Role of NINJA in root jasmonate signaling

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Wound responses in plants have to be coordinated between organs so that locally reduced growth in a wounded tissue is balanced by appropriate growth elsewhere in the body. We used a *JASMONATE ZIM DOMAIN 10* (*JAZ10*) reporter to screen for mutants affected in the organ-specific activation of jasmonate (JA) signaling in *Arabidopsis thaliana* seedlings. Wounding one cotyledon activated the reporter in both aerial and root tissues, and this was either disrupted or restricted to certain organs in mutant alleles of core components of the JA pathway including *COI1*, *OPR3*, and *JAR1*. In contrast, three other mutants showed constitutive activation of the reporter in the roots and hypocotyls of unwounded seedlings. All three lines harbored mutations in *Novel Interactor of JAZ* (*NINJA*), which encodes part of a repressor complex that negatively regulates JA signaling. These *ninja* mutants displayed shorter roots mimicking JA-mediated growth inhibition, and this was due to reduced cell elongation. Remarkably, this phenotype and the constitutive *JAZ10* expression were still observed in backgrounds lacking the ability to synthesize JA or the key transcriptional activator MYC2. Therefore, JA-like responses can be recapitulated in specific tissues without changing a plant's ability to make or perceive JA, and MYC2 either has no role or is not the only derepressed transcription factor in *ninja* mutants. Our results show that the role of NINJA in the root is to repress JA signaling and allow normal cell elongation. Furthermore, the regulation of the JA pathway differs between roots and aerial tissues at all levels, from JA biosynthesis to transcriptional activation.

root growth | herbivory | plant fertility | mapping by sequencing

In response to mechanical damage or herbivory, plants rapidly accumulate the lipidic prohormone jasmonic acid (JA) and biologically active jasmonoyl-L-isoleucine (JA-Ile), which inhibits growth and activates defense responses to promote plant fitness (1). In the current model of JA perception and signaling, JASMONATE ZIM DOMAIN (JAZ) proteins block the function of JA-responsive transcriptional activators in the absence of bioactive JA-Ile (2, 3). JAZ proteins form a repressor complex by recruitment of the general corepressor TOPLESS (TPL) and TPL-related proteins either directly or indirectly through interaction with the adaptor protein Novel Interactor of JAZ (NINJA) (4, 5). When JA-Ile accumulates, it acts as a bridging ligand between the F-box protein CORONATINE INSENSITIVE 1 (COI1) and JAZ proteins to form the JA coreceptor complex (6). This leads to ubiquitylation and degradation of JAZs, setting the JA-responsive transcriptional activators free to function (2, 3, 6).

Recent studies of the plant wound response and other JA-signaling processes have relied on measuring the accumulation of JAs and/or JA-dependent transcripts in entire tissue extracts (7, 8). Although such approaches have contributed to our current understanding of JA responses, a better spatiotemporal resolution could facilitate the study of pending questions on JA and wound signaling through novel cell biological and genetic means. Because reliable methods for in situ detection of jasmonates have rarely been implemented (9), transcriptional reporter lines based on robust JA-dependent promoters are one of the best tools to accurately localize JA signaling *in planta*. Another common feature of previous wound response studies in *Arabidopsis* is the use of adult rosettes, although experiments on the effects of exogenously applied JA have additionally used in vitro-grown seedlings (10). Small seedlings offer a convenient system to follow the spatiotemporal localization of JA signaling. Furthermore, visualizing JA signaling in all seedling tissues opens avenues to investigate how JA responses are temporally and spatially organized between organs in the plant body.

Arabidopsis seedlings have previously been the system of choice in many forward genetic screens that successfully identified JA-signaling components. These screens chiefly used the reduction in root growth caused by exogenously applied JA as a phenotypic output (10–12). However, specific screens for JA-mediated wound signaling have not been reported for *Arabidopsis*, and only one such screen was performed in tomato (13). Other genetic screens have used JA-responsive reporter lines to recover mutants with constitutive JA signaling (14), but in only two cases the corresponding mutant gene was identified and characterized (15, 16).

As part of a continuing effort to identify organ-specific activators or repressors of wound signaling, we screened seedlings from an EMS-mutagenized population of *JAZ10-GUSPlus*, a JA-responsive reporter, for either impaired activation after cotyledon wounding or constitutive expression without mechanical stimulation. The screen yielded mutant alleles of known JA biosynthesis and signaling components including three *ninja* alleles. Here we report the unexpected finding that NINJA is indispensable in repressing JA signaling in roots and in maintaining normal root growth. Even in a null JA synthesis background, these mutants display growth phenotypes normally observed when seedlings are wounded or treated with JAs. Our results provide evidence for a previously unrecognized organ specificity of JA responses, and we extend this observation to JAR1, which is involved in the synthesis of biologically active JA-Ile, and MYC2, a transcriptional activator of JA signaling.

Results

JA-Responsive Reporter for Spatial Localization of Wound-Induced Signaling. Mechanical wounding rapidly and strongly induces a well-characterized set of JA-dependent genes such as *JAZ10* (17, 18).

