NINJA connects the co-repressor TOPLESS to jasmonate signalling

Laurens Pauwels[1,2,*], **Gemma Fernández Barbero**[3,*], **Jan Geerinck**[1,2,*], **Sofie Tilleman**[1,2], **Wim Grunewald**[1,2,4], **Amparo Cuellar Pérez**[1,2], **José Manuel Chico**[3], **Robin Vanden Bossche**[1,2], **Jared Sewell**[5], **Eduardo Gil**[6], **Gloria García-Casado**[6], **Erwin Witters**[7,8], **Dirk Inzé**[1,2], **Jeff A. Long**[5], **Geert De Jaeger**[1,2], **Roberto Solano**[3,6], and **Alain Goossens**[1,2]

[1] Department of Plant Systems Biology, Flanders Institute for Biotechnology (VIB), Technologiepark 927, B-9052 Gent, Belgium

[2] Department of Plant Biotechnology and Genetics, Ghent University, Technologiepark 927, B-9052 Gent, Belgium

[3] Departamento de Genética Molecular de Plantas, Centro Nacional de Biotecnología-CSIC, Campus Universidad Autónoma, 28049 Madrid, Spain

[4] Department Molecular Biotechnology, Faculty of Bioscience Engineering, Ghent University, Coupure links 653, B-9000 Gent, Belgium

[5] Plant Biology Laboratory, The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, California 92037, USA

[6] Genomics Unit, Centro Nacional de Biotecnología-CSIC, Campus Universidad Autónoma, 28049 Madrid, Spain

[7] Department of Biology, Centre for Proteome Analysis and Mass Spectrometry, University of Antwerp, Groenenborgerlaan 171, B-2020 Antwerpen, Belgium

[8] Flemish Institute for Technological Research, Boeretang 200, B-2400 Mol, Belgium

Abstract

Jasmonoyl-isoleucine (JA-Ile) is a plant hormone that regulates a broad array of plant defence and developmental processes[1–5]. JA-Ile-responsive gene expression is regulated by the transcriptional activator MYC2 that interacts physically with the jasmonate ZIM-domain (JAZ) repressor proteins. Upon JA-Ile perception, JAZ proteins are degraded and JA-Ile-dependent gene

expression is activated[6],[7]. The molecular mechanisms by which JAZ proteins repress gene expression remain unknown. Here we show that the JAZ proteins recruit the Groucho/Tup1-type co-repressor TOPLESS (TPL)[8] and TPL-related proteins (TPRs) through a previously uncharacterized adaptor protein, designated Novel INteractor of JAZ (NINJA). NINJA acts as a transcriptional repressor of which the activity is mediated by a functional TPL-binding EAR repression motif. Accordingly, both NINJA and TPL proteins function as negative regulators of jasmonate responses. Our results point to TPL proteins as general co-repressors that affect multiple signalling pathways through the interaction with specific adaptor proteins. This new insight reveals how stress- and growth-related signalling cascades use common molecular mechanisms to regulate gene expression in plants.

JA-Ile signal transduction is controlled by a Skp1/Cullin/F-box (SCF) E3 ubiquitin ligase of which the F-box subunit is encoded by *CORONATINE INSENSITIVE 1* (*COI1*)[9]. Recently, the JAZ proteins have been identified as the targets of SCF^{COI1}[6],[7]. In the presence of (+)-*7-iso*-JA-L-Ile, the endogenous bioactive hormone, JAZ proteins interact with COI1 and are subsequently degraded by the 26S proteasome[6],[7],[10],[11]. In an unelicited state, JAZ proteins bind the transcription factor MYC2 and inactivate its function[6]. Hormone-triggered removal of JAZ proteins releases MYC2, enabling the transcriptional activator to regulate the expression of early jasmonate responsive genes, including the *JAZ* genes[6],[7],[12],[13].

Here, tandem affinity purification (TAP) was applied to isolate the core jasmonate signalling module and to find new JAZ interactors. We used a recently established TAP technology platform that allows the efficient isolation and identification of protein complexes from *Arabidopsis* cells[14]. For the simultaneous visualization of the dynamics of JAZ complex assembly, TAP was done with cell cultures elicited or not with jasmonic acid (JA). As JAZ degradation upon JA treatment has been reported to be very rapid[15], we first investigated JAZ degradation kinetics in our model system. A cycloheximide chase experiment revealed an estimated half-life of 1.4 minutes for a JAZ1-firefly luciferase (fLUC) fusion protein upon treatment with JA (Supplementary Fig. 1). Next, JAZ1 was C-terminally fused to a TAP-tag and expressed under a CaMV 35S promoter in *Arabidopsis* cells. To avoid full degradation of the tagged bait, cell cultures producing JAZ1-TAP were harvested 1 minute after JA treatment. Several proteins already shown to interact with JAZ1 were identified (Fig. 1a), validating the approach (for the mass spectrometry-driven identification of candidate interactors, see Supplementary Table 1). Firstly, JAZ1 formed a complex with JAZ12, matching reported dimerization of JAZ proteins[16],[17]. Secondly, interaction of JAZ1 with MYC3, a close relative to MYC2, was observed. Finally, COI1 occurred in a complex with JAZ1, but only in the presence of JA, showing that TAP is applicable for the identification of ligand-mediated protein-protein interactions.

We focused on an uncharacterized protein isolated in the TAP analysis (Fig. 1a), hereafter designated NINJA, and encoded by the locus At4g28910. NINJA is related to the ABI-FIVE BINDING PROTEIN (AFP) family, which consists of four members in *Arabidopsis*[18]. Whereas abscisic acid (ABA) induces the expression of *AFP* genes in *Arabidopsis* seedlings[18], *NINJA* expression is induced by methyl jasmonate (MeJA) within 1 h and for at least 12 h after elicitation (Supplementary Fig. 2). This pattern corresponds with that of the

previously identified *NINJA* homologue of tobacco (*Nicotiana tabacum*) in a screen for MeJA-inducible transcripts[19].

