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Report

The AP2/ERF Transcription Factor WIND1 Controls Cell Dedifferentiation in *Arabidopsis*

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Summary

Many multicellular organisms have remarkable capability to regenerate new organs after wounding. As a first step of organ regeneration, adult somatic cells often dedifferentiate to reacquire cell proliferation potential, but mechanisms underlying this process remain unknown in plants. Here we show that an AP2/ERF transcription factor, WOUND INDUCED DEDIFFERENTIATION 1 (WIND1), is involved in the control of cell dedifferentiation in *Arabidopsis*. *WIND1* is rapidly induced at the wound site, and it promotes cell dedifferentiation and subsequent cell proliferation to form a mass of pluripotent cells termed callus. We further demonstrate that ectopic overexpression of *WIND1* is sufficient to establish and maintain the dedifferentiated status of somatic cells without exogenous auxin and cytokinin, two plant hormones that are normally required for cell dedifferentiation [1]. In vivo imaging of a synthetic cytokinin reporter [2] reveals that wounding upregulates the B-type *ARABIDOPSIS* RESPONSE REGULATOR (ARR)-mediated cytokinin response and that WIND1 acts via the ARR-dependent signaling pathway to promote cell dedifferentiation. This study provides novel molecular insights into how plants control cell dedifferentiation in response to wounding.

Results and Discussion

WIND1 Expression Is Rapidly Induced by Wounding

Cellular dedifferentiation, in which adult somatic cells change from differentiated to less differentiated states and regain cellular proliferative competence, occurs widely in multicellular organisms. Recent studies in mammals have demonstrated that forced expression of defined transcription factors can induce pluripotent stem cells from adult somatic cells [3], indicating that cell dedifferentiation is a genetically controlled process. One of the most profound examples of cell dedifferentiation is provoked by wounding and is vital to promoting regeneration of new tissues and organs [4]. Wound-induced cell dedifferentiation is widespread in various organisms including fish, amphibians, mammals, and plants, but it remains to be determined whether these naturally occurring dedifferentiation events are also stimulated by transcription factors. Compared to animals, plants have a remarkable capacity to change their cell fate, and the first examples of in vitro plant cell dedifferentiation, unorganized cell proliferation to form callus, were reported as early as the 1930s [5]. It was later discovered that the ratio of two plant growth regulators, auxin and cytokinin, is critical to promote dedifferentiation and subsequent redifferentiation of plant explants [1]. It is well established in *Arabidopsis*, for instance, that auxin-rich medium promotes callus formation whereas subsequent culture of callus on high-auxin or high-cytokinin medium permits regeneration of roots and shoots, respectively [6]. Similar physiological manipulation of cell dedifferentiation and redifferentiation has been employed extensively in other plant species [7], but surprisingly little is known about how plants regulate cell dedifferentiation at the molecular level [8–10].

As reported for many other plants, *Arabidopsis* seedlings undergo cell dedifferentiation and form callus in response to wounding (Figure 1A). To identify key regulators mediating plant cell dedifferentiation, we previously isolated a set of genes differentially expressed between *Arabidopsis* seedlings and cultured cell lines [11]. Among them, *WOUND INDUCED DEDIFFERENTIATION 1* (*WIND1*), a putative AP2/ERF-family transcription factor previously called *RAP2.4* (*At1g78080*) [12], is preferentially expressed in cultured cells [11]. To explore the possibility that *WIND1* is involved in wound-induced cell dedifferentiation, we first tested whether the *WIND1* expression is induced by wounding. Previous microarray experiments implicated *WIND1* as a wound-responsive gene [13], and consistent with this, transgenic plants carrying *WIND1* promoter-driven β-glucuronidase (*Pro$_{WIND1}$:GUS*) and green fluorescent protein (*Pro$_{WIND1}$:GFP*) showed highest promoter activity at the wound site, whereas in intact plants, its activity was visible only in some cell types such as root pericycle and meristematic stem cell niche (Figures 1A and 1B; see also Figure S1 available online). Quantitative RT-PCR analyses of 30-day-old wild-type leaves revealed that *WIND1* transcript levels were increased up to 3-fold within a few hours after wounding (Figure S1). Consistently, the *Pro$_{WIND1}$:GFP* marker showed that *WIND1* promoter activity was strongly enhanced within several hours after wounding (Figure 1D). *WIND1* expression persisted in pericycle and surrounding cells beyond the first 24 hr and was also detected in proliferating callus cells (Figures 1A and 1C). Similarly, the WIND1-GFP fusion proteins, expressed under the *WIND1* promoter, accumulated within the nucleus of cells close to the wound site (Figure S1). These data clearly demonstrate that *WIND1* is rapidly induced at the wound site and is constitutively expressed during postwounding callus development.