Significance

Plant growth, the basis of agricultural production, is compromised when plants defend themselves against herbivores. Wound-induced growth reduction is coordinated between organs by hormones termed "jasmonates." We developed a sensitive assay that marks tissues where wounding activates jasmonate function in seedlings. This assay showed that a key repressor of jasmonate responses is active mainly in roots where it permits normal growth. A deeper understanding of cell-size control is crucial in successfully engineering plants that display reduced growth restriction under stress.

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We used the promoter of this gene to drive the transcription of a secretable β-glucuronidase (GUS)–based reporter as a tool to visualize JA signaling in *Arabidopsis*. In untouched 5-d-old (d.o.) wild-type (WT) reporter seedlings, we detected weak *JAZ10-GUSPlus* (*JGP*) expression under our staining conditions (Fig. 1). However, piercing one cotyledon with a needle 2 h before GUS staining resulted in strong *JGP* activation not only in the wounded cotyledon but also in the shoots, the hypocotyl, and the root. The undamaged cotyledon displayed limited and more variable reporter staining, which suggests that long-distance wound signaling to this organ is weaker and/or takes more time to arrive. Wound-induced *JGP* expression was confined to the vasculature in the roots (Fig. 1, *Inset*) whereas in the aerial organs it spanned other tissues in addition to the vasculature. We have never observed *JGP* expression reaching the meristematic zone (MZ) of the primary root after cotyledon wounding, although cells of this zone respond strongly to exogenous methyl jasmonate (MeJA) (*SI Appendix*, Fig. S1), which is de-esterified *in planta* into the prohormone JA. Quantification of *JAZ10* transcripts in roots and aerial organs of seedlings independently confirmed the strong induction of *JAZ10* in both organ types after cotyledon wounding (*SI Appendix*, Fig. S2). We also crossed the *JGP* reporter into the JA-deficient mutant *aos* (19); wounding of this *aos JGP* line neither increased GUS activity (Fig. 1) nor induced high levels of *JAZ10* transcripts (*SI Appendix*, Fig. S2), validating the JA dependency of the reporter line.

***coi1* and *opr3* Mutant Alleles.** To search for genetically encoded activators of the wound response mediated by JA, we screened for mutant seedlings impaired in *JGP* reporter activation after cotyledon wounding. Five independent mutants (34A, 44B, 77A, 82B, 87A) were insensitive to wounding in the entire seedling, and four of them displayed insensitivity to MeJA treatment (*SI Appendix*, Fig. S1), suggesting that they were mutated in the JA receptor gene *COI1*. However, only lines 44B and 87A were completely male sterile as expected of *coi1* null mutants, whereas in lines 34A and 82B male sterility was limited to the first few flowers (*SI Appendix*, Fig. S3). Complementation tests with the *coi1-1* mutant (11) containing the *JGP* reporter and sequencing of the *COI1* gene demonstrated that all four lines carried *coi1* mutant alleles (*SI Appendix*, Table S1), hereafter designated *coi1-32* and *coi1-33* (fully male sterile) and *coi1-8*$_{Lausanne}$ (*coi1-8*$_L$) and *coi1-34* (partially male sterile). Note that the mutation in *coi1-8*$_L$ is identical to the previously reported *coi1-8* (20).

The fifth wound-insensitive mutant (77A) was fully responsive to MeJA treatment (*SI Appendix*, Fig. S1) but displayed a slight sterility phenotype similar to that of *coi1-8*$_L$ and *coi1-34* (*SI Appendix*, Fig. S3). The mutation causing this phenotype was identified by whole-genome sequencing and shown to lie in a splice donor site of the JA biosynthetic gene *OXOPHYTODIENOATE-REDUCTASE 3* (*OPR3*) (21, 22). This mutant allele, designated *opr3-2*, expressed a longer-than-wild-type *opr3* transcript that included an 81-nt intron (*SI Appendix*, Fig. S4 and Table S1). This does not disrupt the normal reading frame of the transcript but is predicted to add 27 amino acids between A116 and V117 to the final protein. The strongly impaired wound induction of *JAZ10* transcripts in *coi1-34* and *opr3-2* seedlings was confirmed (*SI Appendix*, Fig. S5).

Roots Are the Main Activity Domain of *JAR1*. Next, we isolated mutant line 61E, where cotyledon wounding resulted in relatively normal *JGP* activity in the aerial organs but no or very little activity was observed in the roots (Fig. 2*A*; *SI Appendix*, Fig. S6). Concomitantly, we crossed the *JGP* reporter into the *jar1-1* mutant (10) and observed a very similar phenotype, except that the lack of root response was fully penetrant (Fig. 2*A*; *SI Appendix*, Fig. S6). Crossing *jar1-1* with line 61E did not complement the phenotype, indicating that 61E carried a mutant allele of *JAR1*, which we name *jar1-13* (*SI Appendix*, Table S1). Quantification of *JAZ10* transcripts showed that cotyledon wounding induced high *JAZ10* levels in the aerial organs of the *jar1* mutants, although they accumulated three times fewer transcripts than the WT (Fig. 2*B*). In contrast, roots of the wounded *jar1* mutants did not induce *JAZ10* transcription (*jar1-1*) or just barely (*jar1-13*). This phenotype could be due to a delay in long-distance JA signaling from the wounded aerial organs to the root. Therefore, we tested the *JGP* reporter at several late time points after wounding (up to 8 h) in WT and *jar1-1* seedlings (*SI Appendix*, Fig. S7). In WT, *JGP* activity was saturated at 3 h postwounding in all responsive tissues. In *jar1-1* aerial organs, reporter staining was weaker than WT at 2 h postwounding but also reached saturation after 3 h. Nevertheless, *jar1-1* roots did not display reporter activity at any of the time points considered. Additionally, *jar1-1* seedlings treated with MeJA for 2 h activated *JGP* in aerial organs but not in the root (*SI Appendix*, Fig. S8). In all these treatments *jar1* mutants showed a relatively sharp separation of responsive vs. insensitive tissue around the hypocotyl–root interface.

