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Letter

Regulation of touch-stimulated de novo root regeneration from Arabidopsis leaves

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The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (https://academic.oup.com/plphys/pages/general-instructions) is: Kalika Prasad (kalikasaksham11@gmail.com).

Dear Editor,

Among several of the plant's lateral organs, leaves show versatile regenerative responses, be it natural, mechanical-injury induced, or tissue culture-mediated. Regeneration of entire plants from various species of *Kalanchoe* leaves is an example of natural regeneration from leaf (Smith et al., 2019). In tissue culture-mediated regeneration, small leaf explants can give rise to entire shoot and/root system via callus in the presence of hormonal supplements. The incised mid-vein of an undetached growing leaf, and the cut end of detached leaves exhibit regenerative responses, both of which fall under mechanical injury-induced regeneration. Although mid-vein regeneration in growing leaves was investigated only recently, mechanical injury-induced regenerative responses at the cut end of detached leaves have been studied for several years (Chen et al., 2014; Ikeuchi et al., 2016; Bustillo-Avendaño et al., 2018; Zhang et al., 2019; Radhakrishnan et al., 2020). Studies in Arabidopsis (*Arabidopsis thaliana*) reported the emergence of adventitious roots from the cut end of detached leaves, be it the base of leaf blade or the petiole via *de novo* root regeneration (DNRR; Chen et al., 2014; Bustillo-Avendaño et al., 2018). This ability of part of a tissue to produce an organ, whose identity is different from its parent tissue, is rather intriguing. However, DNRR is not the only response observed at the cut end of a detached Arabidopsis leaf; wound healing in the form of callus formation occurs at the cut end of leaves that do not undergo DNRR. With the available data, it was unclear if the decision to make callus or DNRR is random or if any external inductive cues favor one over the other. It was therefore imperative to investigate this differential regenerative response to the same injury in the same organ. Using various experimental approaches, we show that the factor favoring DNRR over callus formation is the direct physical contact of the cut end to any solid or liquid surface. Interestingly, the plant hormone auxin shows elevated accumulation in response to touch to the wound site. We further show that PLETHORA (PLT) genes, which are essential as well as sufficient for DNRR, regulate this process via a mechanism distinct from PLT-regulated lateral root (LR) formation or other PLT-regulated regenerative responses.

We repeated the previously established DNRR assay using leaves collected from seedlings 7 d post germination (Bustillo-Avendaño et al., 2018). When the leaves were placed abaxial-side down with the cut end of the petiole touching the surface of the hormone-free solid Murashige and Skoog-Agar medium (MS-agar medium), we observed

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DNRR, consistent with the previous studies (Bustillo-Avendaño et al., 2018; Figure 1, A, A′, B, B′). Here, only 30% (n = 93) of the leaves regenerated mature root and the remaining showed neither DNRR nor callus formation (Figure 1, O). However, when placed adaxial-side down with the petiole in the air, the leaves failed to regenerate root, and instead callus formation was observed at the cut end in 62% of samples (n = 95; Figure 1, C, C′, D, D′, O). At a glance, three factors appeared to be different between the two responses: (i) Nutrient availability at the cut end (minimal MS and sucrose), (ii) orientation of leaf on the MS-agar (abaxial or adaxial), and (iii) physical contact of cut end to the agar surface.