In parallel, we performed yeast two-hybrid (Y2H) library screens using JAZ2 and JAZ3 as baits, and both identified NINJA as a direct interactor. Systematic Y2H analysis of the specificity of NINJA for JAZ proteins revealed interactions with most JAZ proteins, except JAZ7 and JAZ8 (Supplementary Fig. 3a). Furthermore, other ZIM domain proteins, containing the TIFY (TIF[F/Y]XG) motif and belonging to the group-II TIFY proteins[20], such as PEAPOD 1 (PPD1), PPD2 and TIFY8, interacted with NINJA (Supplementary Fig. 4a). Reversibly, we checked whether AFP proteins could bind JAZ proteins, but neither AFP2 nor AFP3 interacted with JAZ1 (Supplementary Fig. 4b), suggesting the specificity of JAZ proteins for NINJA.

Besides the TIFY motif, JAZ proteins have a C-terminal conserved Jas domain, which is involved in the interaction with both MYC2 and COI1[6],[10],[11],[21]. Y2H with a JAZ1 deletion series revealed that NINJA was only capable of binding JAZ1 fragments containing the TIFY motif. Moreover, a 39-amino-acid fragment holding the conserved TIFY motif was sufficient for binding of NINJA (Fig. 1b and Supplementary Fig. 4c). Consistently, NINJA did not interact with a JAZ1 protein fusion lacking the TIFYAG sequence (amino acids 128-133, tify) (Fig. 1b). Pull-down experiments with JAZ-MBP fusion proteins and extracts of transgenic plants expressing NINJA-GFP confirmed the Y2H observations. NINJA was pulled-down by all JAZ-MBP fusion proteins, except JAZ7, JAZ11 and JAZ12 (Supplementary Fig. 3b). Moreover, by using JAZ3 derivatives, only the fragment containing the TIFY motif (JAZ3 C) was able to pull-down NINJA-GFP (Supplementary Fig. 3b). NINJA-GFP did not interact with NINJA-MBP, COI1-MBP or MYC2-MBP in these pull-down experiments (Supplementary Fig. 3b).

NINJA and all AFP proteins are characterized by three conserved protein domains designated A, B and C. A functionality has been described only for the C domain, that has been shown to be necessary and sufficient for binding of the AFP proteins with the bZIP transcription factor ABSCISIC ACID INSENSITIVE 5 (ABI5)[18]. A deletion series of NINJA was constructed (Fig. 1c) and tested for JAZ1 interaction, demonstrating that the C domain of NINJA was responsible and sufficient for JAZ protein interaction. Finally, the 39 amino-acid protein fragment harbouring the TIFY motif of JAZ1 directly interacted with the C domain of NINJA (Supplementary Fig. 4d).

Analysis of seedlings producing a C-terminal GFP fusion with NINJA revealed a clear nuclear localization for NINJA (Supplementary Fig. 5), as already shown for both AFP and JAZ proteins[6],[7],[15],[18]. Furthermore, NINJA-GFP levels remained constant for 3 h after jasmonate treatment, whereas a JAZ1-GFP fusion was nearly undetectable only 5 minutes after jasmonate application (Supplementary Fig. 5), suggesting that, unlike JAZ proteins, the stability of NINJA is unaffected by jasmonates.

Knock-out T-DNA insertion lines for NINJA were not available from the *Arabidopsis* database (http://www.arabidopsis.org). Hence, we generated transgenic *Arabidopsis* lines expressing full-length *NINJA* or *NINJA* hairpin RNAi constructs, both under control of the

CaMV 35S promoter (Supplementary Fig. 6a,b). *NINJA* overexpression (OE) significantly decreased jasmonate sensitivity, as reflected by an impaired inhibition of root growth in the transgenic plants (Fig. 2a and Supplementary Table 2). An *in vitro* degradation experiment with extracts from *NINJA* OE lines demonstrated that this phenotype could not be explained by protection of JAZ proteins from degradation (Supplementary Fig. 7), which could account for the phenotype caused by truncated JAZ3 proteins in the *jai3* mutant[6]. Conversely, *NINJA* knock-down (KD) lines showed a derepressed response to jasmonates. Microarray analysis revealed that even without exogenous hormone treatment *NINJA* KD lines overexpressed many known early jasmonate-responsive genes (Fig. 2b and Supplementary Table 3). This effect was even more prominent when plants were treated with the JA-Ile mimic coronatine (Supplementary Fig. 8 and Supplementary Table 4). Consistently, a slight increase in JA mediated inhibition of root growth was observed in *NINJA* KD lines (Fig. 2a and Supplementary Table 2). Together, these results indicate that NINJA functions as a negative regulator of jasmonate signalling.

To investigate NINJA function in JA signalling, we set up a new TAP experiment with NINJA as bait. NINJA was present in a complex with the Groucho/Tup1-type co-repressor TPL and its homologues TPR2 and TPR3, independently of JA elicitation (Supplementary Table 5). Furthermore, the group-II TIFY proteins JAZ12 and PPD2 and MYC3 were identified as well, confirming the previous interaction data (Fig. 1a and Supplementary Fig. 3 and 4).

