A role for the root cap in root branching revealed by the non-auxin probe naxillin

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SUPPLEMENTARY RESULTS

Supplementary Table 1: Small molecule screening data.

Category	Parameter	Description
Assay	Type of assay	Reporter gene assay (p*CYCB1;1*::GUS), whole organism-based (*Arabidopsis thaliana*)
	Target	Phenotypic, changes in *CYCB1;1* expression pattern in root tissue
	Primary measurement	β-glucuronidase activity
	Key reagents	Substrate for β-glucuronidase (X-Gluc)
	Assay protocol	Described in the Methods section (Compound screening and growth conditions)
	Additional comments	
Library	Library size	10000
	Library composition	DIVERSet; a diverse set of small synthetic molecules
	Source	ChemBridge Corporation
	Additional comments	http://www.chembridge.com/screening_libraries/diversity_libraries/index.php
Screen	Format	96-well plate
	Concentration(s) tested	50 μM
	Plate controls	NAA, NPA
	Reagent/ compound dispensing system	Manual
	Detection instrument and software	Stereo-microscope (Ceti)
	Assay validation/QC	Qualitative assay, intraplate comparison of *CYCB1;1* expression pattern with positive (NAA) and negative (NPA) control
	Correction factors	n.a.
	Normalization	n.a.
	Additional comments	Screening performed at the VIB Compound Screening Facility
Post-HTS analysis	Hit criteria	*CYCB1;1::GUS* expression in the primary root
	Hit rate	0.88%
	Additional assay(s)	Root phenotypic analysis
	Confirmation of hit purity and structure	Compounds were repurchased (ChemBridge Corporation)
	Additional comments	Only non-auxin-like hits were retained for further analysis

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Supplementary Table 2: Overview of the primers used (5' to 3').

mapping nar1/ibr3	
F22F7-FOR	GCTTCATATTGTCACCACATTAATTC
F22F7-REV	AACCCCTGAACTCTGCAAC
F2O10-FOR	TTTGGTTATGTAGGGTTTGAT
F2O10-REV	TTGGTAGATAATGTGAAACCA
F24P17-FOR	CACTGCACATTGCACGAAC
F24P17-REV	AGGATTTGAGGCCATTGGAT
F17A9-FOR	TCCAAATTCAAAAGCCACAA
F17A9-REV	TCCAGAATCTTGAACGATTTCAT
T1B9-FOR	CTTTTTATGGCCCAAATCCA
T1B9-REV	CACGCTGTGTCGTTTTTCTG
T1B9-FOR	TTCTTTCTCTCGCGCTCTTC
T1B9-REV	TGCATCGCTGATCTAATCCTC
MUO22-FOR	CCCTGCTCTTCTTGTTGTCA
MUO22-REV	TGCAGCAGGATAGGTTGGT
T8P19-FOR	GCATGAGAACACGCAAAAGGA
T8P19-REV	TCCAGCGATAAATGTGCTGTA
sequencing primers	
IBR3-FOR1	CTCTGAGGAGCAGAGCGAGA
IBR3-REV1	ACCTAAAGCCCGAAGCACCT
IBR3-FOR2	CCGTTTTGAAATGCAGAATCA
IBR3-REV2	CCTTCACTTGTTGAAGCCAAA
IBR3-FOR3	GCGAGGCAATTACTGCAAAA
IBR3-REV3	CTGGCATCGAAAGCATTCCT
IBR3-FOR4	TTCATTTGTGTTGGCAGCATT
IBR3-REV4	ATGAATCTCCCGCTTCCATC
IBR3-FOR5	TCCAGAACACCCTCCATCTG
IBR3-REV5	TGGAACGGTAAACAACATCAGG
IBR3-FOR6	GGCCTCACGAACCTGGAGTA
IBR3-REV6	TTGGGGCGTTGAAATCAGTT
IBR3-FOR7	ATGGATCCCAGGTGCAGAGT
IBR3-REV7	CCAACAATCGGGTTCCTTCT
IBR3-FOR8	AGCGTGGAATGGAGCTAATG
IBR3-REV8	TTTGTACTAAAAGACCCAAGAACAA
cloning primers	
pIBR10::GUS-FOR	ggggacaagtttgtacaaaaaagcaggcttcgaaggagatagaaccaaactctcattgtcttgttgggag
pIBR10::GUS-REV	ggggaccactttgtacaagaaagctgggtcaccgcctccggatcggtggtgatcggaggaagaagaag
pIBR1::3nGFP-FOR	TAGTTGGAATGGGTTCGAAggaagcaaatgaagtagcagatgg
pIBR1::3nGFP-REV	TTATGGAGTTGGGTTCGAAttcaatctttttttttctttttgc
pIBR3::3nGFP-FOR	TAGTTGGAATGGGTTCGAAccataatcctaataaactccatcc
pIBR3::3nGFP-REV	TTATGGAGTTGGGTTCGAAgatgttccttcactctcctctatctcg
pAIM::3nGFP-FOR	TAGTTGGAATGGGTTCGAAggagacaaaaagattaatatgg
pAIM::3nGFP-REV	TTATGGAGTTGGGTTCGAAagcttaatttctctctgatgag
pMFP2::3nGFP-FOR	TAGTTGGAATGGGTTCGAAcctctttgacaaaccacttgagaagc
pMFP2::3nGFP-REV	TTATGGAGTTGGGTTCGAAtttcgagcttcgttgagattttgcg

