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# WIND1-based acquisition of regeneration competency in Arabidopsis and rapeseed

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## WIND1-based acquisition of regeneration competency in Arabidopsis and rapeseed

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**Abstract** Callus formation and *de novo* organogenesis often occur in the wounded tissues of plants. Although this regenerative capacity of plant cells has been utilized for many years, molecular basis for the wound-induced acquisition of regeneration competency is yet to be elucidated. Here we find that wounding treatment is essential for shoot regeneration from roots in the conventional tissue culture of *Arabidopsis thaliana*. Furthermore, we show that an AP2/ERF transcription factor WOUND INDUCED DEDIFFERENTIATION1 (WIND1) plays a pivotal role for the acquisition of regeneration competency in the culture system. Ectopic expression of WIND1 can bypass both wounding and auxin pre-treatment and increase *de novo* shoot regeneration from root explants cultured on shoot-regeneration promoting media. In *Brassica napus*, activation of Arabidopsis WIND1 also greatly enhances *de novo* shoot regeneration, further corroborating the role of WIND1 in conferring cellular regenerative capacity. Our data also show that sequential activation of WIND1 and an embryonic regulator LEAFY COTYLEDON2 (LEC2) enhances generation of embryonic callus, suggesting that combining WIND1 with other transcription factors promote efficient and organ-specific regeneration. Our findings in the model plant and crop plant point to a possible way to efficiently induce callus formation and regeneration by utilizing transcription factors as a molecular switch.

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### Keywords

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Introduction

Plant cells exhibit high developmental plasticity when they face severe environmental stresses. *De novo* organogenesis after wounding is one of the most remarkable examples of cell fate change in plants as well as other multicellular organisms (Birnbaum and Sánchez Alvarado 2008). Like blastema formation in amphibian limb regeneration (Maden 1976), plants often form mass of proliferating cells called callus at the wound site, which often serves as a source for subsequent tissue regeneration (Bostock and Stermer 1989; Stobbe et al. 2002; Ikeuchi et al. 2013). Callus formation is also observed in *in vitro* tissue culture. Groundbreaking research in mid 20th century has shown that two plant hormones, auxin and cytokinin, are key inducers for callus formation and subsequent regeneration (Skoog and Miller 1957). In the conventional tissue regeneration system, segments of plant explants are incubated on hormone-containing media. In the case of *Arabidopsis thaliana* (*Arabidopsis*), two-step culture method is most commonly adopted, where root and/or hypocotyl explants are preincubated on an auxin-rich callus induction medium (CIM), then transferred to a cytokinin-rich shoot induction medium (SIM) or auxin-rich root induction medium (RIM) (Valvekens et al. 1988; Ozawa et al. 1998; Che et al. 2002). Enormous studies using the plant tissue culture system have contributed to the advancement of basic and applied science (Thorpe 2012) but the molecular mechanisms of how plant cells modify developmental plasticity in response to various environmental stimuli, especially wounding and hormonal treatment, is just beginning to be understood.

By comparing differentially expressed genes between seedlings and cultured cell lines in *Arabidopsis* (Iwase et al. 2005), we identified an AP2/ERF transcription factor WOUND INDUCED DEDIFFERENTIATION 1 (*WIND1*) and its close homologs *WIND2–4* as wound-responsive master regulators of callus formation (Iwase et al. 2011a; Iwase et al. 2011b). Plants ectopically overexpressing *Arabidopsis WIND1* (*AtWIND1*) develop callus on phytohormone free media, not only in *Arabidopsis* but also in other species such as *Brassica napus* (rapeseed), *Nicotiana tabacum* (tobacco) and *Solanum lycopersicum* (tomato) (Iwase et al. 2011a; Iwase et al. 2011b; Iwase et al. 2013). In addition, calli generated by the chemical induction of *WIND1* regenerate tissues when transferred to the chemical inducer-free medium where *WIND1* is no longer activated (Iwase et al. 2011a). Therefore, *WIND* proteins have roles in modifying the developmental plasticity, which is conserved, at least to some extent, in plant kingdom. In the CIM-SIM two-step culture system, cells in explants that undergo callus formation during CIM incubation, are believed to acquire the competence to respond to shoot induction signals in the subsequent step (Sangwan et al. 1992; Che et al. 2006 ; Duclercq et al. 2011; Perianez-Rodriguez et al. 2014). It is plausible that *WIND1* participates in the

acquisition of competency and *de novo* organogenesis in tissue culture but this possibility is yet to be addressed. Recent studies have pointed out that callus formation in CIM is mainly generated from xylem pole pericycle cells and the competence for shoot regeneration can be considered as the ability to induce lateral root meristem (Atta et al. 2009; Sugimoto et al. 2010). Whether WIND1 affects callus induction from lateral root meristem is also one of the important questions to be answered.