TPL interacts directly with the N-terminal ERF-associated amphiphilic repression (EAR) motif of the AUX/IAA protein IAA12[8]. The EAR motif is a hallmark of transcriptional repressors and is encountered in most AUX/IAA proteins[22]. Correspondingly, scanning of the NINJA amino acid sequence revealed an EAR motif in domain A (Supplementary Fig. 9), suggesting a direct interaction between the EAR protein NINJA and TPL, a hypothesis that was confirmed by Y2H and bimolecular fluorescence complementation (BiFC) (Fig. 3a and Supplementary Fig. 10). Removal of domain A did not affect the interaction with JAZ1 (Fig. 1c), but abolished that with TPL, as was also the case when three Leu residues were mutated to Ala in the NINJA EAR motif (mEAR, Fig. 3a). Moreover, domain A of NINJA was sufficient for the interaction with TPL (Fig. 3a). The association of TPL with the JAZ protein complex was further supported by phenotypic analysis of the *tpl-1* mutant[23], which exhibited hypersensitivity to JA (Fig. 3b and Supplementary Table 2). Together, these results indicate that NINJA connects the JAZ proteins with the TPL co-repressors.

Considering the association with TPL, we assessed the transcriptional repressor capacity of NINJA. First, a protein fusion of NINJA with the GAL4 DNA-binding domain (DBD) could repress the basal activity of a promoter containing upstream activation sequence (PUAS) elements in tobacco protoplasts (Fig. 3c). Second, a NINJA fragment containing the EAR motif, but lacking a JAZ interaction domain, was sufficient for repression. Conversely, deletion of the domain A or mutation of three conserved Leu residues in the EAR motif abolished the repression capacity of NINJA (mEAR, Fig. 3c). Third, the transcriptional activation of the PUAS by a DBD-MYC2 fusion protein could be counteracted by co-expression with NINJA (Fig. 3d). Together, these results establish NINJA as a transcriptional repressor of jasmonate responses.

Our findings expand the parallelism between auxin and jasmonate signalling pathways: the hormones (indole-3-acetic acid and (+)-7-*iso*-JA-L-Ile, respectively) are perceived by F-box proteins (TIR1 and COI1, respectively) that target repressor proteins (AUX/IAA and JAZ, respectively) for rapid degradation by the 26S proteasome, which finally releases the transcription factors (ARFs and MYCs, respectively) that activate the downstream hormone responses[24],[25] (Fig. 4). We show that transcriptional repression in jasmonate signalling is mediated as in auxin signalling, by a protein containing an EAR repression motif that recruits the TPL co-repressors to the complex[8] (Fig. 4). However, in jasmonate signalling, the EAR motif is not present on the SCF degradation targets, but on a separate, stable protein, i.e. NINJA. In this manner, extra features might have been added to the complex, allowing crosstalk with other hormone signalling pathways in the cell and fine-tuning of the jasmonate response.

The TIFY motif of JAZ proteins is necessary and sufficient for binding to the NINJA/TPL repressor complex, corresponding to the essential role of this domain in repression of jasmonate signalling by JAZ10.4[17]. The TIFY motif is also necessary for dimerization of JAZ proteins[16],[17]. Future research should reveal whether binding of NINJA and JAZ proteins to the TIFY motif can occur simultaneously and how this dual functionality shapes the repressor activity of the JAZ proteins. The TIFY motif is also present in ZIM domain proteins, such as PPD1, PPD2 and TIFY8, that are not inducible by jasmonates but that nonetheless interact with NINJA (Supplementary Fig. 4a). As the phenotypes associated with the PPD proteins do not seem to be related to jasmonate signalling[26], NINJA might play a larger role than mediating jasmonate-responsive gene expression only (Fig. 4d). The EAR motif of NINJA, as well as that of the AUX/IAA proteins, is responsible for the interaction with TPL proteins. This motif is also conserved amongst all NINJA-related AFP proteins, implicated in ABA signalling[18] (Fig. 4e), hinting at a possible implication of TPL proteins in multiple signalling pathways, a hypothesis supported by Y2H analysis that demonstrated the direct interaction of AFP proteins and TPL (Supplementary Fig. 4e). Thus, TPL proteins emerge as general co-repressors in plants that are recruited to different signalling pathways by specific adaptor proteins, such as AUX/IAA proteins (for auxin), AFP proteins (for ABA) and the complex NINJA-JAZ (for jasmonate).

Methods Summary

Generation of DNA constructs

Standard molecular biology protocols and Gateway™ (Invitrogen, Carlsbad, USA) technology were followed to obtain expression clones.

Tandem Affinity Purification

Arabidopsis cell suspension cultures (PSB-D) were transformed as described previously[27] with baits fused to the GS-TAP-tag[14]. TAP was as described[27] with modifications. Peptide fragments were analysed by MALDI TOF/TOF.

Generation of transgenic and mutant *Arabidopsis* plants

Homozygous transgenic *Arabidopsis* T3 plants over-expressing or silencing *NINJA* were generated using floral dip[28].

Protoplast assays

Transient expression assays in tobacco protoplasts were carried out as described previously[13,29] with modifications.

Full Methods

Constructs

Gateway™ pENTRY vectors were obtained from ABRC (www.arabidopsis.org). Alternatively, open reading frames (ORFs) were amplified from a cDNA template with Platinum Taq High Fidelity (Invitrogen) and equipped with Gateway™ attB sites. Deletion and mutant gene variants were constructed with the GeneTailor Site-Directed Mutagenesis system (Invitrogen). The structure and sequence of all used destination vectors were as described[30,31] and accessible on line at http://www.psb.ugent.be/gateway/ or otherwise referenced.

Cell suspension cultivation, transformation and JA treatment

Arabidopsis cell suspension cultures (PSB-D) were maintained and transformed as described previously[27] without callus selection. Stably transformed cell cultures were scaled up and harvested 6 days after subculturing. Cells were mock-treated or elicited with 100 μM JA (Duchefa, Haarlem, The Netherlands).