First, to eliminate absorption of nutrients by the cut end during DNRR, we carried out a simple split-plate experiment with MS-agar in top half and nutrient-free solid MS-Agar (agar-only) in bottom half of the plate. The leaves were placed abaxial-side down such that, only their proximal region with petiole touched the surface of agar-only media. We allowed the distal region of the leaves to be in contact with surface of MS-agar to allow minimal nutrient transport for its sustenance and growth (Figure 1, E and F and Supplemental Figure S1, A). Interestingly, 20.78% (n = 154) leaf explants produced DNRR from the cut end that touched the surface of the agar-only media, while the remaining leaves produced neither DNRR nor callus formation. Second, to examine the role of leaf orientation, we placed the leaves adaxial side down on the MS-agar media and gently pressed them down ensuring the cut end touched the agar surface (Figure 1, G and H). We noticed that 34.2% (n = 76) leaves exhibited DNRR. Third, we designed an agar block experiment which ensured that the leaves were oriented adaxial-side down, the cut sites were devoid of nutrients, and touched the surface of agar-only block (Figure 1, I and J). We noticed that 17.86% (n = 65) leaves exhibited DNRR. We also explored the possibilities of DNRR when the cut end touched the surface of other materials such as water and soil (Figure 1, K and M). Leaves oriented abaxial-side down produced DNRR in liquid medium as well as on soil, with a success rate of 46.5% (n = 78) and 56.76% (n = 74) respectively (Figure 1, L and N). However, the leaves oriented adaxial-side down produced only callus and no DNRR (Supplemental Figure S1, B and C). The results were similar with the observations from solid MS-agar (Figure 1, B′ and D′).

Availability of water is essential for leaf survival, as well as for regeneration. However, it is not the sheer availability of water, but its direct physical contact to the cut end of leaves that triggers DNRR. When the leaves are allowed to float on water with the adaxial-side down and cut end in the air, they do not undergo DNRR, but instead produce callus at the cut end (Supplemental Figure S1, B). Notably, the entire leaves including their petioles are green and healthy enough to undergo callus formation and do not wither away. Direct physical contact of water to Arabidopsis leaves showed an increase in the transcript levels of several touch responsive genes (Braam and Davis, 1990; Van Aken et al., 2016; Van Moerkercke et al., 2019). This shows that water can indeed trigger touch-induced physiological responses in plants. However, which of the touch responsive genes are upregulated during DNRR, and their functions, await further studies. Taken together, the results are in agreement with the hypothesis that touch to a solid or liquid surface is the major factor distinguishing the two regenerative responses at the cut end of a detached leaf namely, DNRR and callus formation.

Auxin is implicated in various regenerative responses (Mathew and Prasad, 2021). We therefore examined the auxin level using a sensitive marker, R2D2 in the two distinct regenerative responses at the cut end of the detached leaf (Liao et al., 2015). We found that the auxin level when the cut end touched the agar was higher than when the cut end did not touch the agar surface (Figure 2 and Supplemental Figure S2). We consistently observed an increase in auxin level when the cut end was in physical contact with soil as well (Supplemental Figure S3). Touch-induced increase in auxin level was further reflected by a mild upregulation in the expression of auxin responsive genes *INDOLE-3-ACETIC ACID INDUCIBLE 9* (*1AA9*) and *MONOPTEROS* (*MP*/*ARF5*) upon RT-qPCR (Figure 2, F). We then looked for transcriptional regulators which could show similar differential expression in response to touch at the cut end. Due to their indispensable and established role in plant regeneration, we chose PLT3, PLT5, and PLT7 (*PLT3-YFP*, *PLT5-YFP*, *PLT7-YFP*) in wild-type (WT) background for investigation (Kareem et al., 2015; Radhakrishnan et al., 2020). Upon examining the expression pattern of PLT7, we found several cells near the cut end showed prominent YFP expression when it continuously touched the MS-agar surface (Figure 3, A–C and Supplemental Figure S4, A). However, when the cut end failed to touch the MS-agar, the YFP expression was faint and limited to few cells (Figure 3, D–F and Supplemental Figure S4, B). PLT3 as well as PLT5 showed similar differential expression in response to touch near the cut end (Supplemental Figure S4, C–P). It should be noted that, hereafter all the leaf explants were cultured on hormone-free solid MS-agar media. Leaves from *plt3;plt5-2;plt7* failed to yield any DNRR or even root primordium-like structure despite the cut end being in contact with the MS-agar surface (Figure 3, G–I). Rather the triple mutant produced a micro-callus at the cut site (Supplemental Figure S4, Q). The micro-callus here is an inconspicuous mass of proliferating cells that lack root-specific markers such as PLT1, PLT2, and WUSCHEL RELATED HOMEOBOX 5 (WOX5; Supplemental Figure S5). This suggests that these cells lack root identity. Interestingly, over-expression (OE) of PLT7(WT;*35S::PLT7-GR*), induced DNRR at a frequency of 52.2% (n = 46) even when the cut end did not touch the MS-agar (Figure 3, J–L and Supplemental Figure S4, R and S). Thus, PLT7-OE can over-ride the need for touch suggesting PLT7 is necessary and sufficient to induce DNRR. Until now PLT3,5,7 has been reported to act through two different transcriptional regulatory modules during several other

