Wounding-induced changes in cellular pressure and localized auxin signalling spatially coordinate restorative divisions in roots

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Wound healing in plant tissues, consisting of rigid cell wall-encapsulated cells, represents a considerable challenge and occurs through largely unknown mechanisms distinct from those in animals. Owing to their inability to migrate, plant cells rely on targeted cell division and expansion to regenerate wounds. Strict coordination of these wound-induced responses is essential to ensure efficient, spatially restricted wound healing. Single-cell tracking by live imaging allowed us to gain mechanistic insight into the wound perception and coordination of wound responses after laser-based wounding in Arabidopsis root. We revealed a crucial contribution of the collapse of damaged cells in wound perception and detected an auxin increase specific to cells immediately adjacent to the wound. This localized auxin increase balances wound-induced cell expansion and restorative division rates in a dose-dependent manner, leading to tumorous overproliferation when the canonical TIR1 auxin signaling is disrupted. Auxin and wound-induced turgor pressure changes together also spatially define the activation of key components of regeneration, such as the transcription regulator ERF115. Our observations suggest that the wound signaling involves the sensing of collapse of damaged cells and a local auxin signaling activation to coordinate the downstream transcriptional responses in the immediate wound vicinity.

Significance

Plants are sessile organisms that cannot evade wounding or pathogen attack, and their cells are encapsulated within cell walls, making it impossible to use cell migration for wound healing like animals. Thus, regeneration in plants largely relies on the coordination of targeted cell expansion and oriented cell division. Here we show in the root that the major growth hormone auxin is specifically activated in wound-adjacent cells, regulating cell expansion, cell division rates, and regeneration-involved transcription factor ERF115. These wound responses depend on cell expansion of the eliminated cells presumably perceived by the cell damage-induced changes in cellular pressure. This largely broadens our understanding of how wound responses are coordinated on a cellular level to mediate wound healing and prevent overproliferation.
promoting and inhibiting wound responses in a dose-dependent manner. Finally, we show that ERF115 expression and wound-responsive cell division are linked spatially and temporary through the perception of damaged cell collapse, indicating a crucial role of turgor pressure and cell wall integrity perception in wound signaling.

Results

Local Auxin Accumulation in Cells Undergoing Restorative Division. To observe potential changes in auxin signaling after single-cell ablation, we used an auxin signaling marker, R2D2, which consists of an auxin-degradable RPS5::DII-Venus component and the stable RPS5::mDII-Tdtomato, allowing detection of increased auxin signaling (18). We used vertical growth confocal imaging coupled with live tracking (19, 20) to follow cells during regeneration over a prolonged period (16 to 32 h). We observed a down-regulation of DII-Venus indicative of auxin signaling increase in wound-adjacent cells approximately 3 h before the first division (Fig. 1 and Movie S1). These changes in DII were absent from neighboring cells not adjacent to the wound in the same roots. Notably, after the first division, daughter cells that were still adjacent to the wound retained the high auxin signaling activity, while the nonadjacent daughter cells quickly regained normal auxin signaling levels (Fig. 1 A and C). These dynamics were not due to any possible bleaching effects of the ablation or propidium iodide staining (SI Appendix, Fig. S1 A–E and Movies S2 and S3), and at least ~50% of all wound-adjacent cortex and ~20% of all wound-adjacent endodermis cells exhibited this down-regulation of DII-Venus (SI Appendix, Fig. S1G), altogether suggesting increased auxin signaling in cells activated by wounding.

To elucidate the source of this increased auxin signaling in the wound-adjacent cells, we performed long-term imaging of roots treated with the auxin biosynthesis inhibitors kynurenine and yucasin, as well as with the polar auxin transport inhibitor naphthylphthalamic acid (NPA) (21–23). Notwithstanding the overall auxin accumulation throughout the root tip after NPA treatment, we still observed consistent DII signal down-regulation specifically in the wound-adjacent cells compared with their direct neighbors (SI Appendix, Fig. S1H and Movie S5). A wounding-induced local increase in auxin signaling also occurred after inhibition of auxin biosynthesis (SI Appendix, Fig. S1H and Movie S5). Even though some role of these processes cannot be completely ruled out, these pharmacologic manipulations suggest that auxin biosynthesis or polar transport are not major contributors to the local increase in auxin signaling during regeneration. Other, as-yet unidentified auxin homeostasis processes, such as release of free auxin from conjugates, may play a role.