Protein degradation assays

The entry clones pEN-L4-2-R1, containing the pCaMV 35S promoter, pEN-R2-fLUC-L3 and pEN-L1-JAZ1-L2 were recombined by MultiSite Gateway LR reaction with pKCTAP as destination vector. Transformed cell cultures were grown for several weeks in the absence of kanamycin and for 1 week after subculturing before the protein degradation assays. For cycloheximide (CHX) chase experiments, 200 μg/mL CHX (Sigma) dissolved in DMSO with or without 100 μM JA (Duchefa) was added to the cells, which were subsequently harvested at multiple time points by vacuum filtration. Proteins were extracted as described[32]. GFP fluorescence was utilized to normalize variations in protein extraction. Half-life was calculated as described[33] with modifications. For each sample individually, the fLUC activity (l)/GFP fluorescence (g) ratio was divided by an average l/g value for the control samples (i.e. the first time point without JA) to generate a normalized l/g value. For graphic presentation, the natural log of the normalized l/g value was determined and plotted in function of time. A linear best-fit line $y = mx + c$ was calculated with linear regression in which $y = \ln(\text{normalized } l/g)$, m the slope, x the time and c the intercept to determine the half life with $y = \ln(0.5)$.

In vitro degradation of JAZ proteins after incubation with cellular extracts from wild-type or NINJA OE plants was performed as described[6]. In brief, JAZ3 protein was obtained and labelled by *in vitro* translation using TNT wheat germ extract systems (Promega) in the

presence of [^{35}S] methionine. Labelled JAZ3 was incubated at 30°C for different times with plant extracts (100 mg of total protein extract in 50mM Tris-HCl, pH 7.0, 5mM MgCl$_2$, 5mM ATP and 50μg/μL ubiquitin) with or without the proteasome specific inhibitor MG132 (200μM) or JA (50μM). The reaction was stopped with loading buffer at 4°C and run on protein gels under denaturing conditions. Quantification of the gels and image analysis was performed on a Phosphorimager and with ImageJ.

Tandem affinity purification

Purifications were done as described[27] with the following modifications. Before centrifugation, crude protein extracts were treated with 3747.8 units DNAse I (AppliChem, Darmstadt, Germany) per 10 ml extraction buffer for 1 h at 4 °C under gentle rotation. A soluble protein fraction was obtained by twice centrifugating at 36,900 × g for 20 min at 4 °C. The GS-TAP-tag was used, which had been reported as superior in this system[14], supplemented with Gateway recombination sites by PCR by means of pCeMM CTAP(SG) (GenBank Accession number EF467048) as template and recombined to pEN-R2-GStag-L3. The entry clones pEN-L4-2-R1, containing the CaMV 35S promoter, pEN-R2-GStag-L3 with a GS-TAP-tag and an entry clone with the ORF were recombined by MultiSite Gateway LR reaction with pKCTAP as destination vector. Peptide fragments were analysed with a 4800 Plus MALDI TOF/TOF Analyzer (Applied Biosystems) and the corresponding proteins were identified with a local database search engine (Mascot 2.1; Matrix Science) and the TAIR 8.0 database[34]. Approximately 40 TAP experiments, mock or with GUS, GFP, or only the tag as baits were performed to assess the experimental background. Peptides that led to the identification of the preys were only found back in the data output of the TAP experiments with the respective bait(s).

Yeast two-hybrid

Gateway-compatible variants of pGAD424 and pGBT9 were used as destination vectors. The *Saccharomyces cerevisiae* yeast strain PJ69-4A was co-transformed with a bait and a prey plasmid and selected on SD-Leu-Trp agar medium. pGAD424 and pGBT9 vectors not carrying a Gateway cassette were used as controls. Three individual transformants were grown for 1 day in liquid culture, dropped in several dilutions on SD-Leu-Trp-His and allowed to grow for 2 days at 30°C. Alternatively, when mentioned, Gateway-compatible vectors pGADT7 and pGBKT7 were used and co-transformed into the yeast strain AH109.

Yeast two-hybrid screen

JAZ2 and *JAZ3* sequences were PCR amplified with the Expand High Fidelity PCR system (Roche) from plasmid templates provided by TAIR as described[6]. PCR products were digested with *Eco*RI and *Pst*I and cloned into *Eco*RI-*Pst*I digested pGBKT7 (with GAL4 DNA binding domain, Clontech) and the constructs sequence-verified. The pGBKT7-JAZ2 and pGBKT7-JAZ3 plasmids were used as baits and transformed into yeast strain Y187. The prey cDNA library, from *Arabidopsis* seedlings (12-days-old) grown on Pi-starved media, was prepared in the plasmid pGADT7 and in yeast mating strain AH109 following BD Matchmaker Library Construction (Clontech, version PR32047; Puga et al., unpublished results). Bait (Y187) and prey (AH109) were mated by growing 50 ml of bait and 500 μl of

prey overnight on 2xYPDA medium at 30°C. Yeast diploids were selected by plating on minimal medium SD lacking His, Leu, Trp and adenine and supplemented with 20 mM 3-aminotriazole at 30°C for 4 days. Respectively 67 and 38 clones containing putative interacting preys were initially selected for JAZ2 and JAZ3 baits and then confirmed, sequenced and retransformed into yeast strain AH109 in order to verify true positives.

Protein extracts and pull-down assays

Ten-day-old *Arabidopsis* seedlings were ground in liquid nitrogen and homogenized in extraction buffer containing 50 mM Tris-HCl (pH 7.4), 80 mM NaCl, 10% glycerol, 0.1% Tween-20, 1 mM DTT, 1 mM PMSF, 50 μM MG132 (Sigma), and Complete protease inhibitor (Roche). After centrifugation (16,000 × g at 4°C), the supernatant was collected. For *in vivo* pull-down experiments, 6 μg of resin-bound MBP fusion protein was added to 1 mg of total protein extract and incubated for 1 h at 4°C. After washing, samples were digested with 1 μl factor Xa (New England Biolabs), loaded on SDS-PAGE gels, transferred onto nitrocellulose membranes and incubated with monoclonal anti-GFP antibodies (Sigma).