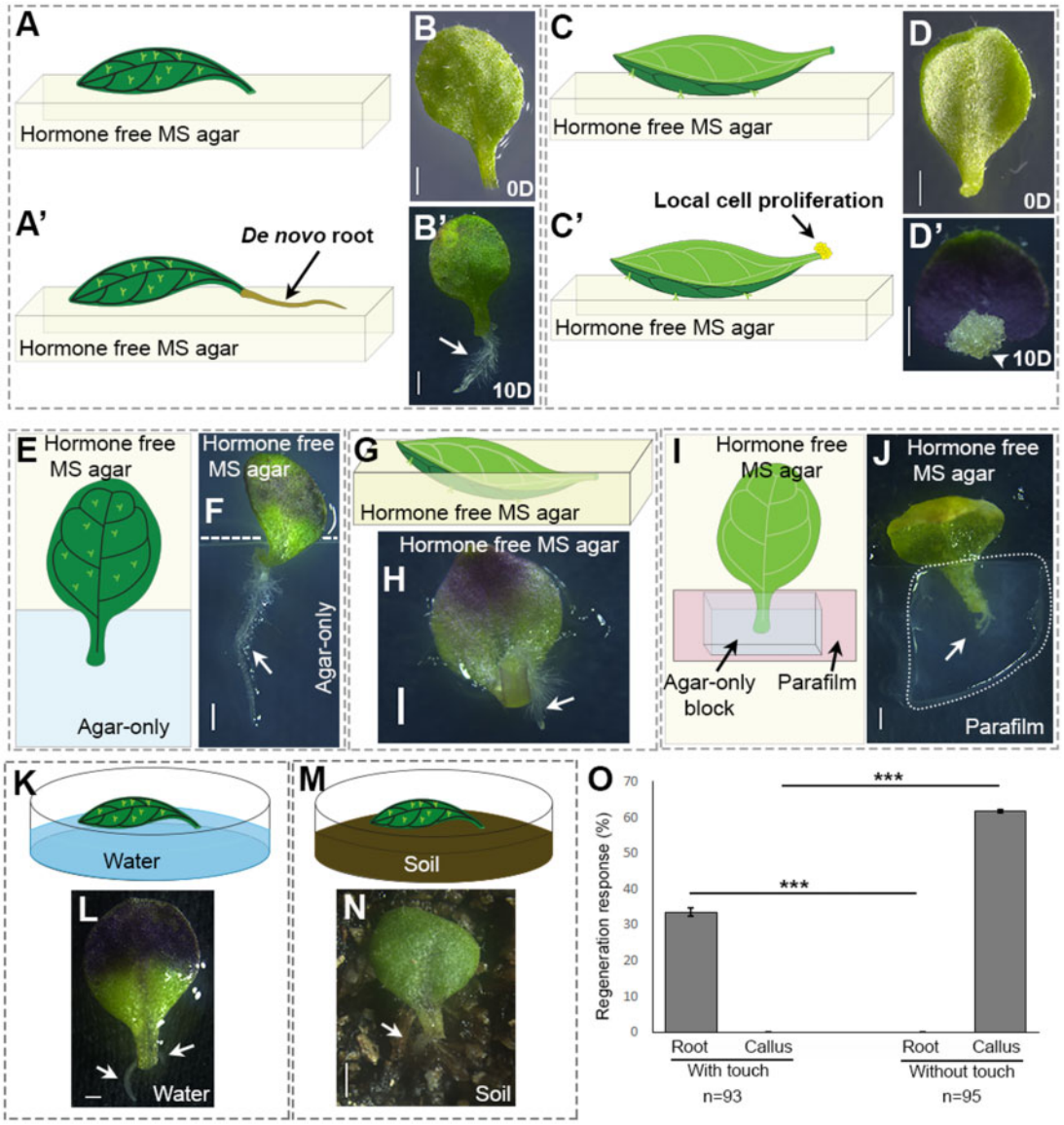

Figure 1 Wound healing response and touch-dependant *de novo* root regeneration at the cut end of a detached leaf: A and A′, A detached leaf when placed abaxial side down on the hormone-free solid MS-agar media (MS-agar media) results in the formation of *de novo* root. B and B′, The stereo-microscopic images of the detached leaf placed abaxial side down that regenerated *de novo* roots. C and C′, A detached leaf when placed adaxial side down on the MS-agar media results in the formation of callus. D and D′, The stereo-micrographs of the detached leaf placed adaxial side down that resulted in callus formation. E, Schematic depicting a "split-plate" where top half of MS-agar medium is insulated from hormone-free solid agar-only medium (agar-only medium). The leaf is placed abaxial side down with its distal end touching the MS-agar medium and its cut end touching the agar-only medium. F, Stereo-micrographs of the leaf showing DNRR on the split plate. G, Schematic showing the detached leaf being pressed into the media with its adaxial side down. H, Stereo-micrograph of the leaf showing DNRR after being pressed into the medium. I, Schematic illustrating the experimental set up where the cut end touches agar-only block but insulated from MS-agar media. Here, the detached leaf is placed adaxial side down on MS-agar medium, and the