In summary, our findings show a local increase of auxin signaling in wound-adjacent cells, specifically those that contribute to the regeneration process by later restorative divisions and expansion. The underlying mechanism for this elevated auxin signaling remains elusive but appears to be independent of both onsite auxin biosynthesis and polar auxin transport.

Dual Auxin Effect on Regeneration Efficiency. The local increase in auxin signaling after wounding implies a role for auxin in restorative patterning. Auxin might accelerate the cell cycle progression and determine the cell division plane or the stem cell fate acquisition, the cell expansion toward the wound, or a combination of these effects. As reported previously (5), the
cortex and endodermis exhibit different rates of restorative, periclinal cell divisions after 12 h; endodermis cells divide rapidly, while division of cortex cells is usually slower. However, after sufficient time (>32 h), all ablated cells are replaced by restorative divisions of wound-adjacent cells.

To test for an auxin effect in wound regeneration, we applied synthetic auxin (1-naphthaleneacetic acid [NAA]) 1 h before the ablation, imaged the wounded roots 12 h after ablation, and determined how many ablation sites triggered periclinal cell divisions in the inner adjacent cell types. These experiments revealed that NAA application increased the division rates at concentrations up to 1 μM (Fig. 2A and SI Appendix, Fig. S2A), particularly in cortex cells, which usually have a relatively low regeneration rate in the first 12 h. On the other hand, the already fast regeneration rate of the endodermis cells (~75%) was not much further increased by auxin (SI Appendix, Fig. S2A). To examine the effects of decreased auxin levels, we treated roots with 10 μM kynurenine and observed that roots with lower auxin levels exhibited decreased restorative division rates in all cell types, again indicating a positive auxin effect on restorative cell divisions (SI Appendix, Fig. S2C).

Nonetheless, at higher concentrations, NAA caused a decrease in division rates, with 1 μM NAA apparently representing a breaking point, where two populations of auxin-treated roots were observed: roots with fast and efficient regeneration similar to plants treated with 250 to 500 nM and roots with strongly inhibited regeneration. The inhibitory auxin effect became more pronounced at 2 μM NAA, and at 5 to 10 μM NAA, regeneration no longer occurred (Fig. 2A and SI Appendix, Fig. S2A). This concentration-dependent auxin effect on restorative division rates suggests a dual, both positive and negative, mode of action for auxin during tissue regeneration.

**TIR1 Auxin Signaling-Mediated, Wound-Induced Cell Proliferation.** To investigate the signaling mechanism underlying this auxin effect, we tested NAA application on the tir1-1 afb2-1 afb3-3 (tir triple) mutant, which is defective in auxin receptors (24, 25). Untreated tir triple mutant showed a regeneration capacity not much different from that of the wild type (WT) (Fig. 2B and SI Appendix, Fig. S2B). However, in the tir triple mutant, treatment with 1 μM NAA, which in WT normally inhibited divisions, increased the restorative division rate of endodermis and cortex cells to almost maximum (Fig. 2B and SI Appendix, Fig. S2B); we even observed repeated divisions within 12 h (SI Appendix, Fig. S2H). This suggests that the dual-phase effect of auxin can be differentiated into an activating effect and an inhibitory effect on restorative divisions, with at least the latter effect depending on the activity of the TIR1 auxin receptors.

Besides its overall effect on the rate of restorative cell divisions, auxin also influenced the direct response of each involved cell toward wounding. Usually, wound sizes one to two cells are filled by one to three adjacent cells at the inner side of the wound, which initiate periclinal divisions (5); however, auxin treatment increased the area of activated cells to nearly all wound-adjacent cells. On some occasions, four or more cells responded to the wound by inducing restorative divisions (Fig. 2C and SI Appendix, Fig. S2 D and E). Auxin also triggered ectopic periclinal cell divisions at the outer wound side or in adjacent upper and lower cells. Usually, these cells undergo normal anticlinal divisions and do not contribute greatly to the wound healing (5) (Fig. 2D and SI Appendix, S2 F and G). This auxin effect on activating more adjacent cells and at all sides also occurred in the tir triple mutant and thus is likely a component of the activating auxin effect that is less dependent on TIR1/AFB-mediated auxin perception.