Bimolecular fluorescence complementation

In planta protein interactions were investigated with bimolecular fluorescence complementation in a tobacco transient expression system as described[8].

Generation of transgenic plants

For subcellular localizations, pEN-L1-NINJA-L2 was recombined with pK7FWG2, yielding a C-terminal GFP fusion. For *NINJA* overexpression and knock-down, the pGWB5 (Invitrogen) and pAGRIKOLA[35] (NASC N245138) vectors were used, respectively. All destination vectors were transformed to the *Agrobacterium tumefaciens* strain C58C1RifR (pMP90). Plants were transformed with the floral dip method[28] and transgenic lines selected as described[36]. Expression analysis was done as described[15],[37].

Confocal imaging

For GFP imaging, T1 seedlings were selected on kanamycin-containing Murashige and Skoog (MS) medium. Roots were briefly incubated in propidium iodide (3 mg/l), washed and subsequently mounted in milliQ water and imaged with a Zeiss LSM 510 confocal microscope.

Analysis of transgenic and mutant *Arabidopsis* plants

For phenotypic analysis, wild-type Columbia-0, *NINJA*-OE and -KD transgenic plants, wild-type Landsberg erecta and mutant *tpl-1* plants were germinated on Johnson's medium with increasing concentrations of JA and grown at 21°C and a 16h light/8h dark cycle. Root length was measured 9 days post germination. For statistical analysis data were ln transformed to achieve homoscedacity. Subsequently, a general linear model was fit using the mixed procedure from SAS (v9.2): log(root length)=μ+G+T+G*T, where G is the fixed genotype effect, T the fixed treatment effect and G*T the genotype*treatment interaction effect. The estimate function was used to estimate the interaction effects. FDR multiple testing was performed to adjust the *P*-values.

Expression analysis

Microarray data of 7-day-old *Arabidopsis* seedlings treated with MeJA and ABA were obtained from AtGenExpress[38]. Raw expression data were processed and statistically analyzed as described[13]. Real Time-qPCR analysis was performed as described[15]. For MeJA time course analysis, wild-type Col-0 seedlings were grown for 7 days on MS medium and transferred to medium containing 25μM MeJA (Duchefa). For microarray analysis, three independent *NINJA*-KD lines were grown for 6 days on MS medium, transferred to soil and grown for 3 additional weeks. Subsequently, plants were treated or not for 6 hours with 0.5 μM coronatine. KD-lines were pooled and 4 biological replicates were independently hybridized per transcriptomic comparison. RNA amplification and labelling were performed basically as described[37]. Hybridization was performed on Agilent Arabidopsis Oligo Microarrays v4 (ref. 021169) following manufacturer specifications. Images from Cy3 and Hyper5 channels were equilibrated and captured with a GenePix 4000B (Axon) scanner and spots quantified using GenPix software (Axon). The statistical significance of the results was evaluated by the non-parametric algorithm 'Rank Products'[39] and available as "RankProd" package at Bioconductor (http://www.bioconductor.org)[40]. This method detects genes that are consistently high ranked in a number of replicated experiments independently of their numerical intensities. The results are provided in the form of *P*-values defined as the probability that a given gene is ranked in the observed position by chance. Genes with an FDR-corrected *P*-value < 0.05 and a fold-change >2 and <−2 were selected for further investigation.

Transient expression assays

Transient expression assays were carried out as described previously[13,29]. Protoplasts were prepared from a Bright Yellow-2 tobacco cell culture and co-transfected with a reporter plasmid containing the fLUC reporter gene driven by a promoter containing five GAL4-binding sites[41], a normalization construct expressing Renilla luciferase (rLUC) under control of the 35S promoter[29] and effector constructs. Effector constructs were constructed by Gateway LR cloning with p2GW7 for overexpression. GAL4DBD fusions were created by combining pEN-L4-2-R1 (35S promoter), pEN-R2-GAL4DBD-L3 and an entry clone holding the ORF combined by MultiSite Gateway LR reaction with pm43GW7 as destination vector. Alternatively, for MYC2, the p2GAL4DBGW6 destination vector was used[13]. For each experiment, 2 μg of each plasmid was used unless mentioned otherwise. In the latter case, total effector amount was equalized in each experiment with p2GW7-GUS mock effector plasmid. After transfection, protoplasts were incubated overnight, lysed and fLUC and rLUC activities determined by the Dual-Luciferase reporter assay system (Promega). Variations in transfection efficiency and technical error were corrected by normalization of fLUC by rLUC activities. For statistical analysis, a general linear model was performed with the General Linear Model procedure of SAS (v9.2). The Dunnett's method was used for multiple comparisons with the indicated reference group.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Browse J. Jasmonate passes muster: A receptor and targets for the defense hormone. Annu Rev Plant Biol. 2009; 60:183–205. [PubMed: 19025383]

2. Kazan K, Manners JM. Jasmonate signaling: toward an integrated view. Plant Physiol. 2008; 146:1459–1468. [PubMed: 18390489]

3. Wasternack C. Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. Ann Bot (Lond). 2007; 100:681–697.

4. Browse J, Howe GA. New weapons and a rapid response against insect attack. Plant Physiol. 2008; 146:832–838. [PubMed: 18316637]

5. Pauwels L, Inzé D, Goossens A. Jasmonate-inducible gene: what does it mean? Trends Plant Sci. 2009; 14:87–91. [PubMed: 19162528]

6. Chini A, et al. The JAZ family of repressors is the missing link in jasmonate signalling. Nature. 2007; 448:666–671. [PubMed: 17637675]