Overexpression of WIND1 Is Sufficient to Induce Unorganized Cell Proliferation and to Maintain the Dedifferentiated State

To further investigate the involvement of *WIND1* in cell dedifferentiation, we ectopically expressed *WIND1* under the

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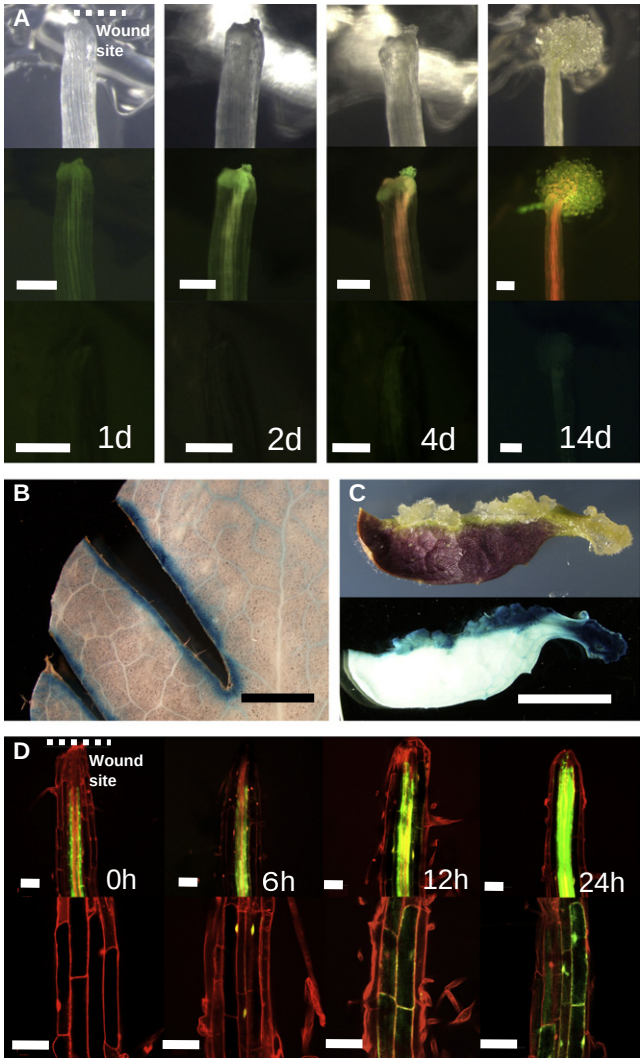

Figure 1. *WIND1* Expression Is Rapidly Induced at the Wound Site

(A) Wounding induces callus formation in etiolated hypocotyls (upper panels), displaying strong Pro_{WIND1}*:GFP* expression (green, middle panels). Lower panels show wild-type control visualized using a filter set to remove red autofluorescence from chlorophyll. Images of representative hypocotyls at 1 to 14 days (d) after wounding are shown.

(B and C) Pro_{WIND1}*:GUS* marks *Arabidopsis* leaf cells at the wound site (B) and proliferating callus cells (C, lower panel). A light micrograph (C, upper panel) shows corresponding callus.

(D) Pro_{WIND1}*:GFP* is upregulated within several hours after wounding. Images of representative roots at 0 to 24 hr (h) after wounding are shown. Confocal optical sections show upregulation of Pro_{WIND1}*:GFP* in pericycle (upper panels) and surrounding cells (lower panels). Cellular boundaries are highlighted by propidium iodide (PI, red).

Scale bars represent 500 μm in (A), 1 mm in (B), 5 mm in (C), and 50 μm in (D). See also Figure S1.

control of the cauliflower mosaic virus 35S (35S) promoter (Figure S2A). Among the 106 transformants we recovered, plants with the most severe morphological alterations (type III, ~20% of all transformants) aborted normal postembryonic development within a few days after germination and initiated callus-like unorganized cell proliferation around the shoot meristem (Figures 2A, 2D, and 2E; Figure 4C). When these masses of cells were excised and transferred to fresh Murashige-Skoog (MS) medium, they continued to proliferate vigorously in the absence of exogenous auxin and cytokinin (Figures S2C and S2D). Similarly, ~60% of seedlings displayed strong morphological defects with severely stunted leaves (type II; Figure 2C; Figure 4C), and when these plants were incubated further on MS medium, they formed callus-like structures from various parts of plants including hypocotyls and roots (Figures 2F and 2G). Around 20% of plants displayed only mild distortion of leaf shapes (type I; Figure 2B; Figure 4C), but compared to wild-type, their T3 progenies formed callus at higher rates after wounding (Figure 3A). We found that these morphological defects correlated positively with the level of *WIND1* transcripts (Figure S2B). The overall morphology of *35S:WIND1* cells strongly resembled that of callus cells induced by exogenous auxin (2.2 μM 2,4-dichlorophenoxyacetic acid [2,4-D]) (Figures S2E and S2F), and the gene expression profile of *35S:WIND1* overlapped significantly with that of 2,4-D-induced callus and another auxin (1-naphthalene acetic acid [NAA])-induced T87 callus [14] ($p < 2.2^{-16}$; Figures 2O and 2P; Tables S1 and S2).