These effects of auxin on the increase in restorative division rate, the spreading of wound responses to all adjacent cells, and the ectopic division plane switch from anticalinal to periclinal were regularly manifested in an overproliferation of the whole wounded area. Roots treated with 1 μM NAA sometimes contained wound-responsive cells that did not stop the periclinal cell divisions and continuously divided and expanded to overfill the induced wounds with new daughter cells. This caused a strong swelling of the root meristem (Fig. 2G), which occurred in ~20 to 50% of WT plants. However, in the tir triple mutant, most ablation sites (60 to 70%) triggered this strong overproliferation phenotype, likely because of a reduction in the TIR1 signaling-mediated inhibitory auxin effect (Fig. 2E and F and SI Appendix, Fig. S2J).

To further test the involvement of TIR1 in this regulation, we used the synthetic CCV-TIR1–convex IAA (CVX-IAA) pair, in which a modified auxin ligand, CVX-IAA, can bind only to a mutated form of TIR1 receptor, CCV-TIR1, but not to WT-TIR1 (26). CVX-IAA triggered swelling only in the CCV-TIR1 plant, but not in the WT plants (SI Appendix, Fig. S2K).

This causally links the auxin effect on wound regeneration to the TIR1/AFB pathway and suggests that both inhibiting and activating auxin effects are mediated by this pathway. In this view, the reduced inhibitory effect in the tir triple mutant can be explained by the overall shift in sensitivity toward higher auxin concentration due to lack of some of the TIR1/AFB auxin receptors.

**Auxin-Induced Cell Expansion in Wound Regeneration.** The overproliferated, swollen roots observed after auxin treatment are the consequence of an increased number of restorative cell division and their consequent cell expansion. Given that auxin has a well-established role in growth both in roots and shoots (27–30), we analyzed changes in cell expansion during wound regeneration. We performed long-term imaging experiments in which we quantified the width of wound-adjacent cells as a proxy for cell expansion. After ablation, the size of the inner adjacent cells remained constant for a short “lag phase” (tlag) of 0 to 4 h, followed by a phase of rapid expansion (texp), usually for 10 h, in which the cells grew continuously toward the wound and bulged toward the inner intact neighbor, suggesting increased turgor pressure. The expansion paused shortly during the time of division (tdiv) and continued afterward (texp) (Fig. 3A and B; SI Appendix, Figs. S3 A–C and F; and Movie S6).

Auxin treatment reduced the expansion rate strongly in WT endodermis cells from 0.80 ± 0.3 μm/h to 0.42 ± 0.3 μm/h and slightly in cortex cells, from 0.68 ± 0.1 μm/h to 0.58 ± 0.2 μm/h (Fig. 3 C and D and SI Appendix, Figs. S3D and S4 A–C). The decreased expansion rates lead to a delay in cell size increase, which correlated with the decreased speed of restorative division induction (SI Appendix, Fig. S3E). In contrast, auxin treatment in the tir triple mutant strongly increased expansion in both the endodermis (1.0 ± 0.2 μm/h) and cortex (1.1 ± 0.3 μm/h) (Fig. 3 C and D and SI Appendix, Figs. S3D and S4 B–D), again correlating with higher rate of regeneration in these conditions. Collectively, these observations suggest that cell expansion is an important component of regeneration after wounding.

Notably, wound-adjacent cells in untreated WT plants exhibited stable cell expansion rates, which decreased only during mitosis. In contrast, cell expansion in auxin treated tir triple cells strongly fluctuated; phases of extremely high expansion (~2 μm/h) were followed by a complete collapse (<0.5 μm/h) long before the onset of mitosis (SI Appendix, Fig. S4E). Steady cell expansion usually requires coordination of cell wall properties and turgor pressure, suggesting that this may be uncoupled in the case of tir triple on 1 μM NAA.