Interestingly, WIND1-overexpressing plants and callus show various regeneration phenotypes. Established callus lines in tobacco by constitutive *AtWIND1* expression display shooty phenotype and chemical-induced *AtWIND1* activation in tomato also form shooty callus on hypocotyls (Iwase et al. 2013). These observations are consistent with our previous findings that WIND1 upregulates cytokinin signaling (Iwase et al. 2011a). However, regeneration of roots or somatic embryos is occasionally observed in *AtWIND1* overexpressors and WIND1 ortholog in an Arabidopsis relative, a salt cress *Thellungiella halophila* (*ThWIND1-L*) overexpressors as well (Iwase et al. 2011a; Zhou et al. 2012; Ikeuchi et al. 2013), raising the possibility that WIND1 renders developmental plasticity and other factor(s) determine the direction of regeneration. Therefore, it is of great interest if combining WIND1 with other growth regulators will lead to directed regeneration of tissue/organs.

In this study we describe roles of wound stimulus and WIND1 function on the cell fate change in tissue culture, i.e. Arabidopsis shoot regeneration from root explants. Based on our observation in Arabidopsis, we also apply the WIND1 function to rapeseed and tomato shoot regeneration process. In addition, we test co-expression of *WIND1* and another developmental regulator to establish an efficient way for plant regeneration.

## Material and methods

### Plant materials

Arabidopsis lines used in this study, wild-type, *35S:WIND1*, *XVE-WIND1*, *Pro<sub>WIND1</sub>:WIND1-SRDX* (Iwase et al. 2011a), and *35S:LEC2-GR* (Ledwoń and Gaj 2009) are all in ecotype Columbia (Col-0) background. *35S:AtWIND1-GR* rapeseed (*Brassica napus* cv Westar) and *35S:AtWIND1-GR* tomato (*Solanum lycopersicum* cv Micro-Tom) plants were previously reported (Iwase et al. 2013). Arabidopsis and tomato were grown at 22°C with continuous light and rapeseed was grown at 23°C, 16 h light and 18°C, 8 h dark.

### Tissue culture conditions

The Arabidopsis shoot regeneration assay was performed according to the procedure previously reported (Valvekens et al. 1988). For germination, we used media containing Murashige and Skoog basal salt mixture (Wako), 1% sucrose and 0.6% Gellan Gum (Wako), with the pH adjusted to 5.8. The germination and shoot regeneration assays for rapeseed and tomato were performed according to the procedures described by Kohno-Murase et al. (1994) and Sun et al. (2006) respectively, except the Agrobacterium co-culture steps. Regenerated shoots were defined as green structures having two true leaves or leaf primordia, which were observed using a stereomicroscope (Leica M165C). The dexamethasone (Sigma) and the 17 $\beta$ -estradiol (Wako) were dissolved in ethanol and dimethyl sulphoxide, respectively, to make 10 mM stock solutions, and subsequently added to the medium after autoclaving.

**Results**

**Wounding is required for the cell fate change during regeneration**

Most plant tissue culture techniques include a step for tissue wounding but how this stimulus affects plant regeneration is not well characterized. To investigate the importance of the wounding step in tissue culture, we first compared tissue regeneration phenotypes between unwounded and wounded roots using an Arabidopsis shoot regeneration system (Valvekens et al. 1998). Fig. 1 shows Arabidopsis roots grown on auxin-rich callus induction medium (CIM) for 4 days, then cytokinin-rich shoot induction medium (SIM) for 21 days. We found that shoot regeneration never occurs from roots without wounding and instead multiple lateral roots are generated from main roots (Fig. 1a, b), indicating that the primary effect of CIM is to increase the number of lateral root primordial and the wound stimulus is required to initiate shoot regeneration.

Since WIND1 induces callus in response to wounding (Iwase et al. 2011a), ectopic expression of WIND1 may mimic the wounding signal leading to the cell fate change. As expected, 35S:WIND1 plants overexpressing WIND1 and therefore exhibiting a weak gain-of-function phenotype (Iwase et al. 2011a), show shoot regeneration even from unwounded root after the CIM-SIM treatment (Fig. 1c, d). Conversely, in a Pro<sub>WIND1</sub>:WIND1-SRDX line which expresses WIND1 chimeric repressor and therefore shows dominant negative phenotype (Hiratsu et al. 2003; Iwase et al. 2011a), shoot regeneration from wounded roots is completely suppressed (Fig. 1e, f). These results clearly show that WIND1 plays a pivotal role for the cell fate alteration in the Arabidopsis shoot regeneration system.