When *WIND1* expression is induced by 17β-estradiol in 21-day-old seedlings transformed with the LexA-VP16-estrogen receptor (*XVE*)-*WIND1* construct [15], callus-like cell masses are formed in mature shoots and roots (Figures S2G–S2J), indicating that elevated *WIND1* expression in postembryonic tissues is sufficient to promote unorganized cell proliferation. Recent work by Atta et al. [16] and Sugimoto et al. [17] has demonstrated that callus, induced by auxin-rich callus-inducing medium (CIM) [6], is formed via activation of stem cell-like pericycle cells rather than dedifferentiation of adult somatic cells. In contrast, both confocal and light microscopy of individual cells in *XVE-WIND1* plants germinated and incubated on 17β-estradiol-containing medium for 4 to 8 days revealed an induction of callus-like cells from the epidermal cell layer of roots, hypocotyls, and cotyledons whereas cells in *XVE-WIND1* plants grown on control medium continued to undergo differentiation (Figures 2H–2N). These morphological alterations were already visible within a few days after 17β-estradiol treatment (data not shown), suggesting that upon WIND1 activation, adult somatic cells withdraw from normal differentiation programs and start developing unorganized masses of cells. In addition, we found that callus-like cells induced from *XVE-WIND1* root explants by 17β-estradiol redifferentiated into both roots and shoots after transfer to 17β-estradiol-free MS medium (Figures S2K and S2L), indicating that *WIND1*-overexpressing cells have reacquired pluripotency, the competence to develop into more than one type of mature organs. Together, these results demonstrate that *WIND1* promotes cell dedifferentiation in *Arabidopsis*.

WIND1 Controls Wound-Induced Cell Dedifferentiation In Vivo

WIND1 and five other closely related genes form a small subfamily of the AP2/ERF transcription factors, and the expression of these homologs was also wound responsive (Figures S3A and S3B). Furthermore, 35S-driven expression of *WIND2* (*At1g22190*), *WIND3* (*At1g36060*), and *WIND4* (*At5g65130*), but not of *At4g39780* and *At2g22200*, caused extensive cell proliferation (Figure S3B), suggesting that these three genes also promote cell dedifferentiation. To further explore the function of *WIND* homologs in vivo, we identified their T-DNA insertion lines, all of which except *WIND4* were complete null mutants (Figures S3C–S3E). It turned out, however, that none of the single mutants nor the quadruple *wind1,2,3,4* mutant were defective in wound-induced callus formation (data not