cut end is sandwiched between a thin parafilm strip and an agar-only block. J, Stereo micrograph of leaf showing DNRR after the cut end being sandwiched between parafilm and agar-only block. K, Schematic representation of leaf placed in water with half strength MS. L, Image showing *de novo* root formation (white arrow) from the cut end of the leaf when placed in water. M, Schematic showing leaf with cut end of petiole touching soil. N, *De novo* root (white arrow) formed from cut end of the leaf in contact with soil. O, Graph showing distinct kind of regeneration response [root (***$P = 2.24 \times 10^{-08}$, Pearson's χ^2 test) and callus (***$P = 2.2 \times 10^{-16}$, Pearson's χ^2 test)] with ($n = 93$, $e = 4$) and without ($n = 95$, $e = 4$) touching the agar. Error bars represent s.e.m. The black and while arrows indicate *de novo* regenerated root. Scale bars represent 1 mm. *n*, sample size; *e*, number of experiments; *D*, days post cut.

regenerative responses: (i) In tissue culture-induced shoot regeneration, where PLT3,5,7 acts through root stem cell regulators PLT1 and PLT2, and CUP SHAPED COTYLEDON 2 (CUC2) and (ii) PLT-CUC2 regulatory axis which acts in a coherent feed-forward loop to upregulate local auxin biosynthesis gene YUCCA4 during mechanical injury-induced vascular regeneration in growing leaves (Kareem et al., 2015; Radhakrishnan et al., 2020).