Differential Effects of JA-Signaling Mutations in Resistance to a Generalist Herbivore. To investigate whether *jar1* mutations affect defense responses in aerial tissues, we tested the performance of our mutant set when challenged with larvae of the generalist lepidopteran *Spodoptera littoralis*. After 10 d of feeding, the average insect weights gained from the mutants impaired in JA signaling fell into two main categories (*SI Appendix*, Fig. S9). Insects gained the most weight in the full loss-of-function mutants *coi1-1* and *aos*, as expected (23). This class included the *coi1-34* mutant, which was as susceptible to the herbivores as *aos* and almost as susceptible as *coi1-1*.

The second group included *jar1-1*, *jar1-13*, and *opr3-2*, which were partially impaired in insect resistance. A similar intermediate phenotype was observed in *Nicotiana attenuata* lines silenced in *JAR1* homologs (24), and it correlates with our observation that wound-induced *JAZ10* transcription was decreased but still present in aerial tissues of *jar1-1* and *jar1-13* (Fig. 2*B*).

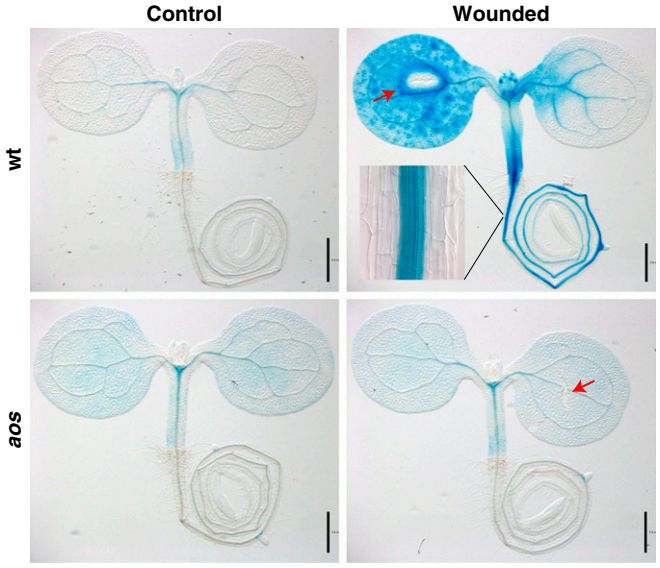

Fig. 1. The *JAZ10-GusPlus* (*JGP*) reporter for JA-mediated wound signaling in 5-d.o. WT and *aos* mutant *Arabidopsis* seedlings. Detection of GUS activity was performed 2 h after wounding. Red arrows indicate cotyledon wounding sites. The reporter in the *aos* mutant background displays a constitutive faint blue staining in the aerial organs not observed in any other genotype and not attributable to a higher basal *JAZ10* transcription (*SI Appendix*, Fig. S2). (*Inset*) Close-up of root. (Scale bars, 0.5 mm.)

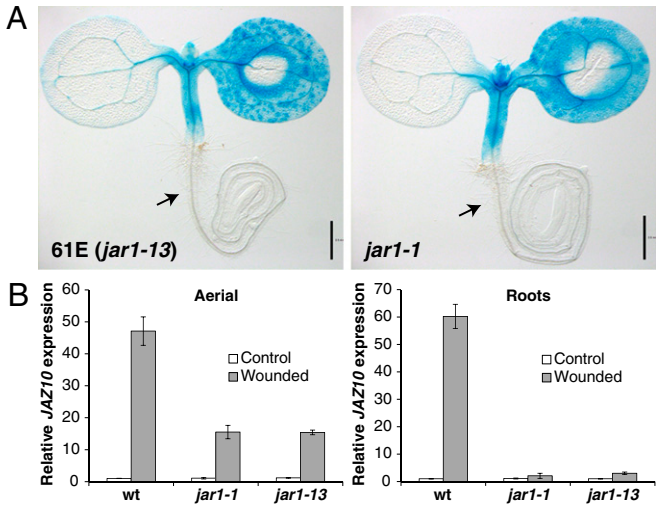

Fig. 2. *jar1* mutations impair wound-induced *JAZ10* expression mainly in roots. (*A*) *JGP* expression in 61E (*jar1-13*) and *jar1-1* seedlings after cotyledon wounding. Black arrows indicate lack of staining in the root of wounded *jar* mutants. (Scale bars, 0.5 mm.) (*B*) Quantitative RT-PCR (qRT-PCR) of *JAZ10* expression 1 h after wounding in aerial organs and roots of WT, *jar1-1*, and *jar1-13* seedlings. *JAZ10* transcript levels were normalized to those of *UBC21* and displayed relative to the expression in the WT unwounded controls. Bars represent the means of three biological replicates (±SD), each containing a pool of organs from ~60 seedlings.

