Auxin-Independent *NAC* Pathway Acts in Response to Explant-Specific Wounding and Promotes Root Tip Emergence during de Novo Root Organogenesis in Arabidopsis[1]

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Plants have powerful regenerative abilities that allow them to recover from damage and survive in nature. De novo organogenesis is one type of plant regeneration in which adventitious roots and shoots are produced from wounded and detached organs. By studying de novo root organogenesis using leaf explants of Arabidopsis (*Arabidopsis thaliana*), we previously suggested that wounding is the first event that provides signals to trigger the whole regenerative process. However, our knowledge of the role of wounding in regeneration remains limited. In this study, we show that wounding not only triggers the auxin-mediated fate transition of regeneration-competent cells, but also induces the *NAC* pathway for root tip emergence. The *NAC1* transcription factor gene was specifically expressed in response to wounding in the leaf explant, but not in the wounded leaf residue of the source plant. Inhibition of the *NAC1* pathway severely affected the emergence of adventitious root tips. However, the *NAC1* pathway functioned independently of auxin-mediated cell fate transition and regulates expression of *CEP* genes, which encode proteins that might have a role in degradation of extensin proteins in the cell wall. Overall, our results suggest that wounding has multiple roles in de novo root organogenesis and that *NAC1* acts as one downstream branch in regulating the cellular environment for organ emergence.

De novo organogenesis, through which detached or wounded plant organs regenerate adventitious roots and shoots, commonly occurs under natural conditions and allows plants to recover from damage (Duclercq et al., 2011; Xu and Huang, 2014). The ability of plants to undergo de novo organogenesis is also widely exploited in agricultural biotechnologies such as tissue culture and vegetative reproduction via cuttings (Sussex, 2008). In tissue culture, adventitious roots and shoots can regenerate from a group of pluripotent cell mass, termed callus. Callus is usually induced by a high level of auxin in the medium, and callus formation was shown to follow the rooting developmental pathway (Sugimoto et al., 2010; He et al., 2012; Liu et al., 2014).

By culturing leaf explants of the model plant Arabidopsis (*Arabidopsis thaliana*) on B5 medium without exogenous hormones, we established a simple method to study de novo root organogenesis mimicking natural conditions (Chen et al., 2014). In this system, adventitious roots can directly regenerate from the leaf explant under the control of endogenous hormones (Chen et al., 2014; Liu et al., 2014). The process of de novo root organogenesis from the leaf explant involves fate transition of regeneration-competent cells (i.e. procambium and vascular parenchyma cells) (Liu et al., 2014). The first fate-transition step from competent cells to root founder cells is controlled by the *WUSCHEL-RELATED HOMEOBOX 11* (*WOX11*) transcription factor gene. The second fate-transition step is from root founder cells to root primordium cells and is marked by *WOX5*. The root apical meristem then differentiates from the root primordium and finally forms the adventitious root tip, which emerges from the leaf explant. Auxin was shown to play a critical role in promoting the fate transition of competent cells (Liu et al., 2014). Upon wounding, auxin is quickly produced in mesophyll cells of the detached leaf explant and is then polar-transported into competent cells near the wound to

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X.C. performed most of the experiments; J.C. analyzed RNA-seq data; Y.Z. and L.X. supervised the experiments; L.C. and G.Z. provided technical assistance to X.C.; X.C., J.C., H.H.,Y.Z., and L.X. analyzed the data; X.C. and L.X. conceived the project and wrote the article with contributions from all the authors.

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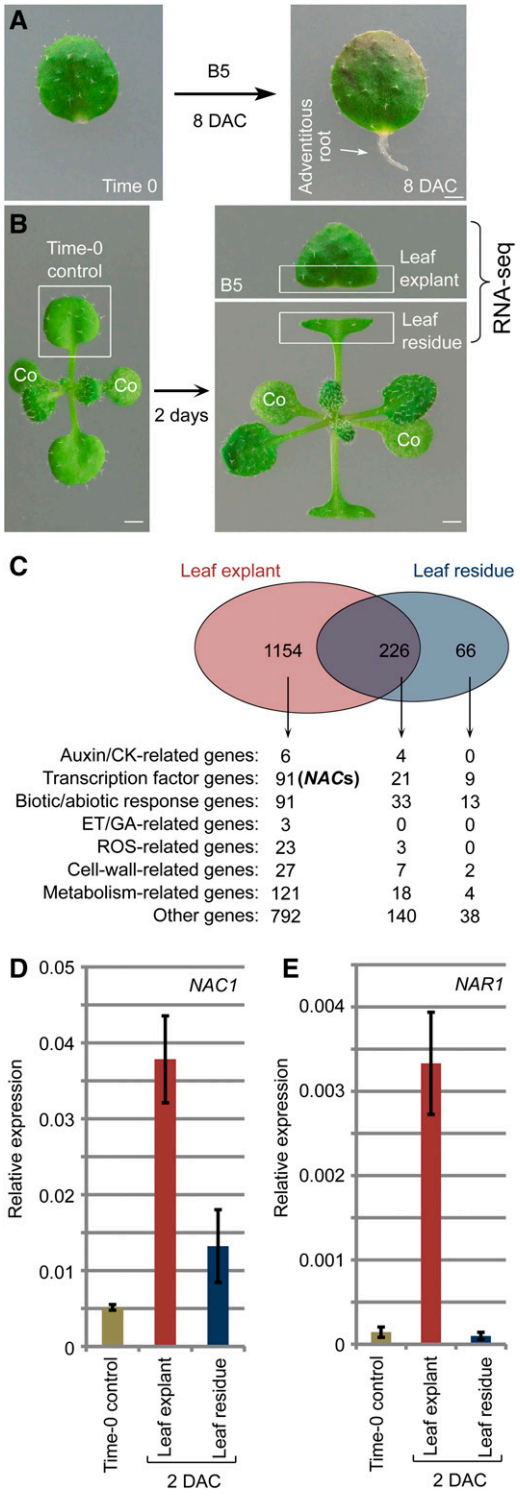

Figure 1. Identification of *NAC* genes induced in response to wounding. A, Regeneration system of de novo root organogenesis. B, Plant tissues used in RNA-seq analysis as indicated by the boxed regions. Leaf explant was cut and cultured on B5 medium without carbohydrates in light conditions. Wounded leaf residue was surrounded by air and did not touch the medium. co, cotyledon. C, RNA-seq data showing identification of *NAC* genes (also see Supplemental Table S1). D and E, qRT-PCR analysis of *NAC1* (D) and *NAR1* (E) transcript levels in leaf explants and leaf residue compared with those in time-0 leaf as indicated in B. Bars show SD from three PCR experiments. Results were confirmed in three biological repeats. Data from one repeat are shown. Bars = 1 mm in A and B.

activate *WOX11* expression, which triggers the first fate-transition step for competent cells. Therefore, activation of the hormone and gene expression networks can be traced back to the very beginning of the wounding event, in which the wound signals were proposed to be the initial molecules for regeneration (Liu et al., 2014; Xu and Huang, 2014). At present, the molecular nature of the wound signal(s) remains unclear.