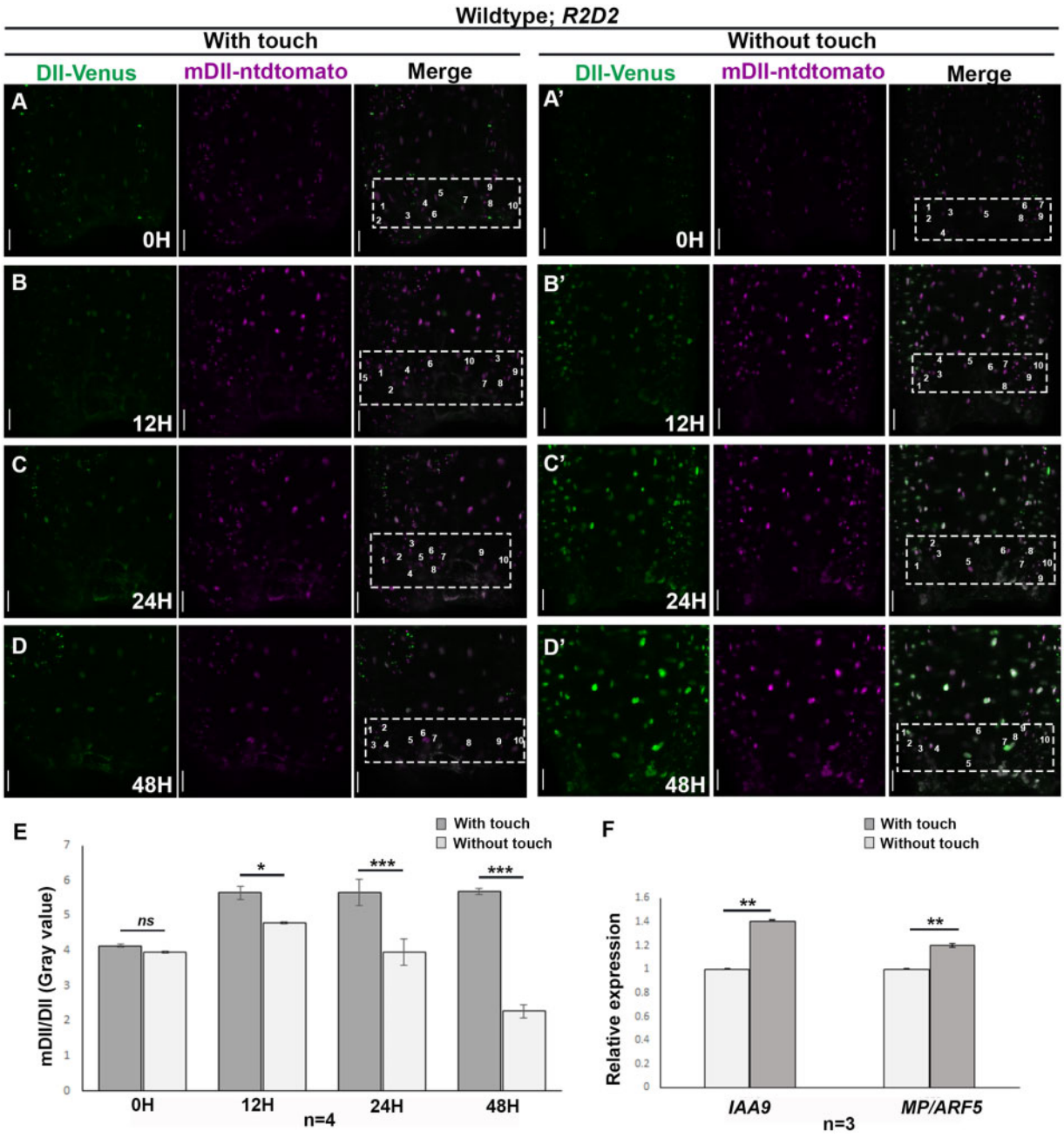

Figure 2 Touch-dependent differential auxin response near the cut ends of detached leaves: A, A′, B, B′, C, C′, D, and D′, Time lapse images showing expression pattern of auxin sensor R2D2 in cut ends of detached leaves which are in contact (A–D)and not in contact (A′–D′) with surface of the MS-agar medium. During time lapse, sequential imaging of the same leaves was done at regular intervals. Decrease in DII-Venus signal (green) indicates the increase in auxin levels. The numbers in the merge panel show nuclei used for quantification. E, Graph showing differential auxin levels in the cut ends of petioles that touched the surface against those that did not touch the surface, at 0 h (ns, $P = 0.8577$, Welch two sample *t* test), 12 h (*$P = 0.04185$, Welch two sample *t* test), 24 h (***$P = 0.0001166$, Welch two sample *t* test), and 48 h (***$P = 3.144 \times 10^{-05}$, Welch two sample *t* test; $n = 4$, $e = 2$). Here, the quantification of auxin level was done using 10 individual nuclei (labelled 1–10) from each leaf. F, Mild upregulation in the expression of auxin response genes *IAA9* (**$P = 0.00251$, Welch's two-sample *t* test) and *ARF5* (**$P = 0.00883$, Welch's two-sample *t* test) (RT-qPCR) transcript levels in detached leaves upon contact with MS-agar media. Each experiment was performed with three biological replicates and each biological replicate contain six leaves. Error bar represents s.e.m. Scale bar: 50 μm, *n*: sample size, *e*: number of experiments, H: hours post cut.

Live imaging using fluorescent-labeled lines did not reveal any rapid upregulation of PLT1, PLT2, or CUC2 (*PLT1-YFP*, *PLT2-YFP*, *CUC2-YFP*) during DNRR in WT. However, PLT1, PLT2, and CUC2 were expressed at later time points in the WT (Figure 3, N and O and Supplemental Figure S6, A–J). Corroborating with lack of DNRR in *plt3;plt5-2,plt7* leaf, we could not detect PLT1 or PLT2, which would be expressed during the onset of the de novo root primordium (Bustillo-