**WIND1 promotes the acquisition of competency for regeneration**

Based on our finding that WIND1 overexpression compensates the wounding process, we further examined whether it also bypasses the CIM treatment. Recent studies suggest that there are at least two types of callus generated from Arabidopsis root explants cultured on CIM, callus derived from wounded site and unwounded site (Iwase et al. 2011; Ikeuchi et al. 2013). In contrast to calli generated from unwounded sites that form from lateral root primordia and highly express root meristem markers (Sugimoto et al. 2010), callus induced at the wound-site does not display such marker expression and its formation is not blocked in *solitary root* mutants defective in lateral root initiation (Iwase et al. 2011a, see supplemental figure; Ikeuchi et al. 2013). Shoot regeneration occurs from calli at both wounded site and unwounded site, referred as ‘wounded site callus’ and ‘unwounded site callus’ hereafter. As shown in Fig. 1e and Fig. 1f, shoot regeneration from both types of callus is completely blocked in *ProWIND1:WIND1-SRDX* plants. In addition, *WIND1* expression is found in both wounded site callus and unwounded site callus (data not shown), indicating that WIND1 also alters the cell fate of unwounded site callus induced by CIM treatment. These results, therefore, suggest that increasing *WIND1* expression level alone might be sufficient to increase the shoot regeneration competency. To test this hypothesis, we used transgenic plants carrying 17 $\beta$ -estradiol (ED)-inducible *LexA-VP16-estrogen receptor (XVE)-WIND1* (Zuo et al. 2000; Iwase et al. 2011a). As we expected, unwounded roots of the *XVE-WIND1* plants treated with ED for 1 day form shoots from unwounded and CIM-untreated roots (Fig. 2d, e). We confirmed this promotion of shoot regeneration is not caused by ED itself (Fig. 2b) or a transgene insertion (Fig. 2c), and similar to the result in Fig. 1a, roots with only a short CIM treatment (1 day) without wounding also fail to produce shoots (Fig. 2a). These results, together with earlier findings, emphasise that *WIND1* can bypass the auxin pre-treatment step and confer a greater competency for regeneration to plant cells.

#### **AtWIND1 increases shoot regeneration competence in rapeseed callus**

Since Arabidopsis *WIND1* (*AtWIND1*) orthologues are found in land plants (Iwase et al. 2013), we next asked if the promotion of tissue regeneration occurs in other species. We have already reported ectopic callus formation in seedlings of rapeseed and tomato from dexamethasone (DEX)-mediated AtWIND1 inducible lines, i.e. utilizing the 35S:AtWIND1-Glucocorticoid Receptor (GR) (Iwase et al. 2013). Normally, rapeseed and tomato transformation are performed through a tissue culture system for regeneration, namely via *de novo* organogenesis from callus using phytohormones (Kohno-Murase et al. 1994; Sun et al. 2006). In these systems, the tissue availability is restricted because of its regeneration capacity, for example, tissue segments close to

shoot apical meristem (SAM) are most often used in rapeseed shoot regeneration (Fig. 3a). We compared shoot regeneration capacity between hypocotyl segments near the SAM (Segment I, not containing the meristem) and segments relatively far from the SAM (Segment II, Fig. 3a), under DEX-treated WIND1 induction or non-DEX treated conditions (Table 1; Fig. 3b). In a condition without DEX, the number of large shoots (having leaf more than 5mm in length) generated from segment I is greater than those generated from segment II (Table 1), and same tendency is also observed in the case of small shoots (having leaf less than 5mm in length) (data not shown), indicating that tissue near the SAM possess higher regenerative competency in this system. Continuous WIND1 activation by DEX treatment (DEX ++, Fig. 3b) seems to have an positive effect on small shoot regeneration from segment II (Table 2), where a 24 to 47 times higher average number of regenerated shoots per segment is observed (Table 1 and Table 2, Fig. 3c, e, f). Importantly, this remarkable promotion of shoot regeneration is reproducibly detected in independent experiments compared to the condition without WIND induction (DEX --, Table 2), suggesting that WIND1 can alter cell fate even in callus cells that are recalcitrant for regeneration. It is also striking that, in the culture of segment II, removal of DEX from culture media which presumably stops the WIND1 activation, positively affects larger shoot regeneration (Table 1 and Table 2, Fig. 3c, d, f), indicating that WIND1 promotes shoot initiation in the culture system but inhibits leaf growth. This tendency is also seen in the segment I, where continuous WIND1 activation by DEX treatment (DEX ++, Fig. 3b) appears to have an inhibitory effect on large shoot regeneration (Table 1), while it promotes regeneration of small shoots (Data not shown). These observations are consistent with the idea that WIND1 promotes shoot initiation in the culture system but inhibit leaf growth. We confirmed that the DEX treatment itself does not affect callus formation and regeneration in this culture condition (Data not shown).

To test whether Arabidopsis WIND1 has similar effects in other crop plants, we used three independent lines of tomato *35S:AtWIND1-GR* which we observed promotion of callus formation in seedling germinated on WIND1 induction condition (Iwase et al. 2013), in a shoot regeneration assay using a method previously reported (Sun et al. 2006). We used segments of cotyledon and hypocotyl, treated with DEX (0.1  $\mu$ M) from the beginning of the culture containing phytohormones. We have, however, not detected any significant promotion of callus formation nor shoot regeneration so far (data not shown).