The results of several studies have suggested that many physical events and chemical signals change quickly in response to wounding (León et al., 2001; Maffei et al., 2007). Other studies have characterized wound-responsive genes induced during plant regeneration, such as the *WOUND INDUCED DEDIFFERENTIATION* (*WIND*) family (Iwase et al., 2011a, 2011b). *WIND* genes are induced by wounding within several hours and are involved in cell dedifferentiation (Iwase et al., 2011a, 2011b). However, it is still unclear how wounding functions at the molecular level in plant de novo root organogenesis, and it is unknown whether wound signals are involved in processes other than the fate transition of competent cells.

In this study, we conducted a genome-wide transcriptome analysis of wounding in the leaf explant and identified that *NAC1* (petunia NAM and Arabidopsis ATAF1, ATAF2, and CUC2), a member of the NAC family of transcription factor genes (Aida et al., 1997), is induced in response to wounding and functions in promotion of root rip emergence.

RESULTS

Identification of *NAC* Genes in Response to Explant-Specific Wounding

We previously established a de novo root organogenesis system using Arabidopsis leaf explants (Chen et al., 2014). In this system, detached leaf explants are cultured on B5 medium without exogenous hormones, and adventitious roots regenerate from the wounded region (Fig. 1A). Detachment of a leaf from a plant causes two wounds: one on the detached leaf explant and one on the leaf residue on the source plant. Adventitious roots usually regenerate from the detached leaf explant but not from the wounded leaf residue, suggesting that the two wounds have different molecular features. To study the effects of wounding on gene transcription during plant regeneration, we performed RNA-seq analyses on the two wounded tissues; the detached leaf explant cultured on B5 medium and the remaining wounded leaf residue at 2 d after culture (DAC). For both tissues, the RNA-seq data were compared with those obtained for the leaf prior to culture

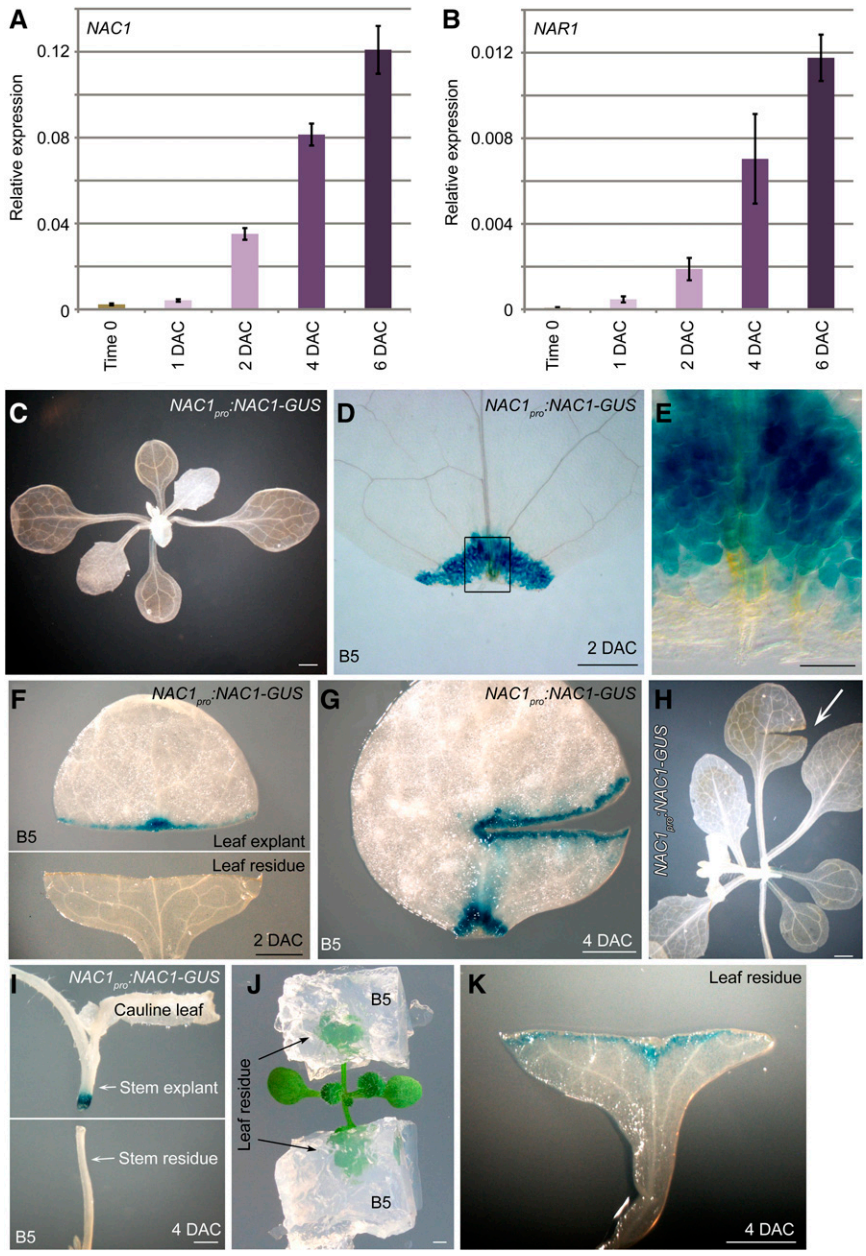

Figure 2. Expression patterns of *NAC* genes. A and B, qRT-PCR analysis of *NAC1* (A) and *NAR1* (B) transcript levels in leaf explants from time 0 to 6 DAC. Bars show SD from three PCR experiments. Results were confirmed in two biological repeats. Data from one repeat are shown. C to E, GUS staining of $NAC1_{pro}$:*NAC1-GUS* seedling (C) and 2-DAC leaf explant cultured on B5 medium (D and E). E, Enlargement of wounded region in D. F, GUS staining of $NAC1_{pro}$:*NAC1-GUS* leaf explant (upper panel) and wounded leaf residue (lower panel) at 2 DAC. Cutting and culture times as indicated in Fig. 1B. G, Large wound on leaf explant from $NAC1_{pro}$:*NAC1-GUS* cultured on B5 medium, showing GUS signal in wounded region. H, Large wound on seedling of $NAC1_{pro}$:*NAC1-GUS*, showing no GUS signal in wounded region. I, Stem of $NAC1_{pro}$:*NAC1-GUS* was cut, and stem explant (distal part) was cultured on B5 medium while wounded stem residue (proximal part) was still exposed to air. GUS signal was observed only in wounded region of stem explant. J and K, Wounded leaf residue was cultured in B5 medium (J), resulting in $NAC1_{pro}$:*NAC1-GUS* signal at wounded region of leaf residue at 4 DAC (K). Bars = 1 mm in C and F to K, 500 μm in D, and 100 μm in E.