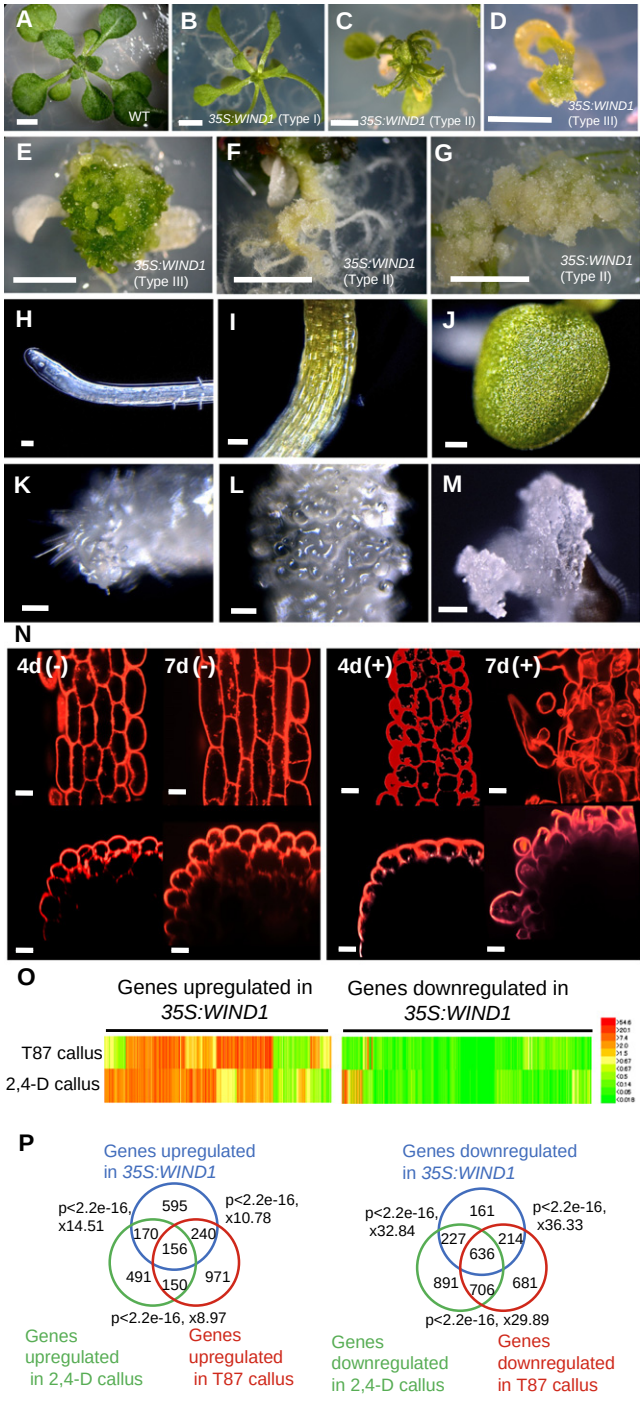

Figure 2. Ectopic *WIND1* Expression Promotes Cell Dedifferentiation in the Absence of Exogenous Phytohormones

(A–D) 21-day-old wild-type (WT, A) and *35S:WIND1* seedlings (B–D) grown on phytohormone-free MS medium displaying weak (type I, B), intermediate (type II, C), and strong (type III, D) levels of morphological alterations.

(E–G) 48- to 60-day-old *35S:WIND1* seedlings forming callus-like structures at shoots (E), hypocotyls (F), and roots (G).

(H–M) *XVE-WIND1* seedlings germinated on phytohormone-free MS medium without (H–J) or with 10 μM 17β-estradiol (K–M). Images of representative 6-day-old roots (H and K), hypocotyls (I and L), and 8-day-old cotyledons (J and M) are shown.

(N) Confocal optical sections of PI-labeled epidermal cells in 4- and 7-day-old *XVE-WIND1* hypocotyls (upper panels) and cross-sections generated from confocal Z stacks (lower panels). *XVE-WIND1* seedlings were germinated in the absence (−) or presence (+) of 10 μM 17β-estradiol.

(O and P) Gene expression profiles in *35S:WIND1* type III callus-like tissue overlap significantly with those of 2,4-dichlorophenoxyacetic acid (2,4-D)-induced callus and 1-naphthalene acetic acid (NAA)-induced T87 callus.

(O) Clustering analysis of 1161 genes upregulated (left, fold change > 5) and 1238 genes downregulated (right, fold change < 0.2) in *35S:WIND1* compared to wild-type.

(P) Venn diagrams showing overlapping genes among different data sets. p values and odds ratios, representing number of overlapping genes/number of genes expected by chance, are listed for each data set comparison.

Scale bars represent 3 mm in (A)–(G); 100 μm in (H), (I), (K), and (L); 250 μm in (J) and (M); and 20 μm in (N). See also Figure S2.

shown), suggesting higher levels of functional redundancies exerted, for instance, by other close homologs. To overcome this, we employed chimeric repressor gene-silencing technology [18] and dominantly repressed WIND1 function by expressing WIND1-SRDX (SUPERMAN repression domain) chimeric proteins under the *WIND1* promoter. As expected, *WIND1-SRDX* plants showed reduced levels of wound-induced callus formation from hypocotyls (Figure 3A) and of exogenous hormone-induced callus formation from hypocotyl explants (Figure 3B), whereas they displayed no other obvious developmental defects under our standard growth conditions. These data clearly demonstrate that WIND1, together with other functionally redundant factors, controls cell dedifferentiation in vivo.

WIND1 Promotes B-Type ARR-Mediated Cytokinin Responses

Given that the balance between auxin and cytokinin is critical to promote callus formation in vitro [1], WIND1 may control this process by modulating their biosynthesis and/or signaling. This idea was supported by the finding that *35S:WIND1* hypocotyl explants displayed increased cytokinin responses, for instance developing callus at low and intermediate cytokinin levels (0.23–2.3 μM kinetin) whereas wild-type explants formed hardly any callus (Figure 3B). High cytokinin-to-auxin ratios promote shoot regeneration in vitro [1, 6], and we observed extensive shoot regeneration from *35S:WIND1* explants (Figure 3B), further suggesting that *35S:WIND1* plants are hypersensitive to cytokinin. Conversely, *WIND1-SRDX* explants resembled those of *arr1,12* double mutants defective in B-type ARR-mediated cytokinin signaling [19], with strong suppression of callus formation at all tested hormone concentrations (Figure 3B). Low cytokinin-to-auxin ratios promote root regeneration [1, 8], and like *arr1,12* mutants, *WIND1-SRDX* explants tended to form roots even in high-cytokinin conditions (Figure 3B), suggesting that cytokinin response is compromised in *WIND1-SRDX* plants.