**Two-step induction of *WIND1* and *LEC2* promotes somatic embryogenesis**

Given that WIND1 increases the competency for regeneration, co-expression of *WIND1* with other developmental regulators might be a method to promote organ or tissue-specific regeneration. To explore this



possibility, we crossed the Arabidopsis *XVE-WIND1* line with an inducible overexpressor of a B3 domain transcription factor *LEC2* (*35S:LEC2-GR*) in which somatic embryogenesis is induced by DEX treatment (Ledwoń and Gaj 2009). We cut etiolated hypocotyls and used plantlets containing the SAM for examining the effect of transgene induction. When only DEX is applied and *LEC2* is solely induced, embryonic callus are generated from very limited loci such as those around the SAM and wound sites (Fig. 4a, b, c). When only ED is applied and thus *WIND1* is solely induced, callus formation is observed at around the SAM and broad region in hypocotyls but organ regeneration does not take place (Fig. 4d, e, f). Interestingly, *WIND1* induction followed by *LEC2* induction results in vigorous embryonic callus formation in nearly all tissues in the plantlets (Fig. 4g, h). When these somatic embryos generated in hypocotyls are transferred to a media without DEX and ED, they regenerate whole plants (Fig. 4i). These results strongly suggest that *WIND1* increases the number of competent cells that are responsive to regeneration stimuli and that combining *WIND1* with other developmental regulators offers novel opportunity for efficient regeneration.

## Discussion

In this study we found overexpression of *WIND1* in Arabidopsis and rapeseed greatly enhances *de novo* shoot regeneration from explants as well as callus cultured on media for promoting shoot regeneration. Expressing another transcription factor *LEC2* after the activation of *WIND1* also induces efficient somatic embryogenesis from tissue that hardly forms embryonic callus under single *LEC2* expression condition. These results reinforce our model that *WIND1* increases cellular plasticity in plants, which subsequently promotes *de novo* organogenesis.

## Roles of wounding in tissue culture

Generally, explants, not intact plants, are used as a starting material in tissue culture. However, roles of wounding in tissue culture system have not been explicitly acknowledged thus far. In this study we observed extensive lateral roots generated from unwounded roots after the CIM-SIM treatment (Fig. 1a, b). This observation clearly shows that cell fate change requires wounding treatment. It is well known that lateral root formation is induced by auxin (Yadav et al. 2010) which is transported from shoots (Robert and Friml 2009), therefore extensive lateral root development observed in this study might be explained by endogenous auxin which comes from aerial tissue. It is conceivable that disturbance of these tissue-tissue or cell-to-cell communication caused by wounding is one of the triggers for the cell fate change such as callus formation

and/or shoot regeneration in the tissue culture system. Surprisingly, WIND1 ectopic overexpression achieves shoot regeneration from roots even from unwounded explants (Fig.1c, Fig.2d) and conversely, reduction of shoot regeneration is seen in the wounded explants of WIND1 chimeric repressor line (Fig.1e, f). We previously showed that *WIND1* is expressed at the wound site of root explants (Iwase et al. 2011b), together indicating that one role of the wounding step is to increase the expression of key factors such as WIND1 required for the cell fate modification.

**WIND1 plays pivotal roles for the acquisition of regeneration competency in the culture system**

It is well known that regeneration capacity of plant cells varies depending on the tissue or cell origin and type of callus (Guzzo et al. 1995). Calli have various physiological traits even if they are induced under the same culture condition, like unwounded site callus and wounded site callus in the CIM culture (Sugimoto et al. 2010; Iwase et al. 2011; Ikeuchi et al. 2013). In this study we observed that shoot regeneration from both unwounded site callus and wounded site callus in *Arabidopsis* is inhibited in the *ProWIND1:WIND1-SRDX* plant (Fig. 1e, f). Moreover, rapeseed callus derived from hypocotyls close to the SAM (Segment I, Fig. 3a) and those distant from the SAM (Segment II, Fig. 3a) show different capacity for regeneration (Table 1, Table 2). Very strikingly, shoot regeneration competency is increased by AtWIND1 activation in both Segment I and Segment II (Table 1, Table 2), implying that capacity for regeneration in callus, at least in *Arabidopsis* and rapeseed, is dominantly controlled by WIND1 and other functional redundant factors such as WIND2-4. Our observation that the frequency of shoot regeneration in the Segment II is increased more than 20 times also highlights that WIND1 can improve regeneration capacity in tissue having less competency for regeneration. The molecular mechanisms underlying this amazing ability of WIND1 should be elucidated in future studies in order to understand the acquisition of competence at cellular and molecular levels. It is important to note that our trial of WIND1 overexpression in tomato tissue culture using cotyledon and hypocotyl explants does not result in any obvious positive effects on callus and shoot regeneration so far. The main differences between these experiments and those we reported previously (Iwase et al. 2013) are timing of WIND1 induction and presence of phytohormones in culture medium. Since WIND1 induction from germination promotes callus formation and successive shoot regeneration from hypocotyls on phytohormone free medium (Iwase et al. 2013), reactions of tissues against WIND1 activation must be different depending on, for instance, tissues and their developmental stages. It will be therefore interesting to further explore how WIND1 induction is influenced by various factors, such as timing and strength of WIND1 induction, tissue used for induction as