(time 0; Fig. 1B). All the tested materials were cultured under a 16-h-light/8-h-dark photoperiod.

The RNA-seq data revealed changes in gene expression patterns that were specific to the leaf explant, specific to the leaf residue, and common to both tissues (Fig. 1C; Supplemental Table S1). Because de novo root organogenesis usually occurs only in the leaf explant, we analyzed the leaf explant wounding-specific gene expression in more detail. Among many transcription factor genes showing changes in expression levels, we identified 12 NAC domain transcription factor genes (Ooka et al., 2003) that were significantly upregulated specifically in the leaf explant but not in the wounded leaf residue (Fig. 1C; Supplemental Table S1). We focused on the *NAC* family because this family is known to be involved in plant regeneration and lateral root formation (Xie et al., 2000; Asahina et al., 2011). To confirm the RNA-seq data, we performed quantitative RT-PCR (qRT-PCR) analyses of the closely related *NAC* genes, *NAC1* and *NAC1-RELATED1* (*NAR1*). The results showed that the two genes had relatively low transcript levels in time-0 leaf explants, but markedly higher transcript levels in 2-DAC leaf explants (Fig. 1, D and E). In addition, *NAC1* was slightly upregulated in the 2-DAC leaf residue, while *NAR1* was not (Fig. 1, D and E).

To analyze the expression patterns of *NAC* genes in more detail, we performed qRT-PCR analyses to detect *NAC* transcript levels during regeneration of leaf explants. The transcript levels of *NAC1* and *NAR1* were upregulated between 1 and 2 DAC and continuously

upregulated afterward in leaf explants during de novo root organogenesis (Fig. 2, A and B). Next, we constructed the $NAC1_{pro}$:*NAC1-GUS* reporter line. The GUS signal of the reporter line was barely detected in leaves at time 0 (Fig. 2C) but was strong and specific to the wounded region of leaf explants at 2 DAC (Fig. 2, D and E). The GUS signal was only observed in the wound of the leaf explant, and not in the wounded leaf residue on the source plant (Fig. 2F). Also, the GUS signal was present in multiple wounds in the detached leaf explant (Fig. 2G) but not in the wound on the attached leaf (Fig. 2H). To further examine the expression of *NAC1* in the wounded region, we performed thin sectioning to show the GUS signals in the wounded region of the $NAC1_{pro}$:*NAC1-GUS* leaf explant at 4 DAC. The result showed that the GUS signal was in both mesophyll cells and competent cells (Supplemental Fig. S1). In addition, the GUS signal was present in the wounded region of the detached stem explant but not in the wounded region of the stem from which it was derived (stem residue; Fig. 2I).

One of the differences between the detached leaf explant and the leaf residue was that the wound on the leaf explant touched the B5 medium, while that on the leaf residue did not touch B5 medium and was exposed to air. Therefore, we supplied B5 medium to the leaf residue (Fig. 2J) and observed that the wounded region of the leaf residue expressed *NAC1* at 4 DAC (Fig. 2K). This result suggested that exposure of the wound to a wet environment could be very important for regeneration.

Overall, our data suggested that *NAC1*, together with *NAR1* (Supplemental Fig. S2A), might be involved in an explant-specific wound-signaling pathway.

NAC1 Is Involved in de Novo Root Organogenesis

Because *NAC* genes were specifically induced in leaf explants, we explored their roles in de novo root organogenesis. The NAC transcription factors activate gene transcription (Xie et al., 2000). Therefore, we fused the NAC1 protein with the repression domain SRDX (Hiratsu et al., 2003) under the control of the *NAC1* promoter to specifically suppress the expression of *NAC1* target genes. This method also avoided redundancy among the *NAC* family genes. Compared with wild-type leaf explants that normally regenerate adventitious roots, leaf explants from the $NAC1_{pro}$:*NAC1-SRDX* transgenic lines showed partially defective rooting (Fig. 3, A, B, and D). In the $35S_{pro}$:*NAC1-SRDX* lines harboring the chimeric NAC1-SRDX fusion under the control of the *Cauliflower mosaic virus* 35S promoter, rooting was more severely blocked (Fig. 3, C and D). In addition, regeneration of adventitious roots from stem explants was defective in $35S_{pro}$:*NAC1-SRDX* lines,