7. Thines B, et al. JAZ repressor proteins are targets of the SCF^{COI1} complex during jasmonate signalling. Nature. 2007; 448:661–665. [PubMed: 17637677]

8. Szemenyei H, Hannon M, Long JA. TOPLESS mediates auxin-dependent transcriptional repression during *Arabidopsis* embryogenesis. Science. 2008; 319:1384–1386. [PubMed: 18258861]

9. Devoto A, et al. COI1 links jasmonate signalling and fertility to the SCF ubiquitin-ligase complex in *Arabidopsis*. Plant J. 2002; 32:457–466. [PubMed: 12445118]

10. Fonseca S, et al. (+)-7-*iso*-Jasmonoyl-L-isoleucine is the endogenous bioactive jasmonate. Nat Chem Biol. 2009; 5:344–350. [PubMed: 19349968]

11. Katsir L, Schilmiller AL, Staswick PE, He SY, Howe GA. COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine. Proc Natl Acad Sci USA. 2008; 105:7100–7105. [PubMed: 18458331]

12. Lorenzo O, Chico JM, Sánchez-Serrano JJ, Solano R. *JASMONATE-INSENSITIVE1* encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. Plant Cell. 2004; 16:1938–1950. [PubMed: 15208388]

13. Pauwels L, et al. Mapping methyl jasmonate-mediated transcriptional reprogramming of metabolism and cell cycle progression in cultured *Arabidopsis* cells. Proc Natl Acad Sci USA. 2008; 105:1380–1385. [PubMed: 18216250]

14. Van Leene J, Witters E, Inzé D, De Jaeger G. Boosting tandem affinity purification of plant protein complexes. Trends Plant Sci. 2008; 13:517–520. [PubMed: 18771946]

15. Grunewald W, et al. Expression of the Arabidopsis jasmonate signalling repressor *JAZ1/TIFY10A* is stimulated by auxin. EMBO Rep. 2009; 10:923–928. [PubMed: 19575013]

16. Chini A, Fonseca S, Chico JM, Fernández-Calvo P, Solano R. The ZIM domain mediates homo- and heteromeric interactions between Arabidopsis JAZ proteins. Plant J. 2009; 59:77–87. [PubMed: 19309455]

17. Chung HS, Howe GA. A critical role for the TIFY motif in repression of jasmonate signaling by a stabilized splice variant of the JASMONATE ZIM-domain protein JAZ10 in *Arabidopsis*. Plant Cell. 2009; 21:131–145. [PubMed: 19151223]

18. Garcia ME, Lynch T, Peeters J, Snowden C, Finkelstein R. A small plant-specific protein family of ABI five binding proteins (AFPs) regulates stress response in germinating *Arabidopsis* seeds and seedlings. Plant Mol Biol. 2008; 67:643–658. [PubMed: 18484180]

19. Goossens A, et al. A functional genomics approach toward the understanding of secondary metabolism in plant cells. Proc Natl Acad Sci USA. 2003; 100:8595–8600. [PubMed: 12826618]

20. Vanholme B, Grunewald W, Bateman A, Kohchi T, Gheysen G. The tify family previously known as ZIM. Trends Plant Sci. 2007; 12:239–44. [PubMed: 17499004]

21. Yan Y, et al. A downstream mediator in the growth repression limb of the jasmonate pathway. Plant Cell. 2007; 19:2470–2483. [PubMed: 17675405]

22. Kazan K. Negative regulation of defence and stress genes by EAR-motif-containing repressors. Trends Plant Sci. 2006; 11:109–112. [PubMed: 16473545]

23. Long JA, Woody S, Poethig S, Meyerowitz EM, Barton MK. Transformation of shoots into roots in *Arabidopsis* embryos mutant at the *TOPLESS* locus. Development. 2002; 129:2297–2306.

24. Chico JM, Chini A, Fonseca S, Solano R. JAZ repressors set the rhythm in jasmonate signaling. Curr Opin Plant Biol. 2008; 11:486–494. [PubMed: 18653378]

25. Gray WM, Kepinski S, Rouse D, Leyser O, Estelle M. Auxin regulates SCF^{TIR1}-dependent degradation of AUX/IAA proteins. Nature. 2001; 414:271–276. [PubMed: 11713520]

26. White DW. *PEAPOD* regulates lamina size and curvature in *Arabidopsis*. Proc Natl Acad Sci USA. 2006; 103:13238–13243. [PubMed: 16916932]

27. Van Leene J, et al. A tandem affinity purification-based technology platform to study the cell cycle interactome in *Arabidopsis thaliana*. Mol Cell Proteomics. 2007; 6:1226–1238. [PubMed: 17426018]

28. Clough SJ, Bent AF. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. 1998; 16:735–743. [PubMed: 10069079]

29. De Sutter V, et al. Exploration of jasmonate signalling via automated and standardized transient expression assays in tobacco cells. Plant J. 2005; 44:1065–76. [PubMed: 16359398]

30. Karimi M, Inzé D, Depicker A. GATEWAY™ vectors for *Agrobacterium*-mediated plant transformation. Trends Plant Sci. 2002; 7:193–195. [PubMed: 11992820]

31. Karimi M, Depicker A, Hilson P. Recombinational cloning with plant Gateway vectors. Plant Physiol. 2007; 145:1144–1154. [PubMed: 18056864]

32. Salmon J, Ramos J, Callis J. Degradation of the auxin response factor ARF1. Plant J. 2008; 54:118–128. [PubMed: 18088308]

33. Dreher KA, Brown J, Saw RE, Callis J. The *Arabidopsis* Aux/IAA protein family has diversified in degradation and auxin responsiveness. Plant Cell. 2006; 18:699–714. [PubMed: 16489122]

