 4, and 7. Each sample was processed for GUS activity by stain in X-Gluc solution. The arrowhead indicates a SAM-like structure. Scale bars = 100 μm.

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

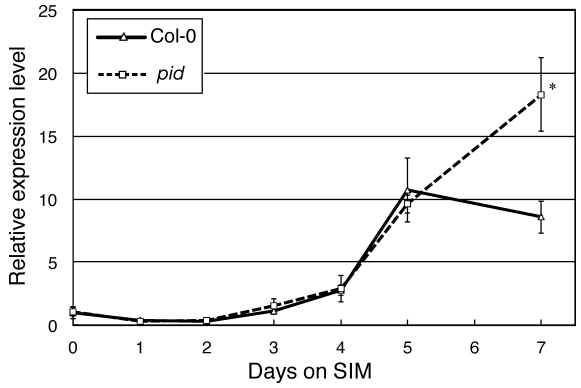

Figure 5. *ESR2* expression patterns during i*n vitro* shoot regeneration. Time courses of *ESR2* expression during *in vitro* shoot regeneration in wild-type (Col-0) and *pid*. Root explants were incubated on CIM for 4 days, and then transferred onto SIM. Expression levels were measured with qRT-PCR. Each value on the vertical axes indicates a relative level to Col-0 on day 0 calculated by reference to the *UBQ5* transcript levels. Numbers on the horizontal axis indicate days after transfer onto SIM. Data represent the average of three independent PCR reactions, and error bars indicate standard deviations. Significant differences from Col-0 are indicated with asterisk (Student's *t* test; $p<0.05$).

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 (Benjamins 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 influorescence 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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