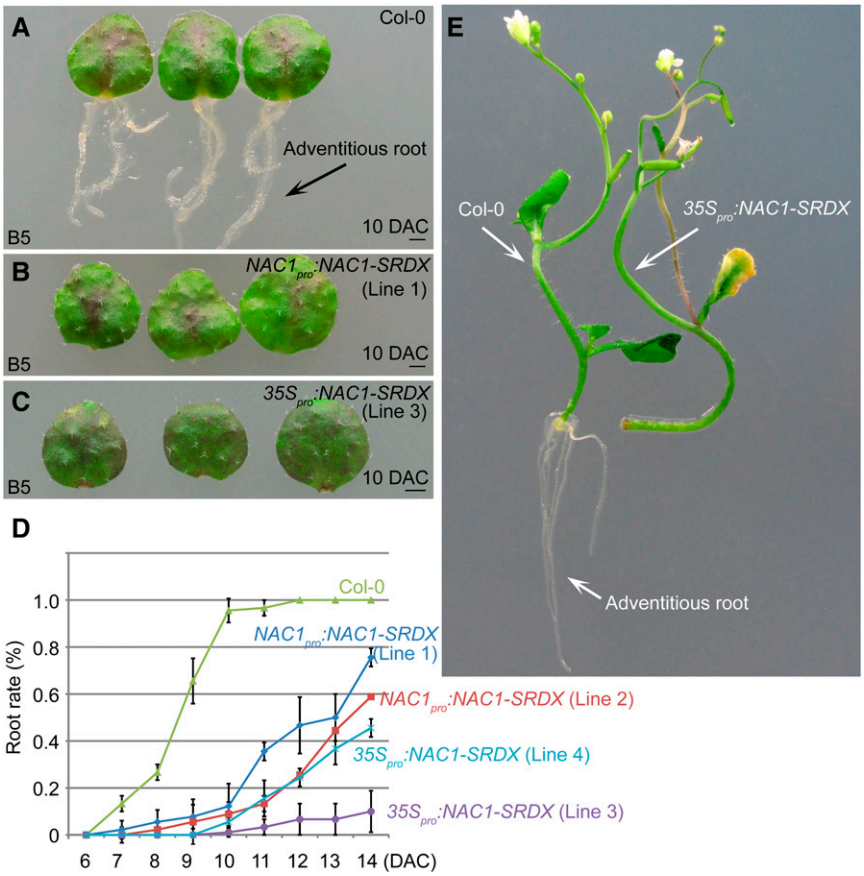

Figure 3. *NAC1* is involved in de novo root organogenesis. A to C, Adventitious rooting from leaf explants of Col-0 (A), $NAC1_{pro}$:*NAC1-SRDX* (B), and $35S_{pro}$:*NAC1-SRDX* (C) on B5 medium. Note that both transgenic lines showed defective rooting at 10 DAC. D, Rooting rate analyses of leaf explants from Col-0, $NAC1_{pro}$:*NAC1-SRDX*, and $35S_{pro}$:*NAC1-SRDX*. Bars show SD from three biological repeats; $n = 30$ in each repeat. E, Adventitious rooting from stem explants of Col-0 (left) and $35S_{pro}$:*NAC1-SRDX* (right) cultured on B5 medium, showing rooting defect in $35S_{pro}$:*NAC1-SRDX*. Bars = 1 mm in A to C.

compared with that in the wild type (Fig. 3E). Regeneration of adventitious roots from leaf explants of $35S_{pro}$:*NAR1-SRDX* lines was also defective (Supplemental Fig. S2B). These data suggested that the *NAC* pathway is involved in de novo root organogenesis in Arabidopsis.

NAC1 Acts Independently of Auxin-Mediated Cell Fate Transition

The results of our previous studies suggested that auxin accumulation in competent cells in the vasculature near the wound initiates cell fate transition to