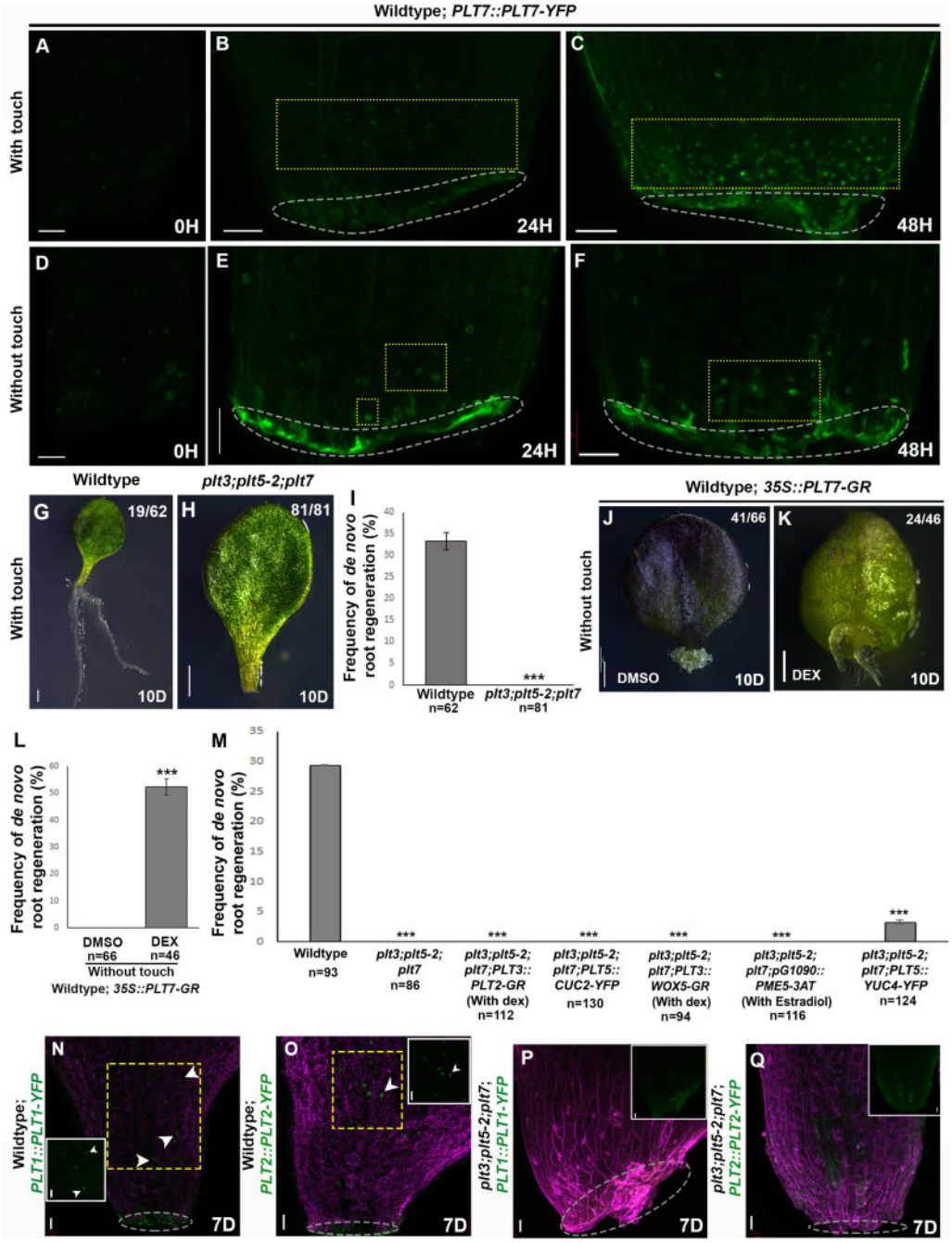

Figure 3 PLT3,5,7 are necessary and sufficient for touch-mediated DNRR: A–F, *PLT7::PLT7-YFP* expression (green) when the cut end is in continuous contact with the MS-agar (A–C) and when the cut end fails to touch the medium (D–F). Yellow dotted area indicates regions with YFP; gray dashed area encloses cut end of the leaf. Note that the green fluorescence seen at the cut end is not the true signal, but rather the reflection from damage. A–F shows brightness-adjusted YFP-channel. G and H, WT leaf explants exhibit DNRR(G) while *plt3;plt5-2;plt7* mutant(H) shows neither callus formation nor DNRR even when the cut end touches the MS-agar medium. I, Frequency of DNRR in WT and *plt3;plt5-2;plt7* mutant (***P-value = 0.0007431, Pearson's χ^2 test). J and K, OE with *35S::PLT7-GR* yields DNRR even when the cut end fails to touch the MS-agar medium. DMSO was used as control. L, Frequency of DNRR upon OE of *35S::PLT7-GR* in WT leaves (***P-value = 7.009 $\times$ 10^{-12}, Pearson's χ^2 test). Scale bars: 