Such clear correlation was not seen for *opr3-2*, where *JAZ10* transcript induction upon wounding was almost completely suppressed (*SI Appendix*, Fig. S5), but herbivore resistance was only partially impaired.

***NINJA* Plays a Major Role as a Repressor of JA Signaling in Roots.** Next, we isolated three mutant lines displaying constitutive *JGP* activity in roots and hypocotyls without previous wounding (Fig. 3*A*; *SI Appendix*, Fig. S10). The staining in the mutant roots was present not only in the vasculature, which is the sole site of root *JGP* expression induced by cotyledon wounding in the WT (Fig. 1), but also in cortex cells of the collet and uppermost root regions. The constitutive activation also tended to reach farther down into the elongation zone (EZ), but it was not observed in the root MZ. Cotyledon wounding of these mutants induced *JGP* expression patterns similar to those in the WT (Fig. 3*A*; *SI Appendix*, Fig. S10). Whole-genome sequencing showed that all three mutants carried lesions in the gene encoding the corepressor NINJA and are designated *ninja-1*, *ninja-2*, and *ninja-3* hereafter.

JAZ10 expression was quantified in *ninja* mutants and a previously characterized RNAi line (4). Nontreated aerial organs of all four lines expressed ~1.7 times more *JAZ10* transcripts than the WT (Fig. 3*B*). These modest but significantly higher *JAZ10* basal levels are in agreement with those reported for adult leaves of *NINJA* RNAi lines (4). In contrast, we observed a stronger effect in *JAZ10* basal levels in the roots of the three *ninja* mutants, where 25 times more *JAZ10* transcripts were detected and, in the *NINJA* RNAi line, with around 10 times more *JAZ10* than WT (Fig. 3*B*).

One hour after wounding *JAZ10* transcripts had accumulated to higher-than-WT levels in the *ninja* lines (Fig. 3*C*). Again, this effect was more prominent in roots (between 3 and 8 times more transcripts with respect to WT) than in aerial organs (~1.8 times higher than the WT). A moderate up-regulation of multiple JA-regulated genes has been previously observed in leaves of a *NINJA* RNAi line (4). These include several defense genes such as *VSP2*, whose up-regulation we also confirmed in our *ninja* mutants (*SI Appendix*, Fig. S11). However, the mild increase in basal and induced JA signaling in *ninja* aerial organs did not result in enhanced resistance against *S. littoralis* larvae under our experimental conditions, even after prolonged feeding times (*SI Appendix*, Figs. S9 and S12).

A *ninja-1 aos* double mutant was mostly indistinguishable from the *ninja-1* single mutant with respect to the constitutive *JGP* expression in hypocotyls and roots (Fig. 3*A*). Therefore, in those tissues loss of NINJA function is sufficient to abolish the repression on JA-responsive promoters, and JA is not required to activate them. The constitutive wound-like *JGP* expression in the