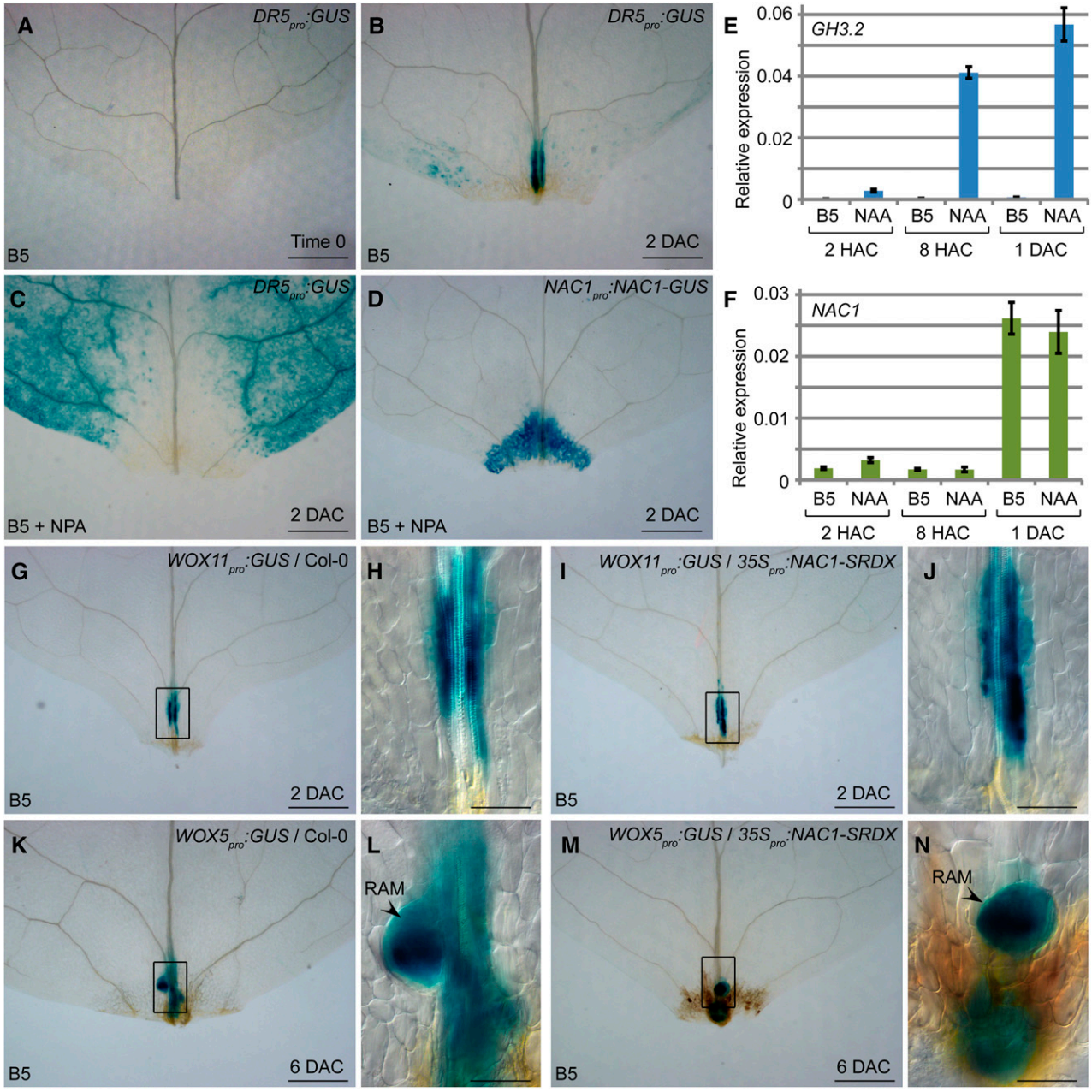

Figure 4. *NAC1* acts independently of auxin-mediated cell fate transition. A and B, GUS staining of $DR5_{pro}$:*GUS* leaf explant cultured on B5 medium at time 0 (A) and 2 DAC (B). C, GUS staining of $DR5_{pro}$:*GUS* leaf explant at 2 DAC on medium containing NPA. Note that the GUS signal did not accumulate in wounded region. D, GUS staining of $NAC1_{pro}$:*NAC1-GUS* in leaf explant at 2 DAC cultured on B5 medium containing NPA. E and F, qRT-PCR analysis of *GH3.2* (E) and *NAC1* (F) transcript levels in leaf explants cultured on B5 medium without or with NAA. Bars show SD from three PCR experiments. Results were confirmed in three biological repeats. Data from one repeat are shown. G to J, GUS staining of $WOX11_{pro}$:*GUS* (G and H) and $WOX11_{pro}$:*GUS*/$35S_{pro}$:*NAC1-SRDX* (I and J) leaf explants cultured on B5 medium at 2 DAC. K to N, GUS staining of $WOX5_{pro}$:*GUS* (K and L) and $WOX5_{pro}$:*GUS*/$35S_{pro}$:*NAC1-SRDX* (M and N) leaf explant cultured on B5 medium at 6 DAC. RAM, root apical meristem. H, J, L, and N, Enlargements of boxed regions in G, I, K, and M, respectively. Bars = 500 μm in A to D, G, I, K, and M and 100 μm in H, J, L, and N.

WOX11-marked root founder cells. Root founder cells further undergo fate transition to become *WOX5*-marked root primordium cells via cell division (Liu et al., 2014). Therefore, we tested whether *NAC1* is involved in this auxin-mediated cell fate transition pathway.

First, we tested whether *NAC1* expression is controlled by auxin. Using the auxin reporter line $DR5_{pro}$:*GUS*, we observed that auxin quickly accumulated in the mesophyll and vasculature in the wounded region at 2 DAC (Fig. 4, A and B). When napthylphthalamic acid (NPA; a polar auxin transport inhibitor) was supplied in the medium, auxin could not accumulate in the wounded region (Fig. 4C). Interestingly, *NAC1* was expressed normally in the wounded region under NPA treatment (Fig. 4D). In addition, expression of *NAR1* was also not affected by NPA treatment (Supplemental Fig. S2C). We conducted qRT-PCR analyses to test whether naphthylacetic acid (NAA; a type of auxin) could induce the transcription of the auxin response gene *GH3.2* (Staswick et al., 2005; He et al., 2012) and *NAC1*. The results showed that the NAA treatment quickly induced *GH3.2* transcription by 2 h after culture (Fig. 4E) but did not induce *NAC1* (Fig. 4F). These data suggested that auxin does not control the expression of *NAC1*.

Next, we tested whether *NAC1* affects the competent cell fate transition in adventitious roots growing from leaf explants. We introduced the $WOX11_{pro}$:*GUS* and $WOX5_{pro}$:*GUS* reporter lines into the $35S_{pro}$:*NAC1-SRDX* transgenic lines to observe expression of the root founder cell marker *WOX11* and the root primordium