34. Swarbreck D, et al. The Arabidopsis Information Resource (TAIR): gene structure and function annotation. Nucleic Acids Res. 2008; 36:D1009–D1014. [PubMed: 17986450]

35. Hilson P, et al. Versatile gene-specific sequence tags for *Arabidopsis* functional genomics: transcript profiling and reverse genetics applications. Genome Res. 2004; 14:2176–2189. [PubMed: 15489341]

36. Harrison SJ, et al. A rapid and robust method of identifying transformed *Arabidopsis thaliana* seedlings following floral dip transformation. Plant Methods. 2006; 2:19. [PubMed: 17087829]

37. Adie BA, et al. ABA is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses in *Arabidopsis*. Plant Cell. 2007; 19:1665–1681. [PubMed: 17513501]

38. Goda H, et al. The AtGenExpress hormone and chemical treatment data set: experimental design, data evaluation, model data analysis and data access. Plant J. 2008; 55:526–542. [PubMed: 18419781]

39. Breitling R, Armengaud P, Amtmann A, Herzyk P. Rank products: a simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments. FEBS Lett. 2004; 573:83–92. [PubMed: 15327980]

40. Hong F, et al. RankProd: a bioconductor package for detecting differentially expressed genes in meta-analysis. Bioinformatics. 2006; 22:2825–2827. [PubMed: 16982708]
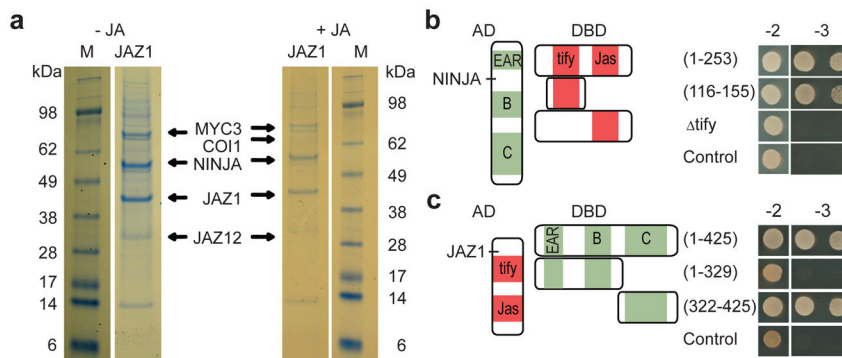
41. Ohta M, Ohme-Takagi M, Shinshi H. Three ethylene-responsive transcription factors in tobacco with distinct transactivation functions. Plant J. 2000; 22:29–38. [PubMed: 10792818]

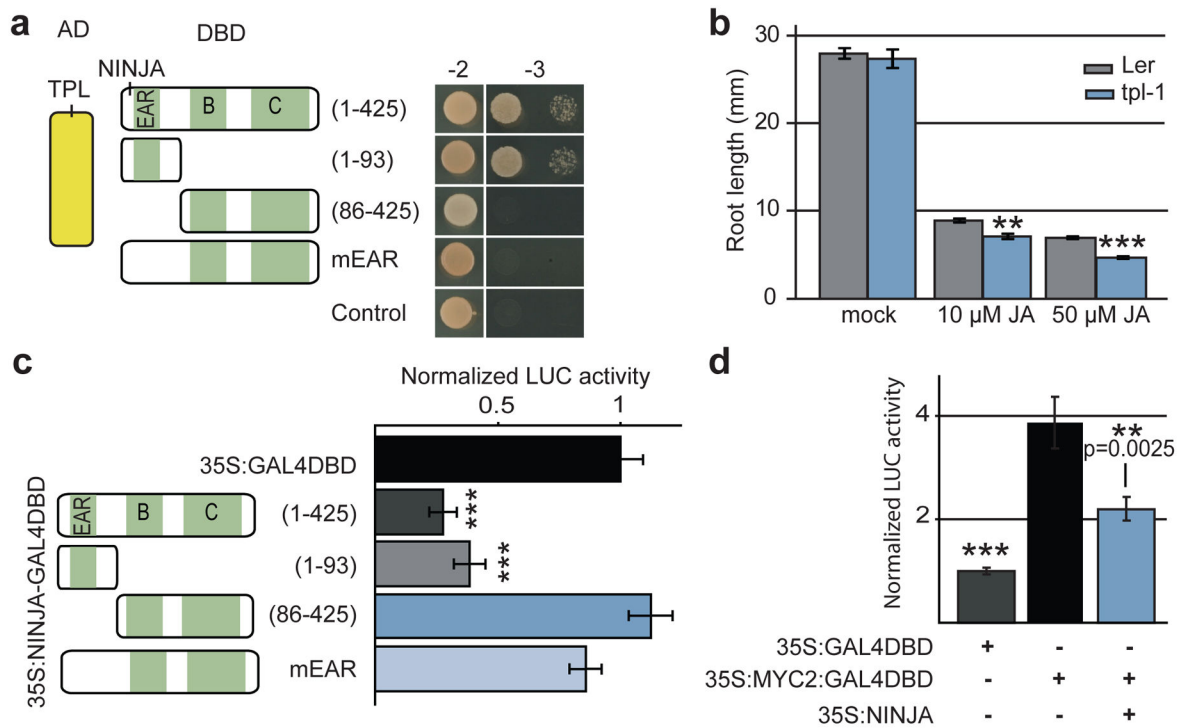**Figure 1. NINJA interacts with JAZ proteins**