well as application of exogenous plant hormones.

### Cell fate manipulation by transcription factors

As reported in the reprogramming of mammalian somatic cells into pluripotent stem cells by Oct4, Sox2, Klf4 and c-Myc so-called Yamanaka-factors (Takahashi and Yamanaka 2006), co-expression of transcription factors has a potential to alter cellular differentiation status dramatically. Gallois et al. (2004) observed that floral organ directly regenerated from root tip when LEAFY (LFY) was co-expressed with WUSCHEL (WUS). LFY is a FLO/LFY family transcription factor that acts as a master regulator of floral development (Weigel et al., 1992) and WUS is a homeodomain containing transcription factor that maintains the shoot apical cells in an undifferentiated state. *WUS* overexpression alone causes ectopic shoot regeneration from root tip but *LFY* overexpression alone never forms floral organs on root. They also showed *WUS* expression combined with auxin induces somatic embryogenesis in roots, suggesting that *WUS* can establish developmental plasticity (Gallois et al. 2004). Similar to this pioneer work, our results reinforce that WIND1 has an ability to increase the developmental plasticity of plant cells from tissue and callus. Our results also show that the combined expression of WIND1 and other developmental regulators is useful for directed alteration of plant cell fate in tissue culture.

### Future perspectives

How plants regulate developmental plasticity is one of the biggest unanswered questions in plant biology (Vogel 2005). In this study we uncovered that wounding or wound-induced transcription factor WIND1 plays central roles in *in vitro* organ regeneration. Recent studies have also started to shed light on understanding this question. For instance, comprehensive gene expression analyses have begun to advance our understandings of molecular networks underlying shoot regeneration from Arabidopsis root explants (Che et al. 2002; Che et al. 2006), cell fate reprogramming from wounded gametophore leaf cells into chloronema apical cells in *Physcomitrella patens* (Ishikawa et al. 2011), tissue reunion in the Arabidopsis inflorescence stem (Asahina et al. 2011) and whole plant regeneration from Arabidopsis mesophyll protoplasts (Chupeau et al. 2013). Several key regulators involved in plant regeneration have also been identified from other studies, for instance, using Arabidopsis cDNA overexpression screening (Banno et al. 2001), Arabidopsis temperature sensitive mutants (Ohtani and Sugiyama 2005; Tamaki et al. 2009; Ohbayashi et al. 2011), and quantitative trait loci analyses based on comparison of Arabidopsis accessions (Motte et al. 2014). Such regulators are

interconnected at multiple levels and further system-level studies of these molecular networks should accelerate our understanding of plant cell totipotency as well as building efficient tissue culture systems.

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Tables

Table 1. Number of shoots (>5 mm in length) regenerated from rapeseed hypocotyl segments I and II

Induction		Number of total shoots	Number of segments	Average Number of shoots per segment
Segment I	DEX - -	7	11	0.65
	DEX ++	5	11	0.45
Segment II	DEX - -	49	126	0.39
	DEX + -	46	63	0.73
	DEX ++	20	63	0.32

Data were scored 2 month after start of culture

Table 2. Number of shoots (including <5mm in length) regenerated from rapeseed hypocotyl segments II

Induction		Number of total shoots	Number of segments*	Average Number of shoots per segment
Experiment 1**	DEX - -	3	10	0.3±2.0
	DEX + -	59	10	5.9±3.9
	DEX ++	74	10	7.4±3.5
Experiment 2**	DEX - -	2	10	0.2±1.3
	DEX + -	75	10	7.5±3.8
	DEX ++	95	10	9.5±4.3

\*10 segments were randomly selected. \*\* Two independent culture experiments



## Figure Legends

### Fig.1. Wounding has a pivotal role on cell fate change.

(a-f) 7-day-old *Arabidopsis* whole seedlings, with or without wound treatments of root, were transferred onto callus induction medium (CIM) and cultured for 4 days. Subsequently, plants were transferred to shoot induction medium (SIM) and cultured for 21 days. Roots of wild-type Col-0 plants (WT) unwounded (a) or wounded (b). Roots of *WIND1* overexpressing plants (*35S:WIND1*), unwounded (c) or wounded (d). Roots of *WIND1* loss-of-function *ProWIND1:WIND1-SRDX* (*WIND1-SRDX*) plants, unwounded (e) or wounded (f). Multiple lateral roots appeared in all unwounded plants, while shoots regenerated only from WT cut roots. Contrastingly, in *35S:WIND1*, both uncut and cut roots regenerated shoots. Note that unwounded explant remains aerial part. Scale bars represent 3 mm.