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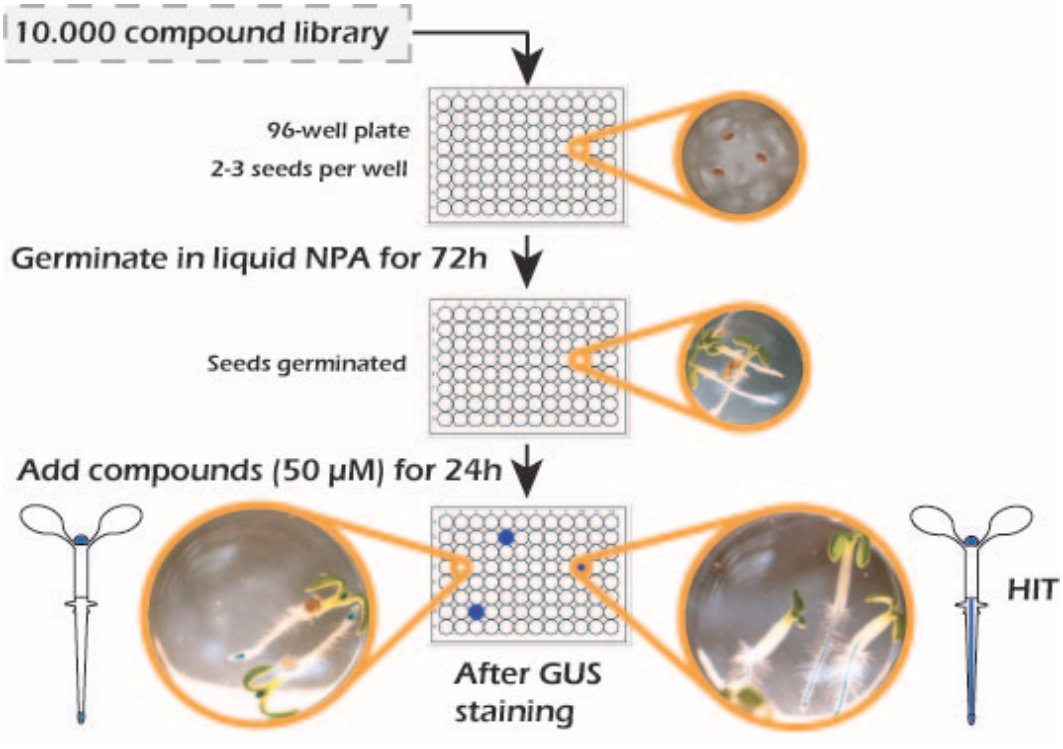