50 μm (A–F), 1 mm (G, H, J, K). M, Graph showing DNRR response in WT, *plt3;plt5-2,plt7* (***P-value = 9.593 $\times$ 10^{-08}, Pearson's χ^2 test), *plt3;plt5-2;plt7; PLT3::PLT2-GR*(with dex) (***P-value = 9.56 $\times$ 10^{-07}, Pearson's χ^2 test), *plt3;plt5-2;plt7; PLT5::CUC2-YFP* (***P-value = 8.853 $\times$ 10^{-11}, Pearson's χ^2 test), *plt3;plt5-2;plt7; PLT3::WOX5-GR* (with dex) (***P-value = 2.631 $\times$ 10^{-08}, Pearson's χ^2 test), *plt3,plt5-2,plt7; pG1090::PME-3AT*(With estradiol) (***P-value = 0.0007, Pearson's χ^2 test), and *plt3;plt5-2;plt7; PLT5::YUC4-YFP* (***P-value = 9.638e−08, Pearson's χ^2 test) leaves (e = 4). N and O, Expression of *PLT1::PLT1-YFP* (green) (N) and *PLT2::PLT2-YFP* (green) (O) in detached leaves of WT, marked by white arrowheads. Inset shows YFP channel. P and Q, Absence of *PLT1::PLT1-YFP* (green) (P) and *PLT2::PLT2-YFP* (green) (Q) in *plt3;plt5-2;plt7* mutant. Error bars represent s.e.m. for I, L, and M. *n*, sample size; *e*, number of experiments; *H*, hours post cut; *D*, days post cut.