In summary, we observed that cell expansion correlates strongly with induction of regenerative divisions. Auxin has a similar dual effect on both cell expansion and regenerative division, presumably balancing turgor pressure and cell wall pressure.
properties to regulate a steady growth toward the wound and permit accelerated periclinal divisions.

**Auxin and Wounding in Activation of ERF115 Expression.** ERF115 is a wound-responsive transcription factor and, so far, the prime candidate for mediating regeneration in plant roots. It is typically not expressed in the undisturbed root meristems but is highly up-regulated after removal of the root tip and is required for its restoration (5, 6, 10). After single-cell ablation, its expression is induced only in those one to three wound-adjacent endodermis or stele cells that later undergo wound-responsive divisions (Fig. 4/4). In contrast, auxin (1 μM NAA) treatment, while having no effect in nonwounded roots, strongly induced ERF115::GFP expression around the wound (Fig. 4 A and B), spreading to all adjacent cells, including cortex cells and those above and below the ablation. Notably, ERF115 expression also expanded to cells nonadjacent to the wound, which normally do not undergo wound-responsive cell expansion and cell division.
Overall, auxin induced an approximate eightfold increment of the ERF115::GFP expression after wounding (Fig. 4C).

We next evaluated ERF115::GFP expression using different auxin concentrations. We found that the minimum concentration of NAA required to enhance ERF115 was 100 nM NAA, which significantly increased the GFP intensity in the few cells undergoing restorative cell division. In contrast, the expression domain expanded only at higher concentrations such as 1 μM NAA (SI Appendix, Fig. S5). To test the involvement of the canonical TIR1 pathway, we evaluated ERF115::GFP expression after wounding in tir triple roots or in the CCV-TIR1 background. Both genetic tools confirmed the involvement of TIR1/AFB signaling (SI Appendix, Fig. S5 B and C). Notably, ERF115::GFP expression was also absent in the untreated tir triple mutant (SI Appendix, Fig. S5C), suggesting involvement of TIR1/AFB auxin signaling in activation of ERF115 expression by endogenous auxin levels.

ERF115 expression also has been proposed to be regulated by other signals, such as methyl jasmonate (MeJA), H₂O₂, or brassinosteroids (6, 11, 12); nonetheless, unlike in the case of auxin, none of these other signals showed a pronounced effect on wound-responsive ERF115::GFP expression (SI Appendix, Fig.
Auxin-Regulated Cell Expansion and Activation of ERF115 Expression.

Since auxin regulates both cell expansion and ERF115 expression after wounding, we performed long-term, live observations to investigate possible correlation between these processes. Indeed, even in absence of auxin treatment, we found that cells with higher expansion rates also had increased ERF115::GFP expression after wounding (SI Appendix, Fig. S6 J and M). These results suggest that auxin is rather a specific signal regulating ERF115::GFP expression after wounding, although not the primary trigger for its activation.

Tissue Integrity and Turgor in Wound Regeneration.

Given the here identified importance of cell expansion for both the regenerative divisions and ERF115 expression, we hypothesized that rapid, nongenetic factors, such as collapse of injured cells and changes in turgor or cell wall tension, may be important early cues for inducing regeneration processes. Therefore, we investigated more closely the immediate responses of the targeted cell and its surrounding neighbors during ablation-induced injury. Following ablation, the cellular content of the killed cells was released to the environment, resulting in a pressure drop, which was visible as a strong bulging of the neighboring cells toward the wound (SI Appendix, Fig. S7A and Movie S7). In those neighboring cells, we observed a rapid displacement of nuclei (SI Appendix, Fig. S7C) and a slow reduction of the initial bulging (SI Appendix, Fig. S7B and Movie S7), suggesting a loss of cell volume in the neighboring cells. Over a longer time (>60 s), pressure built up again