a, Gel separation of proteins co-purified with JAZ1-TAP from *Arabidopsis* cells, after mock-treatment (−JA) or elicitation (+JA). Arrows mark positions of proteins identified by MS. Lane M represents a molecular weight marker (in kDa). **b–c**, NINJA and JAZ interacting protein domains in Y2H assays. Drawings represent JAZ1, NINJA, and derivatives. Numbers indicate ending amino acids. Transformed yeasts were spotted in 10- and 100-fold dilutions on control (−2) or selective medium (−3). Controls are empty vectors. **b**, NINJA interaction with the TIFY motif of JAZ1. **c,** JAZ1 interaction with the C domain of NINJA.
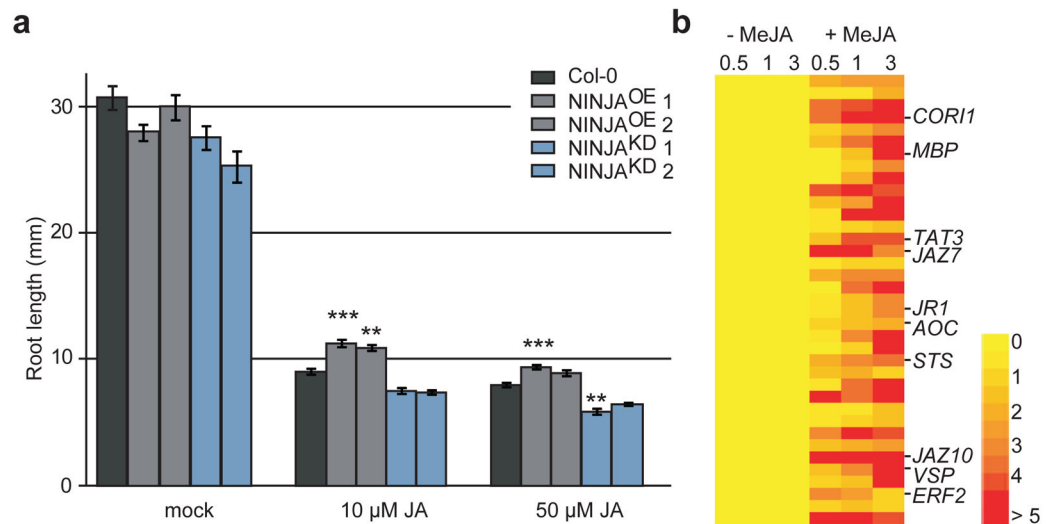
**Figure 2. NINJA negatively regulates JA signalling**

a, Phenotypes of transgenic *NINJA* OE and *NINJA* KD *Arabidopsis* lines. Average primary root length of 9-day-old seedlings germinated on control medium, 10 or 50 μM JA. Data are means ± s.e.m for n 18 (**P 0.01, ***P 0.001, genotype × treatment, see Supplementary Table 2). **b,** Upregulation of MeJA responsive genes in *NINJA* KD plants. Heat maps, generated by the Expression Browser software (http://bar.toronto.ca), represent MeJA modulated expression over time (in hours) of genes significantly induced in *NINJA* KD plants. Scale bar indicates the log fold change.

Figure 3. NINJA interacts with TPL and functions as a transcriptional repressor
a, NINJA and TPL interacting protein domains in a Y2H assay. Drawings represent TPL, NINJA and derivatives. Y2H analysis was performed as in Fig. 1. **b,** JA phenotype of *tpl-1*. Root growth analysis was performed as in Fig. 2. **c–d,** Transactivation activity in tobacco protoplasts transfected with a PUAS-fLUC reporter construct, effector constructs fused or not with GAL4DBD, and a P35S-rLUC normalization construct. Data are means relative to the reference (black bar) ± s.e.m for n=8 (**P 0.01, *** P 0.001; ANOVA, Dunnett's post hoc test). **c,** Functionality of the NINJA EAR repression motif. **d,** MYC2 activity is repressed by NINJA.
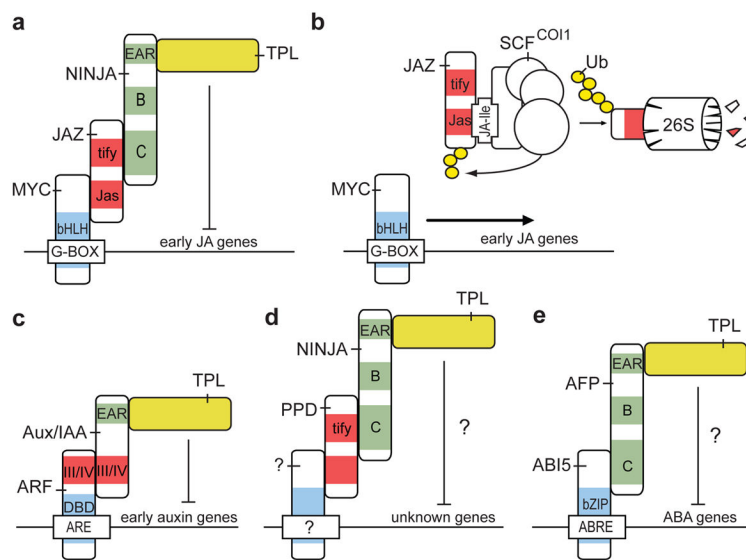
Figure 4. Model for a general function of TPL proteins in plant hormone signalling
a, In the absence of jasmonates, bHLH MYC factors interact with the Jas domain of JAZ proteins that interact through their TIFY motif with domain C of NINJA. The EAR motif of NINJA is essential for interaction with the TPL co-repressors. **b**, In the presence of (+)-7-*iso*-JA-L-Ile, JAZ proteins interact with the ubiquitin ligase SCF[COI1] leading to proteosomal JAZ degradation and subsequent release of the NINJA/TPL complex from the MYC factors and activation of jasmonate-responsive gene expression. **c**, Jasmonate and auxin pathways are built on similar signalling modules. **d**, NINJA interacts with other group-II TIFY proteins which might be recruited by yet unknown transcription factors. **e,** Interaction of the NINJA-related AFP proteins with ABI5 and TPL to regulate ABA responses.