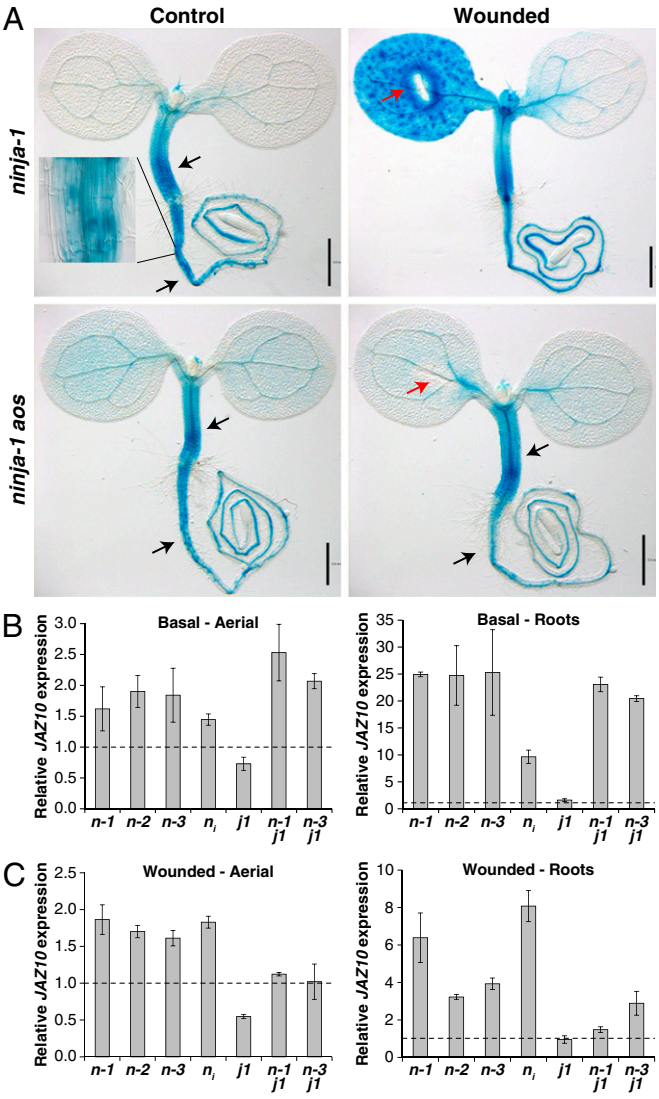

Fig. 3. *ninja* mutants display constitutive *JAZ10* expression. (*A*) *JGP* expression in control and wounded seedlings of *ninja-1* and *ninja-1 aos* (compare with WT and *aos* in Fig. 1). Arrows indicate constitutive reporter activity in hypocotyl and roots (black) and cotyledon wounding sites (red). (*Inset*) Close-up of root. The *ninja-2 aos* double mutant displays the same expression pattern as *ninja-1 aos*. (Scale bars, 0.5 mm.) (*B* and *C*) qRT-PCR of *JAZ10* expression basally (*B*) and 1 h after wounding (*C*) in *ninja-1* (*n-1*), *ninja-2* (*n-2*), *ninja-3* (*n-3*), *ninja* RNAi (n_i), *jin1-7* (*j1*), *ninja-1 jin1-7* (*n-1 j1*), and *ninja-3 jin1-7* (*n-3 j1*). *JAZ10* transcript levels were normalized to those of *UBC21* and displayed relative to the expression of unwounded WT controls (*B*) or wounded WT samples (*C*); thus, WT levels are set to 1 in each plot and indicated with a dashed line. Bars represent the means of three biological replicates (±SD), except for *n-2* and n_i (two biological replicates), each containing a pool of organs from ~60 seedlings. Complete qRT-PCR data are in Dataset S1.

hypocotyl and root of the *ninja-1 aos* double mutant did not change upon wounding, but cotyledons and shoots still failed to activate *JGP* (Fig. 3*A*). Moreover, *ninja-1 aos* and *ninja-2 aos* were as sensitive to herbivore attack as the *aos* mutant (*SI Appendix*, Fig. S13). Therefore, herbivory resistance in aerial organs of *ninja* mutants still required de novo JA biosynthesis and was not detectably influenced by the strong constitutive JA signaling of the roots.

The three *ninja* mutations that we characterized are predicted to cause partial or complete removal of NINJA's C domain (*SI Appendix*, Figs. S14 and S15; Table S1), which is required for interaction with the JAZ repressors in yeast two-hybrid assays (4). To test if the *ninja* mutations impair JAZ repressor function *in planta*, we transformed the *ninja* mutants with constructs overexpressing *JAZ10.3* and *JAZ10.4*, two truncated splice variants of *JAZ10* that are resistant to COI1-mediated degradation in the presence of JA, effectively repressing JA responses (17, 25). As expected, a significant fraction of transformed (T_1) WT seedlings overexpressing *JAZ10.3* and *JAZ10.4* displayed MeJA insensitivity in a root growth assay (*SI Appendix*, Fig. S16). However, T_1 *ninja* seedlings remained more sensitive to MeJA than T_1 WT, suggesting that the *ninja* mutations attenuated the repression mediated by JAZ10.3 and JAZ10.4. The extent of this attenuation remains to be assessed in independent T_2 families.

The deregulated expression of JA response genes in the *ninja* mutants could be attributed to the activity of one or more JA-dependent transcription factors (TFs) normally subjected to JAZ-NINJA-TPL repression. Because the basic helix–loop–helix TF MYC2 is a central activator in several aspects of JA signaling (12, 26), we introgressed the *ninja* alleles into a *myc2* mutant (*jin1-7*) (12). The constitutive *JAZ10* expression was still present in aerial and root organs of *ninja-1 jin1-7* and *ninja-3 jin1-7* double mutants (Fig. 3*B*), suggesting that MYC2 has no role or is not the sole factor promoting deregulated JA signaling in *ninja*. For example, even if MYC2 has a major function in the root response to exogenously supplied JA (12, 26), we found that *JAZ10* induction in roots after cotyledon wounding was not affected in *jin1-2* and *jin1-7* seedlings (Fig. 3*C*; *SI Appendix*, Fig. S17; Dataset S2). Instead, it was abolished only in a triple mutant between *myc2* and two closely related TFs: *myc3* and *myc4* (*SI Appendix*, Fig. S17). On the other hand, *myc2* single mutants displayed reduced *JAZ10* expression in aerial organs after cotyledon wounding (Fig. 3*C*; *SI Appendix*, Fig. S17). This effect was also observed in *ninja jin1-7* aerial organs, where the excess *JAZ10* expression of *ninja* mutants after wounding was eliminated (Fig. 3*C*). A less clear effect was seen in roots of wounded double mutants, where the influence of *jin1-7* on *JAZ10* levels was only mild in *ninja-3* but more prominent in *ninja-1*.