Fig. 4. ERF115 expression is connected to auxin and expansion. (A–C) Auxin application massively increases ERF115 expression around wounds. ERF115::GFP (green) expression on wound-adjacent cells on mock treatment (A) and on 1 μM NAA treatment (B). Asterisks denote ablated cells. Red arrows indicate ERF115::GFP expression expansion to wound-adjacent cortex cells, and the white arrow indicates ERF115::GFP expression expansion to non–wound-adjacent endodermis and stele cells. (C) Quantification of the relative ERF115::GFP fluorescence in wound-adjacent cells (normalized to the ERF115::GFP mean intensity on mock conditions), on mock treatment (green dots) or on μM NAA treatment. Data are represented as relative fluorescence intensity of individual cells. Statistical significance was computed using the two-sample Wilcoxon test. (D–F) ERF115::GFP expression correlates with premitotic expansion. (D) Quantification of the percentage of cells expressing ERF115::GFP, grouped by grade of connection (connected or nonfully connected to the wound). Data are represented as ratios (bars) with error bars indicating upper and lower quartiles. Statistical significance was computed using the χ² test. ERF115 expression was absent from those cells that were only weakly connected to the wound and thus did not trigger cell expansion. However, after treatment with auxin, ERF115 expression coincided with or preceded the onset of expansion (Fig. 4D). Considering the temporal dynamics of upstream transcriptional regulation and GFP maturation, this also ruled out the possibility that ERF115 expression is a direct consequence of cell expansion.

In conclusion, wound-responsive activation of ERF115 expression and cell expansion were strongly correlated spatially and temporally, but did not directly depend on each other, suggesting an as-yet unknown wound-induced upstream signal activating both.

S6 A–E, K, and L). Ethylene, a wound-responsive hormone recently connected to the innate immune system in Arabidopsis root after wounding and single-cell ablation (7, 31), did not influence ERF115 expression (9) (SI Appendix, Fig. S6 F–H) and decreased the amount of restorative divisions in the meristem only slightly (SI Appendix, Fig. S6d). Application of other hormones related with cell wound response, including salicylic acid (SA) and abscisic acid (ABA), also did not change the ERF115::GFP expression after wounding (SI Appendix, Fig. S6 I and J). In addition, we observed that ERF115::GFP expression was absent from those cells that were only weakly connected to the wound and thus did not trigger cell expansion (Fig. 4 E and F).

Given this strong spatial connection between ERF115 expression and wound-responsive expansion, we investigated their time dynamics to gain insight into their interdependency. ERF115 expression was detectable only after the onset of expansion (Fig. 4D), ruling out ERF115 as a major activator of wound-responsive expansion. However, after treatment with auxin, ERF115 expression coincided with or preceded the onset of expansion (Fig. 4D). Considering the temporal dynamics of upstream transcriptional regulation and GFP maturation, this also ruled out the possibility that ERF115 expression is a direct consequence of cell expansion.

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in the neighboring cells, and they again bulged toward the wound (SI Appendix, Fig. S7A). Notably, the release of cellular content and rapid pressure changes as quantified by instant nucleus displacement were absent from roots during treatment with hyperosmotic, 0.5 M mannitol (SI Appendix, Fig. S7 D and G). This suggests that the collapse of killed cells causes a strong turgor pressure shock in the neighboring cells, influencing cell expansion and ERF115 expression.

In support of this idea, treatment with mannitol strongly decreased wound-induced cell expansion and wound-triggered periclinal divisions (Fig. 5 A and B). Notably, the normally occurring, anticlinal (nonrestorative, proliferative) divisions were still ongoing under these hyperosmotic conditions (SI Appendix, Fig. S7F). In addition, the bulging of cell walls toward the inner neighbors, indicative of changes in turgor pressure, was significantly reduced during mannitol treatment (SI Appendix, Fig. S7 G and H). This implies that changes in turgor are specifically required for regenerative, but not proliferative, divisions.

We next evaluated the effect of hyperosmotic treatment on ERF115::GFP expression by applying different concentrations of mannitol to locally injured roots. In contrast to cell expansion, we observed that at mannitol concentrations of 0.23 M and 0.3 M, the ERF115 expression was only slightly reduced after wounding in both mock and auxin (1 μM NAA) treatment. Of note, only hyperosmotic treatment with 0.5 M mannitol inhibited ERF115::GFP expression in the wounded root tips (Fig. 5 C and D), suggesting that cell collapse after wounding, which is absent at treatment with 0.5 M mannitol (see above), is crucial for triggering ERF115 expression.