### Fig. 2. *WIND1* promotes acquisition of competency.

(a, b) 7-day-old *Arabidopsis* wild-type seedlings (WT) were transferred onto CIM (a), or 10  $\mu$ M 17 $\beta$ -estradiol (ED)-containing MS medium (b) for 1 day, and subsequently cultured on SIM for 21 days. (c, d) 7-day-old *XVE-WIND1* plants, harboring a transgene for chemical induction of *WIND1* transcription, were transferred onto CIM (c), or 10  $\mu$ M 10  $\mu$ M ED containing MS medium (e) for 1 day, then transferred to SIM for 21 days. (e) Number of regenerated shoots from the roots of WT and *XVE-WIND1* plants. Shoots were defined as green structure having two true leaves or leaf primordia. Error bars show s.d. (n = 4, Biological replicates). Scale bars represent 1 mm.

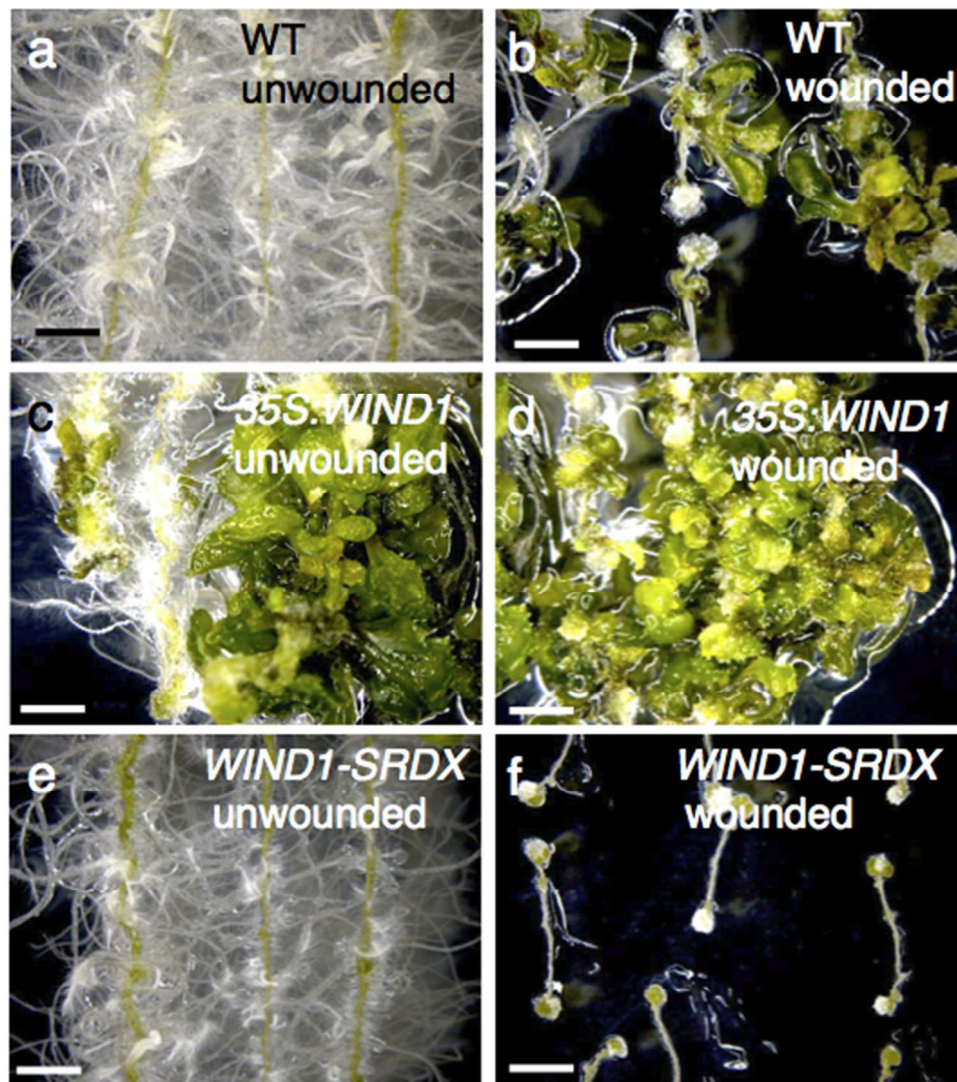
### Fig. 3. *AtWIND1* increases shoot regeneration-competent cells in rapeseed callus.