Our results so far suggested that wounding induces local *WIND1* transcription, which then enhances endogenous cytokinin responses to promote cell dedifferentiation. Previous physiological experiments implied that cytokinin responses are elevated in wounded plant tissues [20], and a synthetic reporter, two-component-output sensor (*TCS*):*GFP* [2], indeed revealed that the B-type ARR-mediated cytokinin response was elevated within 24 hr in pericycle and surrounding cells close to the wound site (Figure 4A; Figure S4A). High levels of *TCS:GFP* and chlorophyll autofluorescence, another physiological indicator of cytokinin response [21], were retained beyond the first 24 hr in wild-type hypocotyls and roots, but these signals were strongly repressed

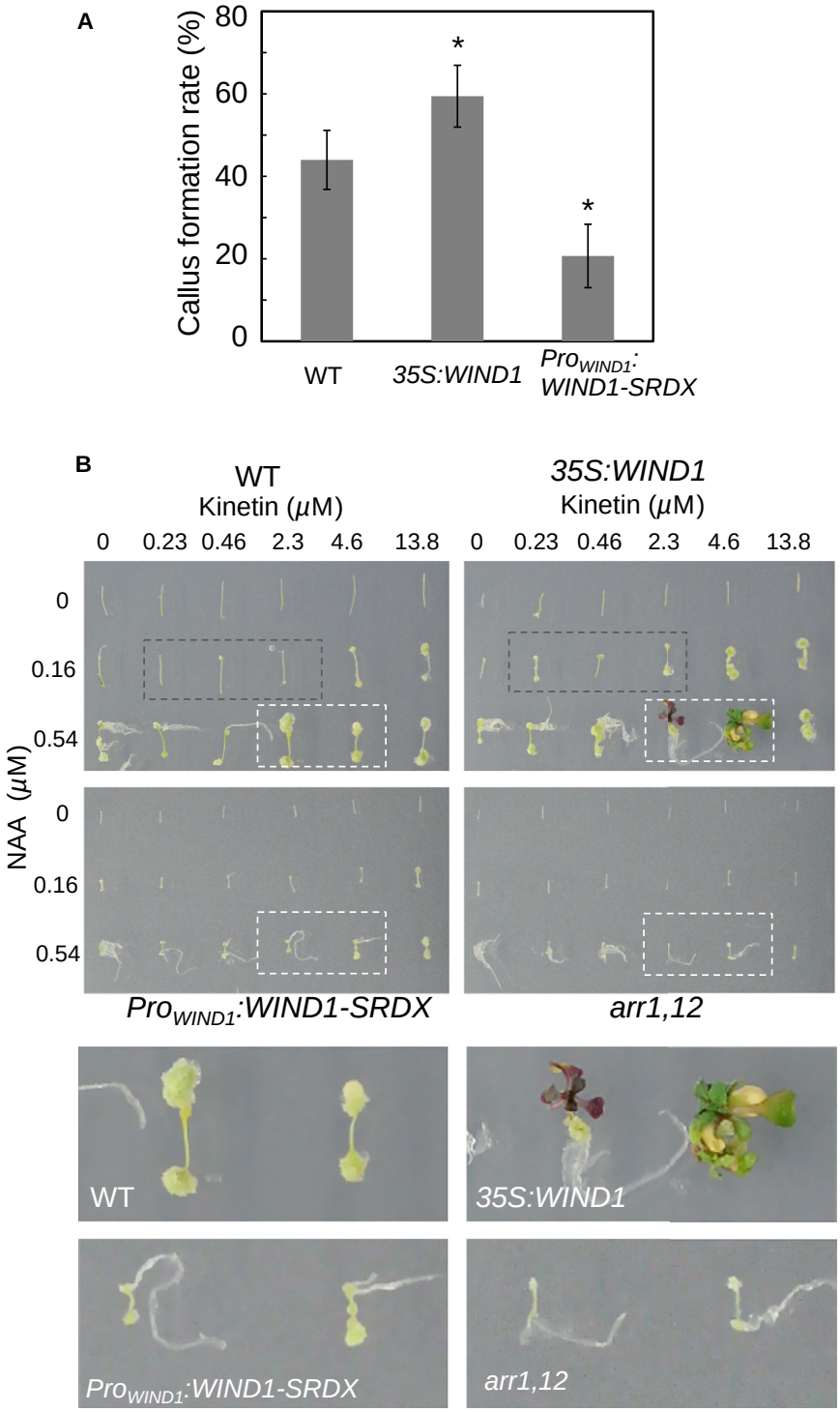

Figure 3. *WIND1* Controls Cell Dedifferentiation In Vivo

(A) Expression of a *WIND1* chimeric repressor impedes wound-induced callus formation. Upper ends of WT, *35S:WIND1* (type I), and *Pro*$_{WIND1}$*:WIND1-SRDX* etiolated hypocotyls were excised, and remaining seedlings were cultured on phytohormone-free MS medium (see also Experimental Procedures). Error bars represent standard deviation (SD). *$p < 0.01$ by t test.

(B) Compared to WT, *35S:WIND1* displays increased sensitivity to cytokinin (dashed gray and white boxes). In contrast, the chimeric repressor *Pro*$_{WIND1}$*:WIND1-SRDX* shows compromised cytokinin responses (dashed white boxes) similar to *arr1,12*. Magnified views of hypocotyl explants marked by the dashed white boxes are shown in the bottom four panels. Etiolated hypocotyl explants were cultured on MS medium containing different levels of auxin (NAA) and cytokinin (kinetin).

See also Figure S3.