This finding prompted us to test the importance of the collapse of the ablated cells for cell expansion, ERF115 expression, and regeneration. To this end, we modified the UV ablation technique by reducing the laser power to 50%. We regularly managed to harm the cell envelope between two cells rather than the whole cells (Fig. 5 E and F). These harmed cells usually did not induce ERF115 expression (Fig. 5G). However, in rare cases, these cells specifically triggered ERF115 induction, which was strongly enhanced by auxin treatment (Fig. 5G and SI Appendix, Fig. S7I). They also induced periclinal cell divisions, accompanied by minor cell expansion on rare occasions (SI Appendix, Fig. S7J), suggesting minor wound signaling without cell collapse.
through cell wall integrity sensing or minor pressure changes after cell envelope harm.

This suggests that cellular collapse after wounding is crucial for ERF115 expression and wound-responsive cell expansion. Furthermore, turgor pressure stress after ablation is a component of the wound response and, together with the local increase in auxin signaling, might influence fast and efficient regeneration.

**Discussion**

In this work, we addressed the question of how plants can effectively regulate the coordination of wound responses on a cellular level to mediate tissue regeneration. We found that local induction of auxin signaling in concert with wound-induced nongenetic mechanisms, such as local expansion of cells in the wound vicinity, play major roles in this process. In summary, our data show how strictly localized auxin signaling coordinates the wound responses by regulating cell division rates, cell expansion rates, and transduction of the wound signal through activation of the ERF115 transcription factor (SI Appendix, Fig. S8).

Our data also show that turgor-driven swelling of the wound-adjacent cells is crucial for the initiation of restorative divisions and subsequent regeneration. This shows interesting similarities/parallels to the initiation of lateral root primordia, where pericycle cells swell before induction of asymmetric divisions (32). This process requires that the overlying endodermis cells shrink or collapse, which can be imitated by local ablation of endodermis cells in the elongation zone (33). The capacity to divide after ablation in the pericycle is independent of auxin signaling; however, local auxin signaling is essential in this context for the induction of a formative and asymmetric first division, leading to the proper formation of lateral root primordia (33, 34). We have observed a similar phenomenon; a local auxin increase in cells adjacent to the wound regulates both their expansion and division during regeneration. An accumulation of auxin could have many sources, for example, release from conjugates or a production from less well known alternative biosynthetic pathways, altered auxin importer activity, or a general release from the plasmodesmata-associated ER as consequence of the disrupted connection to the ablated neighbor. Since such processes are difficult to study, the origin has remained elusive; however, daughters of wound-adjacent cells also exhibit increased auxin signaling activity, implying an active process working throughout the duration of regeneration.

Auxin has a well-established role in expansion on a cellular level, through cell wall remodeling (35), and on a tissue level, through hydraulic pressure regulation (36). The biphasic mode of auxin in cell wall remodeling (27) shares similarities with our observed dual auxin effect of promoting and inhibiting cell expansion and restorative divisions, thus suggesting a negative feedback loop in which prolonged auxin signaling would inhibit wound-responsive division and expansion at the end of the regeneration process. The overproliferation of cells and the tissue swelling after wounding is similar to defects in lateral root primordia initiation during disturbed auxin signaling (37), suggesting that accurate auxin signaling is crucial for the regulation of wound-responsive cell expansion and divisions.

The turgor pressure—or osmotic—stress in the adjacent cells following wounding might be a key component in the wound signaling mechanism, as has been suggested in other tissues (38, 39). Recently, a similar, locally restricted induction of wound responses in the root—the activation of immune signaling—has been proposed to be caused by local pressure changes (31). Notably, the wound-responsive expression of ERF115 is also inducible by osmotic stress (40), and a direct target of ERF115 includes one member of the expansin family (9); cell wall remodeling proteins expressed during osmotic stress to maintain steady growth even during reduced turgor pressure (41). This suggests that ERF115 is a key component of a hydraulic/osmotic stress response after wounding and might be involved in integrating auxin signaling and pressure stress to define the zone of wound response and to both induce and spatially restrict regeneration processes.