Avendaño et al., 2018; Figure 3, P and Q). The defect in LR emergence in *plt3;plt5-2;plt7* was rescued by PLT1-OE or PLT2-OE under the PLT7 or PLT3 promoter (Du and Scheres, 2017; Durgaprasad et al., 2019). However, OE of CUC2, PLT2, or WOX5 under heterologous promoters did not rescue the defect in DNRR in *plt3;plt5-2,plt7* (*plt3;plt5-2,plt7;PLT5:CUC2-YFP*, *plt3;plt5-2,plt7;PLT3::PLT2-GR*, and *plt3;plt5-2,plt7;PLT3::WOX5-GR*) suggesting PLT3,5,7 does not control DNRR by regulating *CUC2*, *PLT2*, or *WOX5* (Figure 3, M). Moreover, we did not observe any YUCCA4 expression (*YUC4-YFP*) in WT during DNRR, but its OE under PLT5 promoter (*PLT5::YUC4-YFP*) could occasionally trigger DNRR from *plt3;plt5-2,plt7* leaves at a very low frequency (Supplemental Figure S7 and Figure 3, M). This suggests it is highly unlikely for PLT3,5,7 to control DNRR via YUCCA4 regulation. We further examined the role of cell wall remodeling enzyme pectin methyl esterase (PME) since it induces LR initiation (Wachsman et al., 2020). However, PME5-YFP was not upregulated in WT during DNRR and PME-OE (*plt3;plt5-2,plt7;pG1090::PME-3AT*) did not trigger DNRR in *plt3;plt5-2;plt7* (Supplemental Figure S8 and Figure 3, M). All things considered we show DNRR requisites a touch-driven, PLT3,5,7-mediated regulatory module, which is distinct from any reported regenerative or developmental pathway.

We show physical contact of the cut end of a detached Arabidopsis leaf either to solid or liquid surface instructs DNRR over callus formation. Moreover, our studies provide possible basis for organ formation from the cut ends of detached leaves of *Dracaena fragrans*, *Peperomia pellucida*, *Episcia cupreata*, *Hoya carnosa*, and *Saintpaulia ionantha*. Although DNRR from leaf necessitates the cut end to touch a surface, the underlying mechanism remains unknown. DNRR may result from a signaling cascade transduced via mechano-sensing which is triggered in response to touch. Regeneration of specific cell types in root was influenced by osmotic pressure, suggesting that mechano-sensing can be instrumental in regeneration (Hoermayer et al., 2020). Nevertheless, other possibilities need to be taken into account. Touch to the surface likely enable inhibitors of DNRR to leach out into the media, which would have otherwise accumulated at the cut end hindering DNRR. It will be interesting to unravel how touch to a surface impacts the PLT-regulated genetic frame-work of DNRR from leaf.

Supplemental data

Supplemental Figure S1. Response of detached leaves when placed on different surfaces.

Supplemental Figure S2. Quantification of auxin levels at the cut ends of detached leaves placed on MS-agar medium.

Supplemental Figure S3. Quantification of auxin levels at the cut ends of detached leaves placed on soil.

Supplemental Figure S4. Expression pattern of PLT3, PLT5 and PLT7 in cut ends of detached leaves.

Supplemental Figure S5. PLT1, PLT2 and WOX5 expression is not detectable in cut ends of *plt3;plt5-2;plt7* mutant leaves.

Supplemental Figure S6. Expression pattern of PLT1, PLT2 and CUC2 in cut ends of detached leaves.

Supplemental Figure S7. Expression pattern of YUC4 in the cut ends of detached leaves.

Supplemental Figure S8. Expression pattern of PME5 in cut ends of detached leaves.

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Conflict of interest statement. None declared.

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