in *WIND1-SRDX* plants (Figures 4A and 4B; Figure S4B). In agreement with these observations, *WIND1*-induced cell dedifferentiation was severely compromised in *arr1,12* mutants (Figures 4C–4E; Figure S4E), further substantiating that WIND1 functions through the ARR-dependent cytokinin signaling pathway. We found that endogenous levels of an active cytokinin, *trans*-zeatin, and its precursors were increased at least by 2-fold in *35S:WIND1* (Figure 4F). Although we cannot rule out the possibility that these changes reflect an indirect consequence of WIND1 activation, these data imply that WIND1 may also activate cytokinin production.

We also explored the possibility that WIND1 influences auxin signaling. To our surprise, however, neither the overall accumulation level of endogenous auxin, indole-3-acetic acid (IAA), nor cellular auxin response as visualized by *DR5:GUS* [22] was elevated in *35S:WIND1* (Figures 4G–4J). In agreement with this, *35S:WIND1* hypocotyl explants failed to regenerate roots under auxin-free culture conditions whereas auxin-overproducing *yucca1D* (*yuc1D*) mutants [23] developed roots (Figure 4K). WIND1 does not appear to act downstream of auxin or cytokinin signaling pathways, because *WIND1* expression was not modified by application of exogenous auxin or cytokinin (Figures S4C and S4D).

WIND1 as a Master Regulator of Cell Dedifferentiation in Plants

This study demonstrates that the AP2/ERF transcription factor WIND1 functions as a key molecular switch triggering cell dedifferentiation in *Arabidopsis*. Our model predicts that wound-induced WIND1 (and its functional homologs) activate the local cytokinin response, which in turn promotes cell dedifferentiation. Given that callus formation is promoted by auxin-rich CIM in *Arabidopsis* [6], the link between WIND1 and cytokinin signaling appears surprising. Previous reports, however, have demonstrated that an excess cytokinin-related response also induces unorganized cell proliferation in *Arabidopsis* [16, 24, 25], illustrating that normal differentiation programs in planta can be altered by multiple physiological conditions with varying ratios of auxin and cytokinin. It is interesting in this context that *ENHANCER OF SHOOT*