Further investigation of the induction of ERF115 expression as a marker for restorative patterning and involvement of other components suggested in wounding responses in the root could help reveal upstream regulators of such local yet distinctive regeneration processes of inducing cell expansion and targeted, restorative cell divisions.

**Methods**

**Plant Material.** Arabidopsis thaliana (L.) Heynh (accession Columbia-0) was used in this work (WT). The following transgenic A. thaliana lines and mutant lines were described previously: RPSSA::mDI1-ntdTomato-RPSSA::Dil-n3Venus (R2D2) (18), HTRS::nLS::GFP (42), tir1-1 afb2-1 afb3-1 (tir triple) (24), CCV-TIR1 tir1-1 afb2-1 (28), ERF15::nLS::GFP-GUS (ERF15::GFP) (10), ERF15::nLS::GFP- GUS cot-1-2 (35), and 35S::MAP4-GFP (43). ERF15::nLS::GFP-GUS was introduced into the tir1-1 afb2-1 afb3-1 and CCV-TIR1 backgrounds by genetic crossing. tir1-1 afb2-1 afb3-1 seedlings were identified by PCR-based genotyping (24) in F2 and F3 generations ERF15::nLS::GFP-GUS, and CCV-TIR1 carrying plants were identified in F2, F3, and F4 generations by kanamycin and basta resistance, respectively.

**Growth Conditions.** Seeds of A. thaliana were sown on Murashige & Skoog (M&S) medium (Duchefa Biochemie) with 1% sucrose and 1% agar, stratified for 1 to 2 d, and grown for 3 to 5 d at 21 °C on a 16-h light/8-h dark cycle.

**Pharmacologic Treatments.** Seedlings were transferred on solid M&S medium containing the indicated chemicals: propidium iodide (PI; 10 μM, Sigma-Aldrich or Thermo Fisher Scientific), NAA (Duchefa Biochemie; final concentration if not indicated otherwise, 1 μM), NPA (final concentration 10 μM,Sigma-Aldrich; final concentration 100 μM, Wako Chemicals); brassinolactone (Sigma-Aldrich, final concentration 10 μM, as indicated), brassinazole (Sigma-Aldrich, final concentration 200 μM), brassinolide (Sigma-Aldrich, final concentration 50 μM), brassinazole (Sigma-Aldrich, final concentration 50 μM, Sigma-Aldrich, final concentration 1 μM, SA (Sigma-Aldrich, final concentration 40 μM), and mavenol (Sigma-Aldrich, final concentration 10 to 0.5 μM, as indicated).

**Sample Preparation.** Seedlings were placed on chambered cover glass (Nunc Lab-Tek; Thermo Fisher Scientific) as described previously (44). With the chamber, a block of solid M&S medium was cut out, and PI solution was added. Once the liquid soaked in, 10 to 15 seedlings were transferred to the agar, and the block was inserted into the chamber.

**Confocal Imaging and Image Processing.** Confocal imaging was performed with Zeiss LSM700/800 inverted microscopes using a 20× or 40× objective or a spinning-disk imaging system. Detection of fluorescence signals was done with Zeiss LSM700 or LSM800 inverted confocal microscope and Zeiss Zen 2.3 software. Where necessary, images were processed by adjusting contrast and lightness.

**Spinning-Disk Imaging.** For the observation of immediate effects during/after ablation, an Andor spining disk microscope (CSU X-1, Ikon 897 camera [back-thinned EMCCD], FRAPPA unit, and motorized piezo stage) with a 63× water immersion objective was used. Videos were acquired with one focal plane every 0.2 s, for 1 to 10 min. All images in a single experiment were captured with the same settings.

**Vertical Stage Microscopy, Root Tracking, and Image Processing.** Vertical stage microscopy for long-term tracking of root meristems was performed as described previously (5, 19). Roots were imaged with a vertically positioned Zeiss LSM700 or LSM800 inverted confocal microscope and Zeiss ZEN 2.3 “Black” or “Blue” software, respectively, with a 20× objective and detection of PI, GFP (see above), and transmitted light. Z-stacks of 30 to 42 μm were set accordingly to image each cell at least once. For root tracking, the TipTracker MATLAB script (Zen Black) or the TipTracker internal macro (Zen Blue) were
used; interval duration was set between 600 s (10 min) and 720 s (12 min). The resulting images were concatenated and analyzed using ImageJ. For registration, Image macros “Correct 3D drift,” “StackReg,” and “Multi-StackReg” were used. Kymographs were generated using the “Reslice” tool and restacked in case of nonregistered videos.