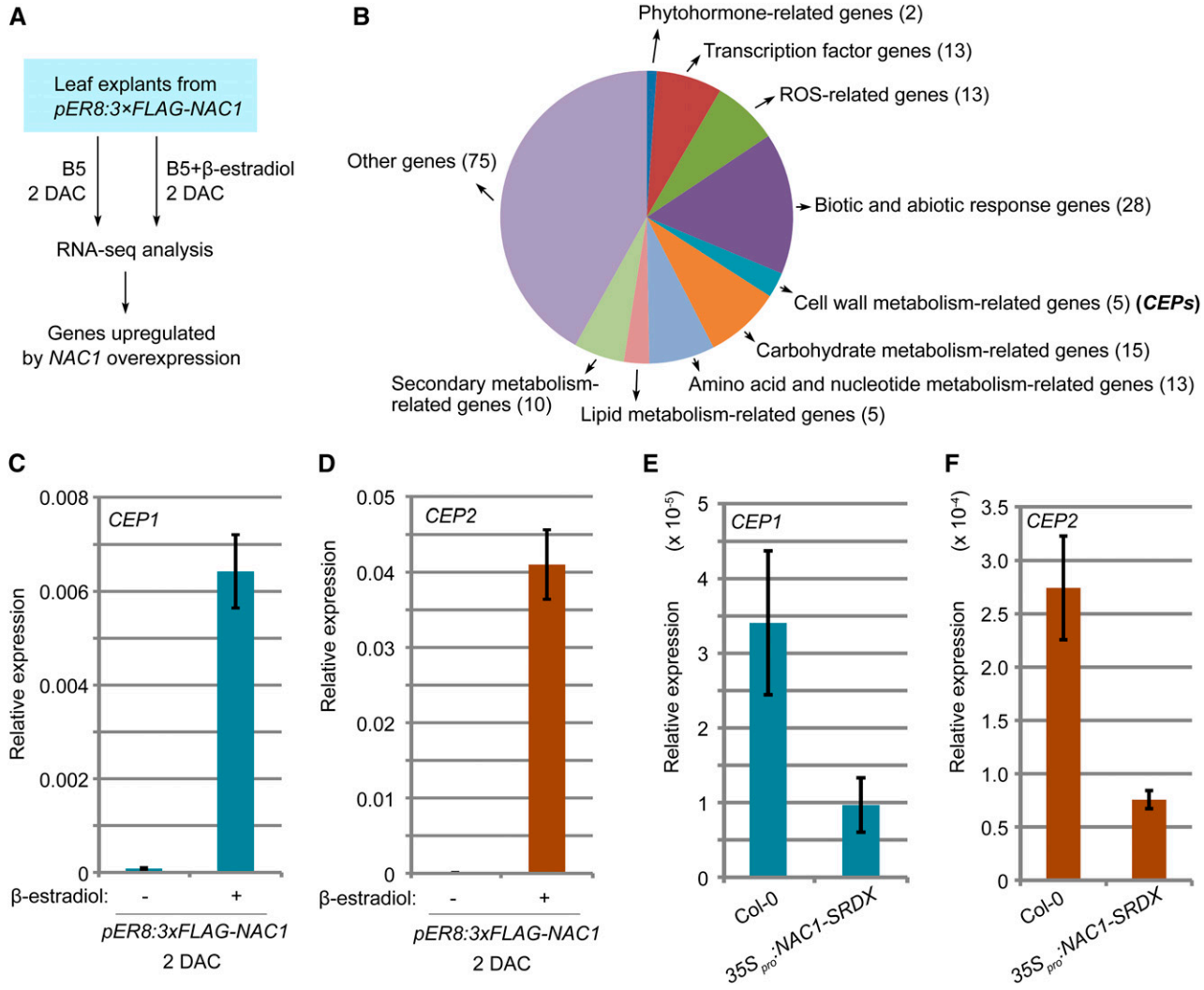

Figure 5. *NAC1* promotes expression of *CEP* genes. A, RNA-seq analysis using 2-DAC leaf explants from *pER8:3×FLAG-NAC1*. Leaf explants were cultured on B5 medium containing β-estradiol/DMSO or DMSO only (control). B, Analysis of upregulated genes in RNA-seq data as indicated in A. Also see Supplemental Table S2. C and D, qRT-PCR analysis of *CEP1* (C) and *CEP2* (D) transcript levels in leaf explants of *pER8:3×FLAG-NAC1* cultured on medium without (−) or with (+) 10 μM β-estradiol at 2 DAC. E and F, qRT-PCR analysis of *CEP1* (E) and *CEP2* (F) transcript levels in leaf explants of $35S_{pro}$:*NAC1-SRDX* at 4 DAC. Bars in C to F show SD from three PCR experiments. Results were confirmed in two biological repeats. Data from one biological repeat are shown.

and root apical meristem marker *WOX5*. The results showed that, similar to their expression patterns in the wild-type background, *WOX11* (Fig. 4, G–J) and *WOX5* (Fig. 4, K–N) were expressed in the $35S_{pro}$*:NAC1-SRDX* transgenic lines. At 6 DAC, *WOX5* expression began to be restricted toward the stem cell niche (Fig. 4, L and N), suggesting that the root apical meristem was forming at this stage in both the wild type and $35S_{pro}$*:NAC1-SRDX*. In addition, *WOX11* and *WOX5* were also expressed during regeneration of leaf explants from $35S_{pro}$*:NAR1-SRDX* (Supplemental Fig. S2D). These data suggested that *NAC* genes act independently of cell fate transition in de novo root organogenesis. Therefore, the block of rooting in $35S_{pro}$*:NAC1-SRDX* and $35S_{pro}$*:NAR1-SRDX* might be caused by the defect in root tip emergence.

NAC1 Promotes Expression of *CEP* Genes

To analyze the possible downstream genes regulated by the *NAC* pathway, we constructed *pER8:3×FLAG-NAC1* lines in which *NAC1* was overexpressed under the control of the β-estradiol-inducible promoter (Zuo et al., 2000). We performed RNA-seq analyses using leaf explants of *pER8:3×FLAG-NAC1* cultured on B5 medium containing β-estradiol for 2 d and leaf explants from the same line cultured on β-estradiol-free B5 medium and compared the data sets (Fig. 5A). The results showed that *NAC1* overexpression induced many groups of genes, including metabolism-related genes (Fig. 5B; Supplemental Table S2).

We noticed that two KDEL-tailed Cys endopeptidase (*CEP*) genes, *CEP1* and *CEP2*, were highly upregulated by *NAC1* overexpression. In previous studies, CEPs were shown to be involved in programmed cell death and to be secreted to the cell wall for degradation of extensin (EXT) proteins (Greenwood et al., 2005; Helm et al., 2008; Hierl et al., 2012). EXT proteins are basic components of the cell wall, and genes encoding these proteins are induced by wounding (Hall and Cannon, 2002; Merkouropoulos and Shirsat, 2003; Cannon et al., 2008). EXT proteins are involved in cell proliferation (Hall and Cannon, 2002; Cannon et al., 2008; Xu et al., 2011) and wound healing (Bostock, 1989; Showalter, 1993; Tiré et al., 1994).

We performed qRT-PCR analyses to confirm the up-regulation of *CEP1* and *CEP2* in the *pER8:3×FLAG-NAC1* background (Fig. 5, C and D). In addition, expression of *CEP1* and *CEP2* was partially inhibited by overexpression of NAC1-SRDX (Fig. 5, E and F). These data suggested that *NAC1* may up-regulate *CEP* expression during de novo root organogenesis.

To determine whether *CEP* and *EXT* are involved in de novo root organogenesis, we analyzed the expression patterns of *CEP1* and *EXT1* using $CEP1_{pro}$*:GUS* and $EXT1_{pro}$*:GUS* reporter lines, respectively. The GUS signal from $CEP1_{pro}$*:GUS* was barely detected at the wounded region in time-0 and 1-DAC leaf explants but began to accumulate in the wounded region at and after 2 DAC (Fig. 6, A–D). In addition, *CEP1* expression was specifically induced in the wounded region of leaf explants but not in the leaf residue of the source plant (Supplemental Fig. S3). The GUS signal from $EXT1_{pro}$*:GUS* was barely detected in time-0 leaf explants; however, its signal quickly became stronger at and after 1 DAC (Fig. 6, E–H), faster than that of $CEP1_{pro}$*:GUS*.