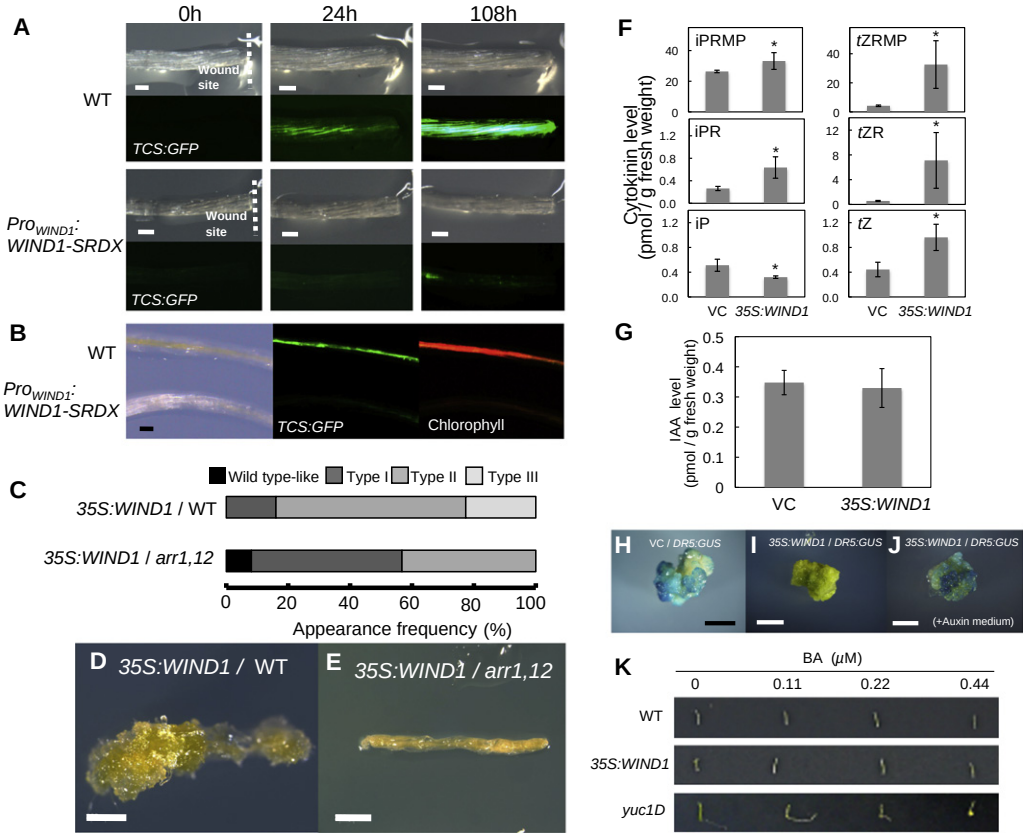

Figure 4. *WIND1* Promotes Cell Dedifferentiation via Cytokinin Signaling Pathways

(A and B) *TCS:GFP* (A and B, green) and chlorophyll autofluorescence (B, red) indicate elevated cytokinin responses in wounded hypocotyls. Both *TCS:GFP* and chlorophyll autofluorescence are strongly repressed in *Pro$_{WIND1}$:WIND1-SRDX*. Photos shown were taken at 0, 24, and 108 hr (A) and 14 days (B) after wounding.

(C–E) *arr1,12* mutations strongly suppress *WIND1*-induced cell dedifferentiation in T1 seedlings grown on MS medium (C) and T3 hypocotyl explants incubated on MS medium containing auxin (0.53 μM NAA) and cytokinin (0.22 μM 6-benzylaminopurine [BA]) (D and E). Phenotypic severity was scored according to the description in Figures 2B–2D. (n = 106 for WT; n = 37 for *arr1,12*.)

(F) Endogenous levels of *trans*-zeatin (*tZ*) and its precursors are increased in *35S:WIND1* type I plants. The following abbreviations are used: iPRMP, isopentenyladenine riboside 5′-monophosphate; *t*ZRMP, *trans*-zeatin riboside 5′-monophosphate; iPR, isopentenyladenine riboside; *t*ZR, *trans*-zeatin riboside; iP, isopentenyladenine; VC, vector control. Error bars represent SD (n = 3). *p < 0.05 by t test.

(G) Endogenous levels of indole-3-acetic acid (IAA) are not altered in *35S:WIND1* type I plants. Error bars represent SD (n = 3).

(H–J) Ectopic *WIND1* overexpression does not modify cellular auxin responses as visualized by the auxin responsive *DR5:GUS* reporter.

(H and I) Empty vectors and *35S:WIND1* vectors were introduced into the *DR5:GUS* reporter line, and the resulting double-transgenic plants were incubated on MS medium with (H, VC plants) or without auxin and cytokinin (I, *35S:WIND1* plants).

(J) GUS expression is visible in *35S:WIND1* callus transferred to MS medium with auxin (2.2 μM 2,4-D).

(K) Cytokinin response assay of WT, *35S:WIND1* type I, and *yucca1D* (*yuc1D*). Hypocotyl explants were harvested from 7-day-old etiolated seedlings and cultured on MS medium containing different levels of BA.

Scale bars represent 200 μm in (A) and (B), 0.5 mm in (D) and (E), and 2 mm in (H)–(J). See also Figure S4.

REGENERATION 1 [25], implicated in cytokinin signaling, is one of the genes highly activated in WIND1-overexpressing cells (Table S1). Intriguingly, our transcriptome analysis detected significant overlaps between genes misexpressed in WIND1-induced callus and those differentially expressed in auxin-induced callus (Figures 2O and 2P), suggesting that these two types of callus have some common characteristics, e.g., being able to proliferate and regenerate new organs. We also noted that the expression of *TUMOROUS SHOOT DEVELOPMENT 1* (*TSD1*)/*KORRIGAN* and *TSD2* genes [26, 27], whose loss of function causes callus-like cell formation, was not markedly modified in WIND1-overexpressing cells (Tables S1 and S2), suggesting that TSD1 and TSD2 do not participate in WIND1-induced callus formation.