Screening library: DIVERSet, ChemBridge Corp.	**10.000** compounds
Primary screen: p*CYCB1;1*::GUS activation in xylem pole pericycle of root (confirmed)	**99** compounds **(88)** compounds
Secondary screen: non-auxin like molecules	**9** compounds
Tertiary screen: minimal effect on primary root growth	**2** compounds

Supplementary Figure 1 Overview of the procedure to screen for activators of lateral root development with the p*CYCB1;1*::GUS marker.

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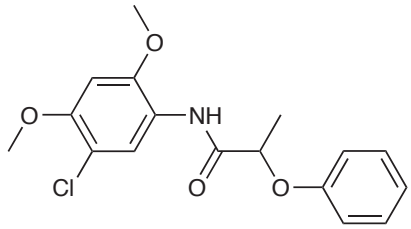

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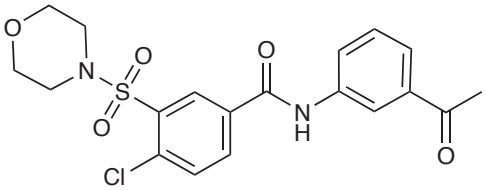

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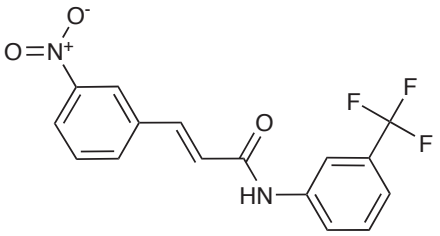

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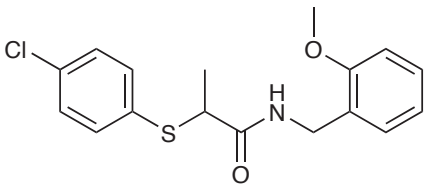

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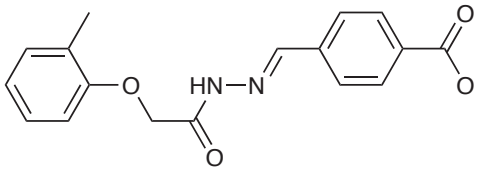

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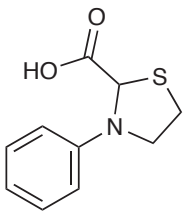

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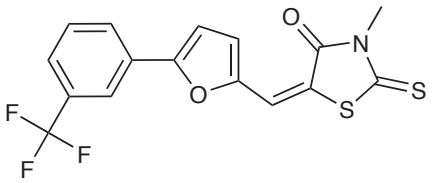

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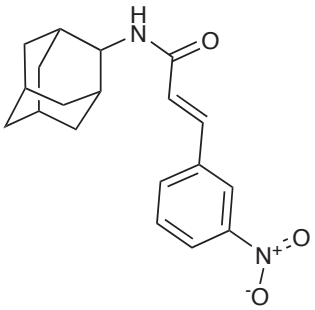

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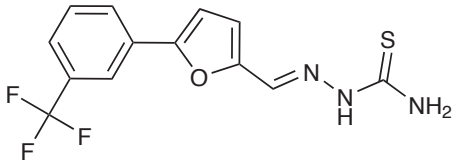

A12 - 5856819

Supplementary Figure 2 Overview of the chemical structures of the nine hit molecules without auxin-like structure and their respective ChemBridge ID number.