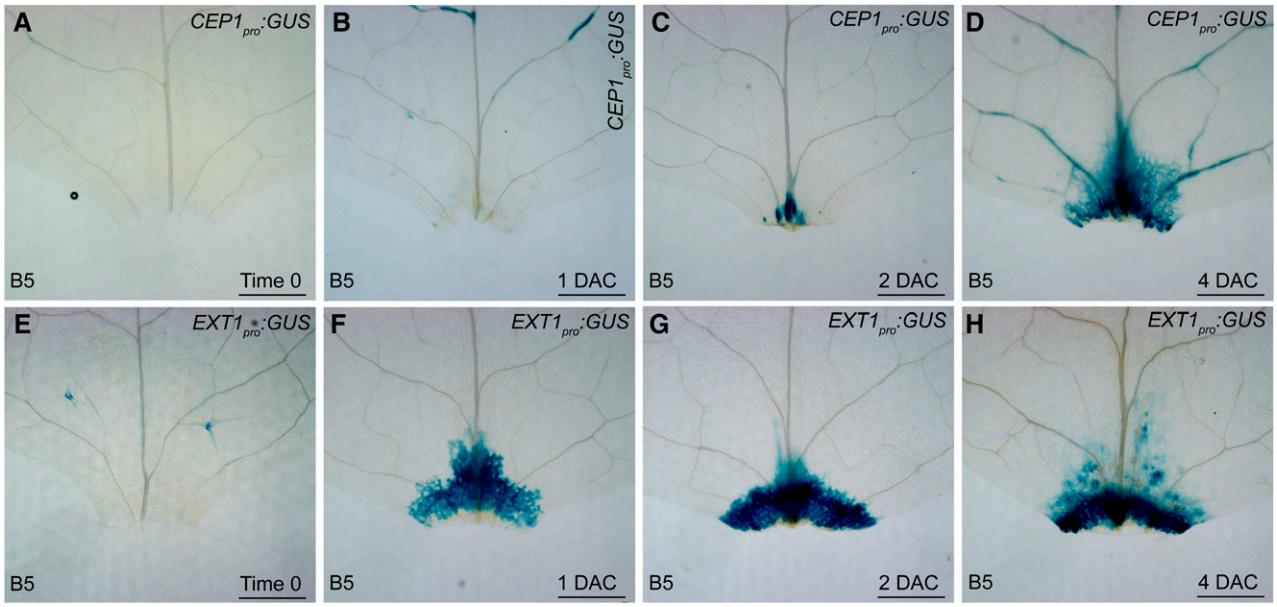

Figure 6. Expression patterns of *CEP1* and *EXT1*. A to D, GUS staining of $CEP1_{pro}$*:GUS* leaf explant cultured on B5 medium at time 0 (A), 1 DAC (B), 2 DAC (C), and 4 DAC (D). E to H, GUS staining of $EXT1_{pro}$*:GUS* leaf explant cultured on B5 medium at time 0 (E), 1 DAC (F), 2 DAC (G), and 4 DAC (H). Bars = 500 μm.

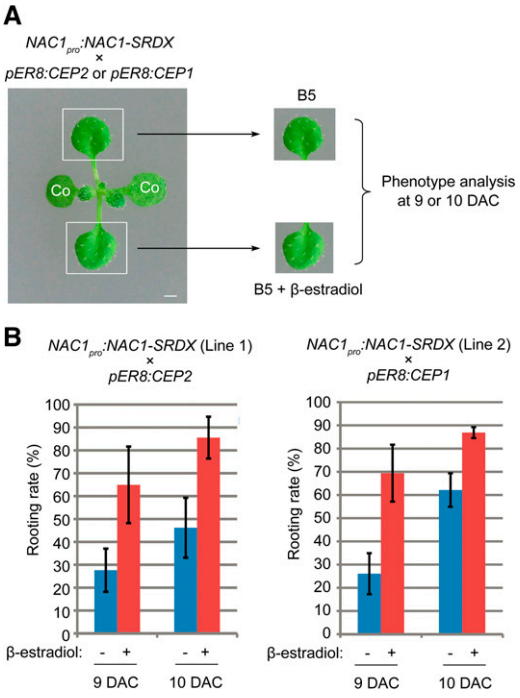

Figure 7. Overexpression of *CEP* partially rescues rooting defect in *NAC1*$_{pro}$*:NAC1-SRDX*. A, Design of rescue experiment. Each leaf explant from the same source plant was cultured on B5 medium containing β-estradiol/DMSO or DMSO only (control). co, Cotyledon. B, Rooting rate analyses of leaf explants from two independent crossed lines (*NAC1*$_{pro}$*:NAC1-SRDX/pER8:CEP2* in the left panel and *NAC1*$_{pro}$*:NAC1-SRDX/pER8:CEP1* in the right panel) cultured on medium without (−) or with (+) 10 μM β-estradiol at 9 and 10 DAC. Bars show SD with three biological repetitions. $n \geq 22$ in each repetition.

Therefore, expression of *CEP1* and *EXT1* was related to wounding during adventitious rooting from leaf explants, and *EXT1* was expressed earlier than *CEP1*.

To test whether the *CEP* pathway contributes to the *NAC1*-mediated de novo root organogenesis, we overexpressed *CEP1* or *CEP2* in the NAC1-SRDX background by crossing *NAC1*$_{pro}$*:NAC1-SRDX* with *pER8:CEP1* or *pER8:CEP2*. Two independent *NAC1*$_{pro}$*:NAC1-SRDX* lines were used in crossing. The F2 plants harboring both transgenic constructs were tested by placing one first-pair leaf on β-estradiol-free medium and the other on medium containing β-estradiol (Fig. 7A). *CEP1* or *CEP2* overexpression partially rescued the rooting defects in the *NAC1*$_{pro}$*:NAC1-SRDX* background (Fig. 7B). These results suggested that the *CEP* pathway at least partly contributes to *NAC1*-mediated regeneration.

DISCUSSION

In this study, we revealed a wounding-induced *NAC* pathway that acts in parallel with auxin-mediated cell fate transition in de novo root organogenesis (see model in Fig. 8). We propose that wounding provides a complex mixture of signals that have multiple roles in controlling the direction of functionally independent molecular pathways. One of the core pathways is the auxin-mediated cell fate transition that occurs in the competent cells of the vasculature within the wounded region. Another pathway revealed in this study involves *NAC* transcription factors that likely function in regulating the cellular environment in both mesophyll and competent cells within the wounded region for promotion of root tip emergence.

Many genes are activated in response to wounding. Previous studies suggested that *WIND1* expression is induced by wounding in plant cell dedifferentiation (Iwase et al., 2011a, 2011b). However, the *NAC* pathway differs from *WIND1*. Expression of *WIND1* is induced by wounding in both detached and attached tissues, whereas *NAC*s expression is induced by wounding only in the detached explant. In addition, induction of *WIND1* occurs rapidly, within hours (Iwase et al., 2011a), whereas up-regulation of *NAC* genes requires 1 to 2 d. Wounding also induces auxin production in de novo root organogenesis (Liu et al., 2014), and the *NAC1* pathway is independent of the auxin-mediated *WOX11* pathway. *NAC1* expression is not induced by auxin, and *NAC1* does not regulate *WOX11* that controls cell fate transition. All these behaviors of *NAC1* suggest that it functions in a novel wounding response pathway. The results of this study suggested that wounding controls many signals that act independently, but cooperatively, for regeneration. However, the molecular nature of wounding is still poorly understood. Further detailed studies on the physical/chemical events during and after wounding, and their molecular functions, will greatly improve our understanding of how regeneration begins.

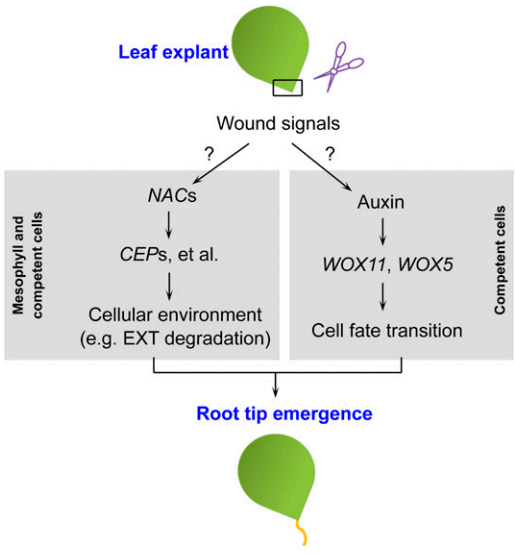

Figure 8. Model for wounding-induced de novo root organogenesis. Wound signals function in at least two separate pathways for de novo root organogenesis. The molecular features of wound signals remain unclear.