Primary Root Shortening in *ninja* as a Result of Reduced Cell Elongation. When grown in vertical plates, *ninja* mutant and RNAi lines had up to 30% shorter roots than WT (Fig. 4 *A–C*), indicating that lack of functional NINJA partly mimicked JA-mediated root growth inhibition. Remarkably, this short root phenotype was also present in *ninja aos* and *ninja jin1-7* double mutants (Fig. 4 *B* and *C*), suggesting that loss of NINJA function bypasses the need of JA to repress root growth and that this may occur in the absence of MYC2. When grown in the presence of MeJA, *ninja* roots are as short as WT, and no hypersensitivity to MeJA was observed under the conditions tested (Fig. 4 *B* and *C*). Similarly, the roots of *ninja jin1-7* were not different from the *jin1-7* single mutant in media containing MeJA (Fig. 4*C*).

Finally, we assessed if *ninja* root shortening was associated with a reduction in cell proliferation or cell elongation or, as when the WT is grown in the presence of exogenous MeJA, a combination of both (27). As an indicator of cell proliferation, we determined cell number in the root MZ of WT and *ninja* mutants, and no significant difference between them was detected

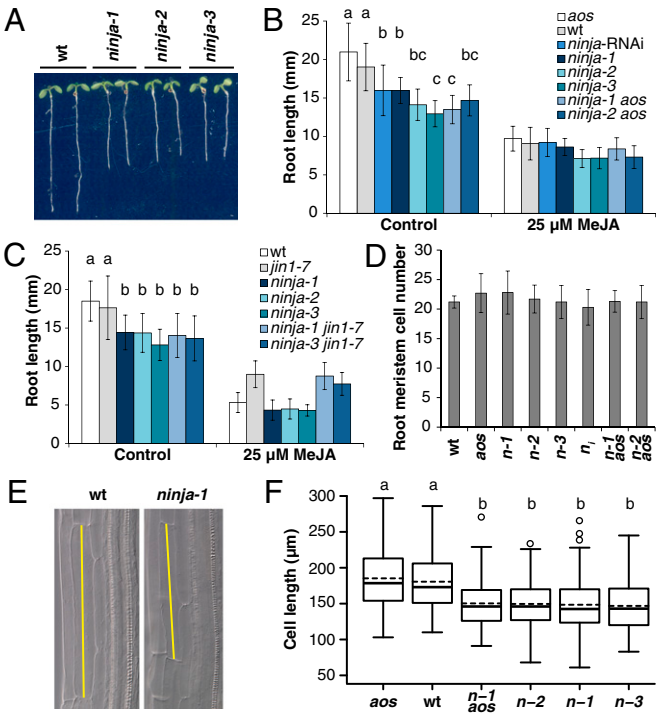

Fig. 4. Reduced root growth and cell length in untreated *ninja* lines. (*A*) Representative 7-d.o. seedlings of WT and *ninja* mutants. (*B* and *C*) Quantification of root growth in WT and mutant lines grown in the absence or presence of 25 μM MeJA for 7 d. Data are the means (±SD) from at least 20 plants. Letters above bars indicate statistically significant differences between pairs of control samples as determined by Tukey's honestly significant difference (HSD) test ($P < 0.02$). (*D*) Primary root meristem cell number in 5-d.o. seedlings of WT and several mutant lines. No statistically significant difference between genotypes was found by ANOVA ($P = 0.475$). (*E*) Representative cortex cells from WT and *ninja-1* mutant roots. Yellow bars indicate cell length. (*F*) Box plot summary of cortex-cell length in 5-d.o. seedlings of WT and several mutant lines grown in the absence of MeJA. Medians and means are represented inside the boxes by solid and dashed lines, respectively. Circles depict outlier data points beyond ±1.5× the interquartile range defined by the whiskers. Letters indicate statistically significant differences between pairs as determined by Tukey's HSD test ($P < 2e\text{-}16$).

(Fig. 4*D*). Conversely, cortex cell length in the differentiation zone (DZ) of *ninja* mutants was on average 20% shorter than in the WT (Fig. 4 *E* and F; *SI Appendix*, Table S2). Therefore, we attribute the root shortening in *ninja* primarily to a reduction in cell elongation.

Discussion

How is the specificity and diversity of JA responses determined throughout the plant? By spatially visualizing the effects of early JA signaling, we provide evidence that part of this diversity can be explained by organ specificity of NINJA, a member of the JA transcriptional repression complex, and of JAR1, the JA-Ile–conjugating enzyme. JAR1 and NINJA are indispensable to, respectively, activate and repress JA signaling in roots, providing a unique opportunity to study a complex network of interactions at an organ level. Roots also rely on a single member of the LIPOXYGENASE family, LOX6, for stress-induced JA accumulation (28). Together, these findings imply that the JA-signaling machinery is less redundant in the roots than in the aerial organs of *Arabidopsis*. How such arrangements evolved and what ecological consequences, if any, they have are open questions.