Recent studies have shown that CIM-induced *Arabidopsis* callus generated at nonwounded sites of plant explants is not dedifferentiated to a ground state and adopts root meristem organization irrespective of its tissue origin [17] (Figure S1I). In contrast, callus induced at the wound site does not display organized expression of root cell markers such as J0121 [28], *Pro$_{SCR}$:GFP-ER* [29], and *Pro$_{WOX5}$:GFP-ER* [30] (Figures S1H and S1I). Our data further reveal that, unlike CIM-induced callus formation at nonwounded sites [17], callus formation at the wound site is not disturbed in *solitary root* (*slr*) mutants [31] defective in lateral root initiation (Figures S1J). These results suggest that wounding induces callus formation through pathways different from those employing the lateral root differentiation program and that resulting callus possesses different levels of cell differentiation and/or dedifferentiation compared to callus at the nonwounded site.

This study shows that the differentiated status of plant somatic cells can change to a less differentiated state by

ectopic expression of transcription factors. It is becoming increasingly clear that there are multiple levels of cellular dedifferentiation status in many multicellular organisms [32, 33]. Although our data clearly show that WIND1 promotes dedifferentiation of relatively young root, hypocotyl, and cotyledon epidermal cells, it remains to be resolved in future studies how far WIND1 can force dedifferentiation, e.g., whether WIND1 can drive dedifferentiation of highly differentiated cells such as trichomes and root hairs. Previous studies have shown that ectopic expression of other AP2/ERF proteins, such as BABY BOOM and PLETHORA, can change cell fate in plants [34, 35]. Our data thus support a role for AP2/ERF proteins in controlling plant cell fate specification, although precise functions of individual AP2/ERF proteins are probably diverse. Putative WIND1 homologs are present in various land plants (data not shown); thus, similar mechanisms might operate to mediate cellular reprogramming in other plant species. In mice, wounding induces expression of the transcription factor Msx1, which then stimulates dedifferentiation of various digit mesenchymal cells [36]. It is therefore plausible that the transcriptional control of cell dedifferentiation represents a general principle underpinning wound-induced organ regeneration across animal and plant kingdoms. Discovery of WIND1 provides a new molecular basis to further dissect how transcriptional regulators reprogram cellular differentiation status.

Experimental Procedures

Plant Material, Growth Condition, and Transformation

Wild-type, J0121 [28], *ProWOX5:GFP-ER* [30], *TCS:GFP* [2], *DR5:GUS* [21], *arr1,12* [18], *yuc1D* [22], *slr* [28], and all other transgenic plants used in this study (except *ProSCR:GFP-ER* [29], which was in the Ws background) were in the Columbia background. T-DNA insertion lines for *WIND1* (SALK_020767), *WIND2* (SALK_139727), *WIND3* (SALK_091212), and *WIND4* (SALK_099481) were obtained from the Arabidopsis Biological Resource Center (ABRC). Plants were grown on soil at 22°C with a photoperiod of 16 hr light/8 hr dark. For plant transformation, we introduced T-DNA vectors carrying an appropriate construct into *Agrobacterium tumefaciens* strain GV3101 by electroporation, and the resultant *Agrobacterium* was infiltrated into *Arabidopsis* by the floral dip method [37].

To induce callus by exogenous phytohormones, we incubated plant explants on MS medium supplemented with Gamborg's B5 vitamin, 2% glucose, 0.05% 2-(N-morpholino)ethanesulfonic acid (MES), 0.8% phytoagar, and various concentrations of auxin and cytokinin. To induce callus formation by wounding, we dissected 7-day-old etiolated seedlings with microscissors at approximately 7 mm from the root-hypocotyl junction, removing an upper end of hypocotyls and cotyledons. Remaining seedlings were incubated on phytohormone-free MS medium supplemented with 0.05% MES, 0.5% sucrose, and 0.8% phytoagar. Callus formation, defined as generation of at least two new cells at the wound site, was scored after 4 days using a Leica M165 C stereomicroscope. An average rate of callus formation was calculated from seven independent experiments, and more than 100 hypocotyls per genotype were tested in each experiment.

Accession Numbers

Microarray data from this study have been deposited at the NCBI Gene Expression Omnibus with the accession number GSE21631.

Supplemental Information

Supplemental Information includes four figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2011.02.020.

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