NAC1 was previously identified as a regulator of lateral roots, and its expression was shown to be related to the auxin-signaling pathway during lateral root development (Xie et al., 2000). *ANAC071*, another *NAC* gene induced by auxin, was reported to be involved in tissue regeneration on the wounded stem (Asahina et al., 2011). In this study, we showed that *NAC1* acts independently of auxin in de novo regeneration of adventitious roots from leaf explants. Therefore, NAC family genes may have divergent roles in organ formation and regeneration, and *NAC1* may have different roles in lateral and adventitious root formation in response to different upstream regulators. Further analyses of how wounding regulates *NAC1* expression and how *NAC1* is regulated posttranscriptionally and post-translationally (Xie et al., 2002; Mallory et al., 2004) will provide new insights into the role of *NAC1* in plant regeneration.

The emergence of adventitious root tips requires cell wall degradation and assembly (Vidoz et al., 2010; da Costa et al., 2013). In grape (*Vitis vinifera*) stem cuttings, expression of the EXT protein was found to be induced during rooting (Thomas et al., 2003). Wounding has been shown to induce *EXT1* expression, suggesting that cells at the wounded site accumulate EXT to strengthen cell walls during wound healing (Bostock, 1989; Showalter, 1993; Tiré et al., 1994; Merkouropoulos and Shirsat, 2003). The results of this study showed that *NAC1* expression is also induced at the wounded site and promotes *CEP* expression. Up-regulation of *CEP*s might probably be related to the degradation of EXT. EXT promotes wound healing, and this might be a barrier for the emergence of regenerated root tips. Therefore, we hypothesize that the NAC1-CEP pathway antagonizes EXT-mediated wound healing, and this allows the emergence of regenerated root tips.

MATERIALS AND METHODS

Plant Materials

Arabidopsis (*Arabidopsis thaliana*) Col-0 was used as the wild type in this study. To produce $NAC1_{pro}$:*NAC1-GUS* and $NAR1_{pro}$:*NAR1-GUS* transgenic plants, 5.0-kb *NAC1* and 3.0-kb *NAR1* genomic sequences including the promoters and coding regions were PCR amplified and inserted into the pBI101 vector (Clontech). $CEP1_{pro}$:*GUS* and $EXT1_{pro}$:*GUS* were constructed by inserting 1.6- and 5.1-kb *CEP1* and *EXT1* promoter sequences, respectively, into the pBI101 vector. $NAC1_{pro}$:*NAC1-SRDX* was constructed by first fusing a sequence encoding the SRDX peptide (Hiratsu et al., 2003) with the 5.0-kb *NAC1* genomic sequence including its promoter and coding region and then inserting the entire fusion construct into pCAMBIA1300 (Cambia). $35S_{pro}$:*NAC1-SRDX* and $35S_{pro}$:*NAR1-SRDX* were constructed by inserting the *NAC1* and *NAR1* cDNA-SRDX fusion constructs into the pMON530 vector, respectively. To construct *pER8:CEP1*, *pER8:CEP2*, or *pER8:3×FLAG-NAC1*, cDNA encoding the full-length CEP1, CEP2, or 3×FLAG-NAC1 was PCR amplified and inserted into the pER8 vector (Zuo et al., 2000). Transgenic plants were obtained by *Agrobacterium tumefaciens*-mediated transformation of each of these constructs into Col-0. $WOX11_{pro}$:*GUS*, $WOX5_{pro}$:*GUS*, and $DR5_{pro}$:*GUS* transgenic plants were described previously (Ulmasov et al., 1997; He et al., 2012; Liu et al., 2014). The primers used for plasmid construction are listed in Supplemental Table S3.

Culture Conditions

Culture conditions were as described previously (Chen et al., 2014). Briefly, Arabidopsis seedlings were first cultured on 0.5× MS medium (Murashige and Skoog, 1962) at 22°C under a 16-h-light/8-h-dark photoperiod. Leaf explants from 12-d-old seedlings or stem explants from 26-d-old seedlings were cultured on B5 medium (Gamborg et al., 1968) without carbohydrates under the same photoperiod.

qRT-PCR and RNA-Seq Analyses

The RNA extractions and qRT-PCR analyses were performed as previously described (He et al., 2012) using gene-specific primers. The qRT-PCR results are shown as relative expression levels normalized against those produced using *Actin*-specific primers. The primers used for real-time PCR are listed in Supplemental Table S3.

For RNA-seq analyses, RNA was extracted using Trizol. Library construction and deep sequencing were carried out using the Illumina HiSeq 2000 platform following the manufacturer's instructions by Genergy Biotechnology. The raw data comprised 100-bp paired-end sequences, and the cleaned reads were then mapped to Arabidopsis genome (TAIR10) using default settings of TopHat v2.0.8 (Kim et al., 2013). The duplicated reads were removed and alignments with MAPQ score > 20 were used for further analysis. RNA-seq alignments were processed using HTSeq-count (Anders et al., 2015), and differentially expressed genes were identified using DESeq (Anders and Huber, 2010) with $|\log_2 \text{fold change}| > 3.5$. The analyzed data are shown in Supplemental Tables S1 and S2.

Histology

We performed GUS staining and thin sectioning as previously described (Chen et al., 2014; Zeng et al., 2015). The differential interference contrast observations were performed using Nikon SMZ1500 and Nikon ECLIPSE 80i microscopes as previously described (Chen et al., 2014).

Accession Numbers

The RNA-seq data obtained in the wounding and *pER8:3×FLAG-NAC1* analyses have been deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under accession numbers GSE74585 and GSE74584, respectively. Sequence data are listed in the Arabidopsis Genome Initiative under the following accession numbers: *NAC1* (At1g56010) and *NAR1* (At3g12977).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. *NAC1* expression in the leaf explant.

Supplemental Figure S2. *NAR1* in de novo root organogenesis.

Supplemental Figure S3. *CEP1* expression responds to wounding of leaf explant.

Supplemental Table S1. RNA-seq analysis of genes expressed in response to wounding.

Supplemental Table S2. RNA-seq analysis of genes upregulated in *pER8:3×FLAG-NAC1*.

Supplemental Table S3. List of primers used in this study.

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