Additional *coi1* and *opr3* Alleles Compromised in JA-Mediated Wound Signaling. The search for mutant seedlings impaired in *JGP* reporter activation after cotyledon wounding yielded one *opr3*, one

jar1, and four *coi1* mutant alleles, confirming the specificity of our screen. *Arabidopsis COI1* has been independently identified in several forward genetic screens (11, 20) because it is a single-copy gene that is necessary for all JA-mediated processes known to date (29). Whereas all *coi1* alleles identified herein showed abolished wound responses and JA sensitivity in seedlings, *coi1-8*$_L$ and *coi1-34* were almost completely fertile. These two mutations cause amino acid substitutions at the end of β-sheets, forming the structurally important leucine-rich repeat (LRR) domains in COI1 (20). In *coi1-8*, this defect was previously reported to partially reduce COI1 protein stability while maintaining the ability to interact with recombinant JAZ1 protein (20). Our results show that this type of hypomorphic COI1 function is sufficient for JA-mediated fertility but insufficient for near-WT defense. Instead, full loss-of-function mutations such as *coi1-33* affect the α-helices of the LRR domains as in the *coi1-4*, *coi1-7*, and *coi1-9* mutants, which fail to accumulate the COI1 protein (20). On the other hand, the JA biosynthetic mutant *opr3-2* is also hypomorphic, but the mutant enzyme is still partially functional in both fertility and defense responses. Even if *JGP* and *JAZ10* expression are not properly induced in *opr3-2* shortly after wounding, the partial herbivore resistance phenotype suggests that some JA signaling may still occur.

***JAR1* Is Indispensable for Activating JA Signaling in Roots After Cotyledon Wounding.** It has been shown previously that JAR1 activity is not strictly required for the wound-induced expression of JA-responsive genes in rosette leaves, where other JA-amino synthetases may exist to account for the remaining JA-Ile levels in null *jar1* mutants (8, 18, 30). Other reports indirectly support a primary role for JAR1 in the roots. First, *JAR1* mRNA levels are highest in seedling roots (30). Second, *jar1-1* displayed a root-specific impairment in the activation of a wound-induced *FAD7p-LUC* reporter (16). Third, *jar1-1* is as susceptible as the JA-deficient mutant *fad3-2 fad7-2 fad8* to *Pythium irregulare*, a soil-borne pathogen that infects roots (31). We found that *jar1-1* and *jar1-13* displayed an intermediate susceptibility to *S. littoralis* between that of the WT and *coi1-1*. This could be attributed to delayed or decreased JA-Ile accumulation that activates slower or reduced downstream defense responses (8, 30). In contrast, cotyledon wounding in *jar1-1* and *jar1-13* failed to activate *JGP* and *JAZ10* expression in roots. *JGP* remained inactive in *jar1-1* roots even at later cotyledon postwounding times as well as after short-term MeJA treatment. Taken together, our data indicate that the seedling wound response partly requires JAR1 in aerial organs whereas the roots are completely dependent on JAR1 activity. Nevertheless, when germinated and grown in medium supplied with exogenous MeJA, *jar1* mutant seedlings retain partial sensitivity to JA (10). Thus, it is probable that exogenous JA overrides endogenous responses. Our results also favor the view that bioactive JA-Ile is not transported from wounded aerial tissues to basal organs. Instead, JA-Ile is most likely synthesized de novo in roots specifically through JAR1 action after the arrival of long-distance signals. This view is consistent with results from recent grafting experiments that demonstrated that roots produce JA and JA-Ile independently of leaves (28).

***NINJA* Is Essential for Repressing Basal JA Signaling and Promoting Cell Elongation in Roots.** Here, we report mutants in NINJA, a transcriptional corepressor of JA response genes that bridges target JAZ proteins to members of the TPL corepressor family (4). The lack of NINJA's C domain in all three *ninja* mutant alleles most likely prevents its interaction with the ZIM domain of JAZ proteins and the recruitment of TPL corepressors to the JA transcriptional repression complex. This prediction is supported by our preliminary evidence that *ninja* mutants are less affected by the JA insensitivity caused by the nondegradable repressors JAZ10.3 and JAZ10.4. Although *NINJA* transcripts were detectable in both aerial organs and roots (*SI Appendix*, Fig. S14; *Arabidopsis* eFP browser, http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi), loss of NINJA function caused ectopic *JGP* and *JAZ10* expression predominantly in hypocotyls and roots, revealing a spatial specificity of the NINJA-dependent complex that represses basal JA signaling.

The major impact of NINJA on the basal repression of root JA signaling could be explained if other as-yet-unknown corepressors act redundantly with NINJA to constitutively inhibit JA signaling in aerial organs (and the root MZ), whereas NINJA is sufficient to repress early JA signaling in the older parts of the primary root. It is also possible that more direct mechanisms repress JA-dependent transcription in aerial organs. First, JAZ8 can act independently of NINJA because it carries an ethylene-responsive element binding factor-associated amphiphilic repression (EAR) motif capable of directly binding TPL (5). This feature is potentially shared by JAZ5, JAZ6, and JAZ7 (32). Second, JAZ1 may directly recruit the chromatin-modifying enzyme HISTONE DEACETYLASE 6 (HDA6) to reduce chromatin accessibility and repress gene expression (33). Alternatively, the tissue-specific differences in constitutive gene expression after losing NINJA-mediated repression may be a reflection of quantitative differences in the activity or basal concentration of specific JA-dependent transcriptional activators between organs.