(a) Schematic diagram of tissue samples used for the rapeseed regeneration assay. Parts of the hypocotyl representing I and II in the figure show segments 2 mm (without meristem), and 1.2~6.2 mm from shoot apical meristem, respectively. (b) Schematic diagram of time periods used for chemical treatments in the regeneration assay. 2,4-D, BA, and DEX are 2,4-dichlorophenoxyacetic acid (1mg/L), 6-Benzylaminopurine (3 mg/L) and dexamethasone (0.1  $\mu$ M), respectively. *WIND1* induction was controlled by culturing on medium with DEX (+) or without DEX (-). wk; week, D; day. (c to e) Shoot regenerating calli generated from the II segment of hypocotyls, cultured without DEX for 13 weeks (DEX --; c), with DEX treatment for 7 weeks followed by DEX-free medium for 6 weeks (DEX +-; d) and with continuous DEX treatment for 13 weeks (DEX ++; e). (f) Three representative calli from each of the DEX (--), DEX (+-) and DEX (++) conditions shows are

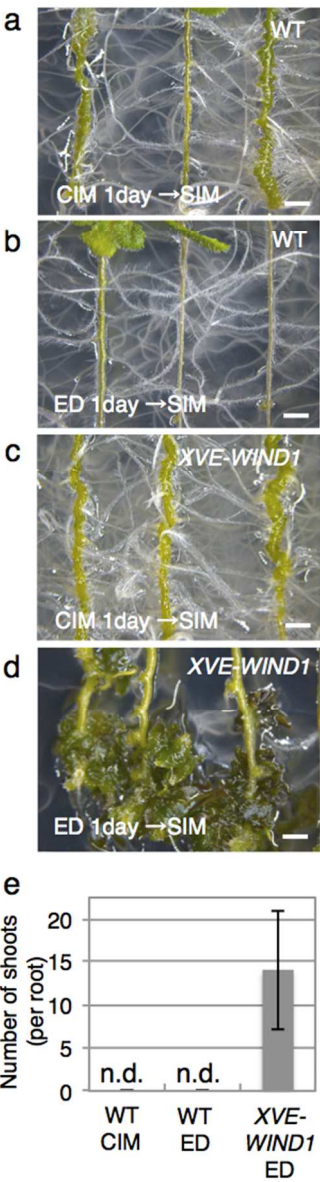
shown in the left, middle and right columns, respectively. Scale bars represent 1 cm (**c, d, e**) and 3mm (**f**).