UV Laser Ablation Setup. UV laser ablation was performed as described previously (5, 33), based on a previously published layout (45). For the cell envelope harming, we used 50% of the laser power needed for the elimination of a full cell. The laser was applied in the upper corner of the outer neighboring cell of the cell of interest (Fig. S5).

Quantification and Statistical Significance. Asterisks illustrate the P value: ***P < 0.001; **P < 0.01; *P < 0.05.

Pericellular Divisions. For counting the outermost (absolute) amount of pericellular divisions after ablation, the number of ablations with and without pericellular divisions (division plane parallel or orthogonal to the growth axis) in any of the adjacent cells (if nothing else indicated, only inner cells) were recorded, and the ratio of positive events to all events was calculated. For the differences, the ratios for each sample experiments were subtracted from the respective control experiment (untreated WT). The mean of these differences from multiple experiments was weighted based on the sample size.

As described previously (5), the binary data were analyzed using conditions (46, 47), pairing the data within individual experiments. The statistical significance was computed using the clogit function from the R package “survival”.

Fluorescence Intensity in Time Series Experiments. The signal intensity of each observed nucleus from multistack three-dimensional (3D) videos in the green (or red, for mDI-ntdTomato) channel was quantified using ImageJ and recorded every time frame. Similarly, data from reference nuclei (neighboring, nonadjacent cells) was recorded, and the ratio of sample to reference value was calculated for each single time point. For EFRI15-GFP, the ratio of each time frame to the first time frame was calculated.

Statistical significance was calculated on the ratios using a Student’s t test using t.test() in R. For calculating the point of expansion collapse (point of the cell wall facing the inner adjacent neighbor) was calculated as the distance between the midpoint of the cell wall segment touching the ablation site and the midpoint of the opposite cell wall at each time point using ImageJ. Expansion rates were calculated between the onset of expansion (end of lag phase) and the initiation of cell division (nuclear envelope breakdown). Statistical significance between samples was calculated with Student’s t test using t.test() in R. For calculating the point of expansion collapse (SI Appendix, Fig. S4E), expansion rates at each time point were calculated from cell width expansion throughout the previous five time frames (50 min). The point of expansion collapse was determined as the first time point at which the expansion rate dropped below 1 μm/h.

Cell Wall Bulging. The distance between the midpoint of a straight line connecting the two cell corners facing the inner adjacent cell and the midpoint of the cell wall facing the inner adjacent neighbor was calculated as inner cell wall bulging. The values obtained from wound-adjacent cells were subtracted from the closest, nonadjacent cells of the same cell type (usually the direct neighbor).

The statistical significance was calculated using the two-sample Wilcoxon test between the mock and manitol treatment values with wilcox.test() in R.

Nucleus Displacement. The distance of nucleus movement/displacement directly after ablation was measured until maximum 5 s after ablation from videos obtained from a spinning disk imaging system (1 frame per 0.2 s). The statistical significance was calculated using a two-sample Wilcoxon test between the mock and the values with wilcox.test() in R.

For qualitative measurements, the signal intensity of all wound adjacent (inner) cells within one focal plane was measured using ImageJ in the green channel. Each obtained value from one experiment/repetition was divided by the mean fluorescence intensity in the reference sample (untreated WT) to obtain (comparable) relative fluorescence intensities.

The statistical significance was calculated using the two-sample Wilcoxon test between the reference and the sample values with wilcox.test() in R.

Data Availability. The R code for computing statistical significance for binary data using a conditional logistic regression is provided in SI Appendix, Code S1. Raw data in the form of microscopy images and compiled videos can be accessed at https://www.ebi.ac.uk/biotudies/biowlomages/studies/5-BIAD23 under accession no. S-BIAD23.

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