Exogenously supplied JA is known to inhibit root growth by reducing both root meristem activity and cell length in the DZ (27). Our results, based on genetic approaches that did not require treatment with exogenous JA, indicate that root shortening in *ninja* mutants is primarily due to a reduction in cell length in the DZ. That is, a major role of NINJA in roots is in cell-size control. This agrees with the pattern of constitutive *JGP* expression observed in *ninja* roots, where derepression of the *JAZ10* promoter encompasses the EZ and the DZ but not the MZ. The growth reduction in *ninja* mutant roots is most likely attributable to increased signaling through transcriptional activators of JA responses such as MYC2, which is normally repressed by a JAZ-NINJA-TPL complex (4, 26). However, a *myc2* mutation did not suppress the ectopic *JAZ10* expression and short root phenotypes in *ninja*. It is possible that, as in the wound response, MYC2 acts additively in the *ninja* mutants with MYC3 and MYC4, two related TFs that also form complexes with JAZ repressors and NINJA in vivo (4, 26). In this case, a combination of *myc2*, *myc3*, and/or *myc4* mutants will potentially suppress the *ninja* phenotype. Alternatively, in seedlings grown under exogenous JA the major role of MYC2 is reducing root meristem cell number by directly repressing *PLETHORA* genes (27). When grown in the presence of JA, *myc2* mutants showed normal root meristem size but retained partial sensitivity to JA-mediated root growth inhibition (27), which can be attributed to reduced cell elongation in the DZ independently of MYC2 function. In this respect, NINJA and MYC2 are likely to regulate different aspects (elongation vs. proliferation) of root growth.

Another possibility is that the *ninja* mutants derepress uncharacterized JA-specific growth factors that may explain the residual sensitivity of *myc234* lines to JA-mediated root growth inhibition (26). The *ninja* mutants provide the opportunity to (*i*) uncover these factors and mediators of hormonal cross-regulation and (*ii*) study TF-specific effects on early JA signaling and cell elongation. However, it remains formally possible that the role of NINJA on root growth is independent of JA signaling.

Role of NINJA in Induced JA Signaling. The higher-than-WT *JAZ10* accumulation in both aerial and root organs of *ninja* mutants (Fig. 3*C*) (4) suggests an active role of NINJA in repressing JA-dependent TFs after cotyledon wounding. These excess *JAZ10* levels were abolished in aerial organs of *ninja jin1-7* double

mutants, implying that NINJA corepressor function is required to attenuate MYC2 signaling after the wounding stimulus in aerial organs. However, unlike aerial organs where cotyledon wounding needs MYC2 to activate normal JA signaling, roots do not absolutely require MYC2 to induce WT *JAZ10* levels because most likely MYC3 and MYC4 can also perform this function (*SI Appendix*, Fig. S17). This, and the finding that the constitutive JA signaling in *ninja* mutants can occur independently of MYC2, is in contrast with the major role attributed to this TF in root responses to exogenous JA (12, 26), emphasizing that the functional hierarchy of JA-signaling components varies according to both tissue and stimulus.

Upon strong and continuous stimulation of signaling in the presence of exogenous MeJA, the role of NINJA in suppressing JA responses becomes less evident. For example, we did not find *ninja* roots to be hypersensitive to JA, suggesting that NINJA exerts its corepression function mainly when JAZ proteins are most stable and not when they are rapidly degraded under a constant exogenous supply of JA. It is plausible that NINJA-independent repression mechanisms—mediated, for example, by JAZ8 (5) or JAZ1-HAD6 (33)—become predominant in such conditions. The increased sensitivity to JA of *jaz10* loss-of-function plants (17, 34) further indicates that JAZ10 can act through NINJA-independent mechanisms to repress induced JA signaling.

This work describes the possibility of root growth inhibition by the JA-signaling machinery in the absence of JA synthesis. Our findings show that (*i*) it may be possible to manipulate what is normally JA-controlled growth inhibition in discrete parts of plants such as hypocotyls and roots, without the necessity of altering either JA synthesis or perception; and (*ii*) the *ninja* mutants will be powerful tools to dissect unexplored cellular events activated downstream of JA signaling to restrict root cell elongation. A deeper understanding of cell-size control will be crucial to successfully engineer plants that display reduced growth restriction under stress.

Materials and Methods

Arabidopsis thaliana accession Columbia was the WT background of the *JGP* reporter and all mutant and knock-down lines. Two different genetic screens were performed on EMS-mutagenized M2 seedlings that were evaluated for *JGP* activity by nondestructive GUS staining. In one screen, we searched for loss of GUS staining in the root after cotyledon wounding, and in the other we screened for constitutive GUS activity in the root of unwounded seedlings. Mutant alleles were identified by whole-genome next generation sequencing (NGS). Detailed experimental procedures of *JGP* reporter line generation, growth conditions, treatments, NGS, and measurements can be found in *SI Appendix*.

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