**Fig. 4. Two-step expression of *WIND1* and *LEC2* promotes somatic embryogenesis in Arabidopsis**

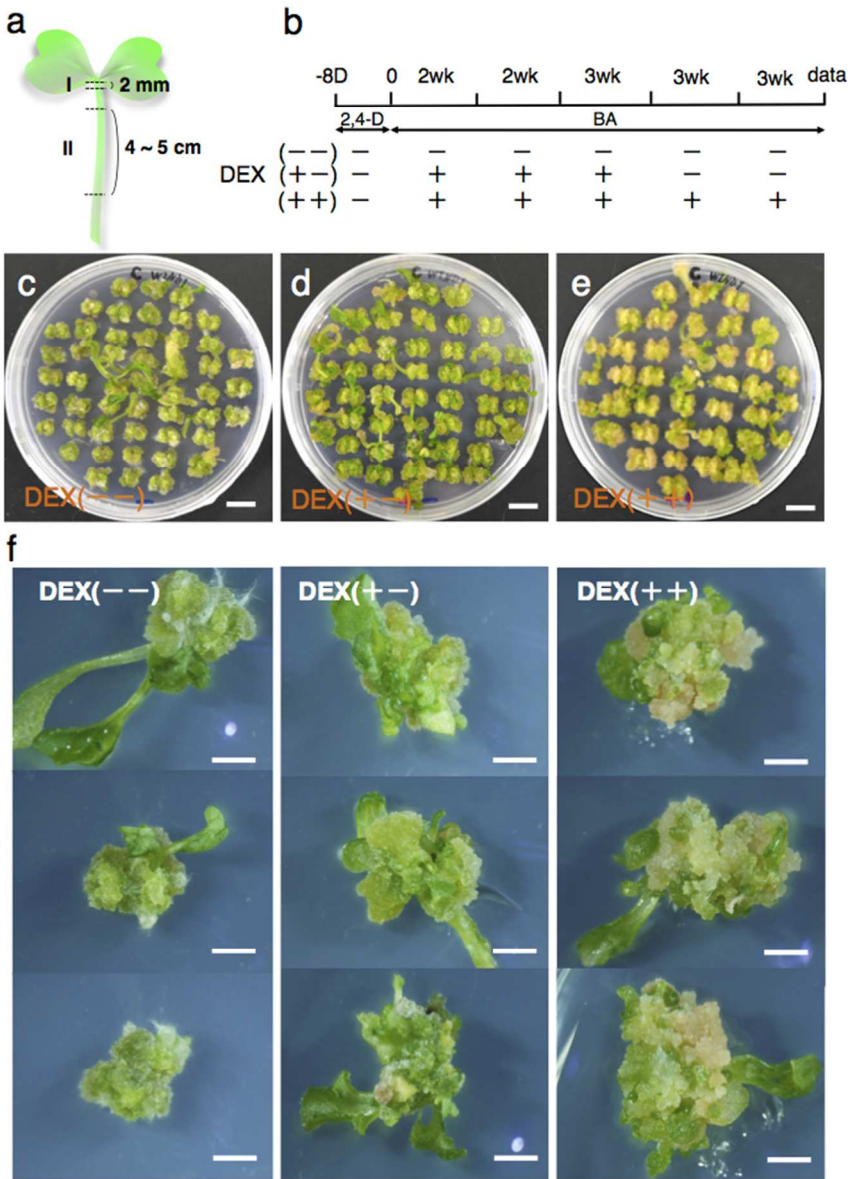
(**a, b, c**) 7-day-old etiolated *35S:LEC2-GR* plant seedlings were cut at positions 7 mm away from the junction and the plantlets with the shoot apical meristem were cultured on 10  $\mu$ M DEX-containing medium for 25 days. Magnification of shoot part and hypocotyl region in a plantlet in (**a**) is shown in (**b**). (**c**) is magnification of hypocotyl part in another individual. (**d, e, f**) 7-day-old etiolated *XVE-WIND1* plant seedlings were cut at positions 7 mm away from the junction and the plantlets with the shoot apical meristem were cultured on 1  $\mu$ M 17 $\beta$ -estradiol (ED)-containing medium for 25 days. Magnification of shoot part and hypocotyl region of a plantlet in (**d**) are shown in (**e**) and (**f**), respectively. (**g, h**) 7-day-old etiolated *35S:LEC2-GR/XVE-WIND1* seedlings were cut at positions 7 mm away from the junction and the plantlets with the shoot apical meristem were cultured on 1  $\mu$ M 17 $\beta$ -estradiol (ED) for 4 days then cultured on 10  $\mu$ M DEX-containing medium for 21 days. Magnification of shoot part of a plantlet in (**g**) is shown in (**h**). (**i**) Embryonic callus generated in hypocotyls were transferred on DEX and ED-free medium then cultured for 30 days. Black and white asterisks show somatic embryo-like structure. Scale bars represent 1 mm.



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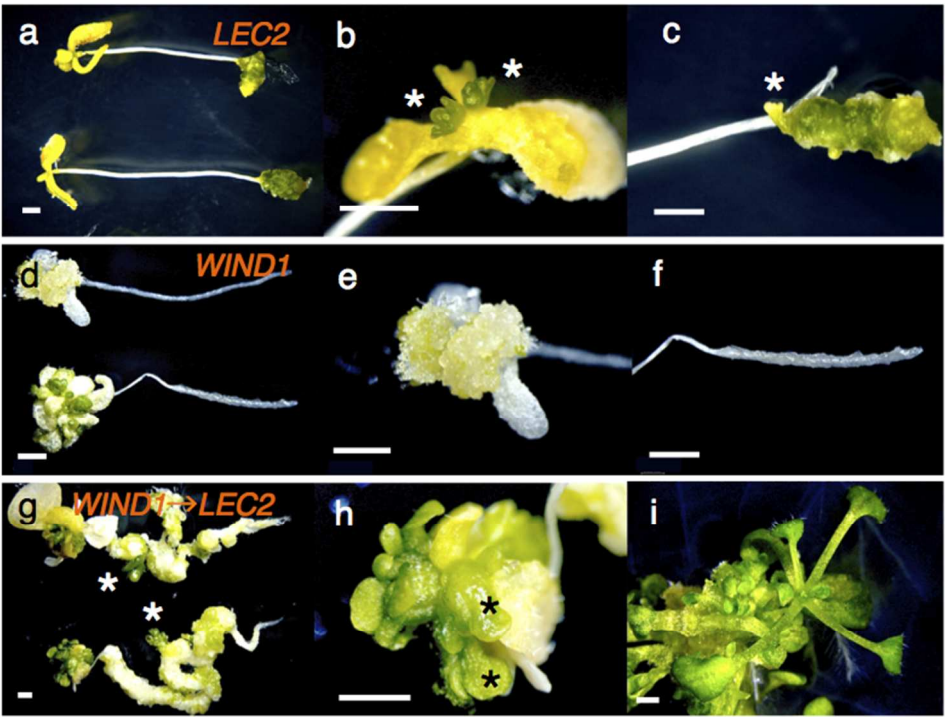


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review