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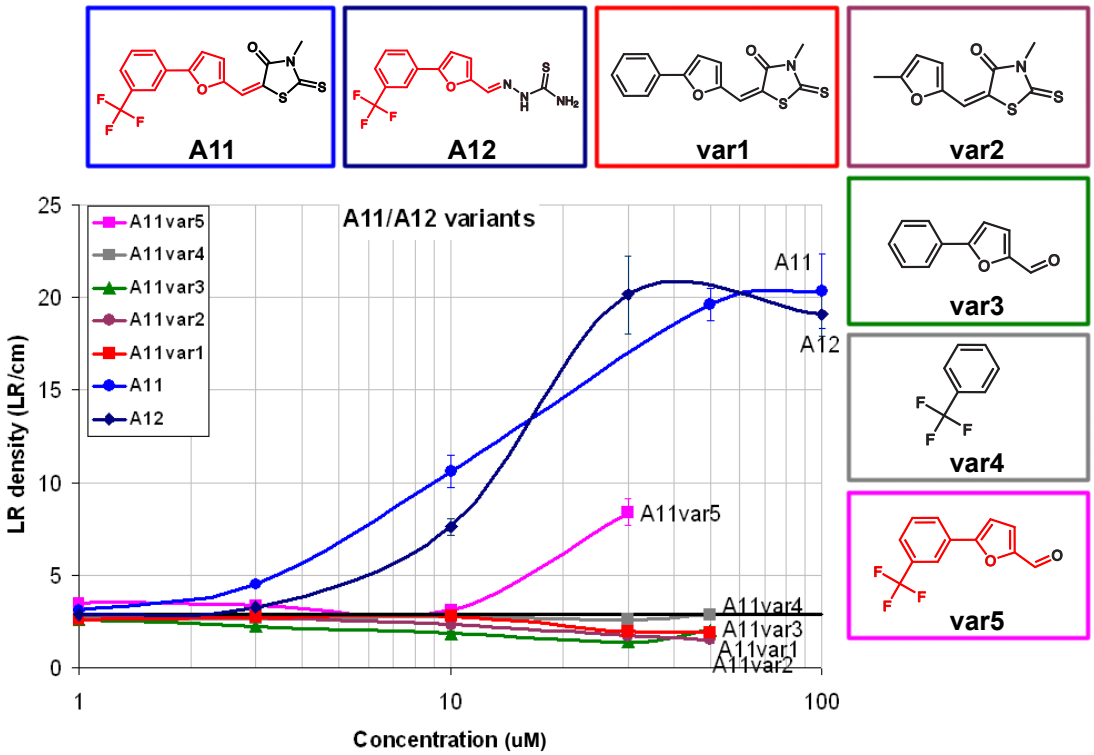
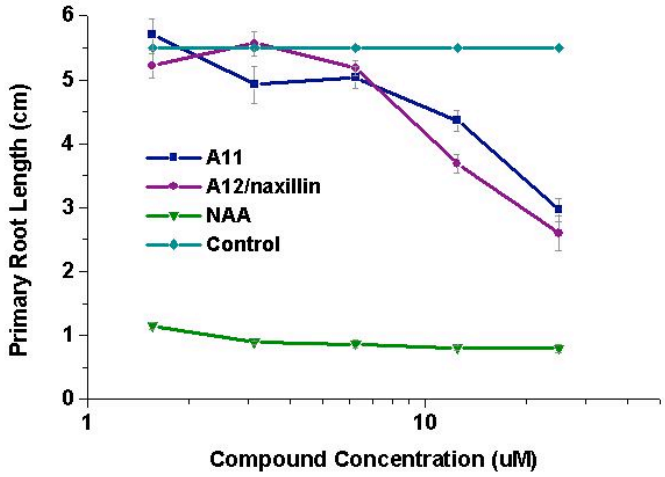


Supplementary Figure 3 Analysis of structural variants of A11 and A12/naxillin to identify the active core structure.



Supplementary Figure 4 Dose-response experiment for inhibition of primary root length by A11 and A12/naxillin in comparison to NAA and mock treatment (DMSO).

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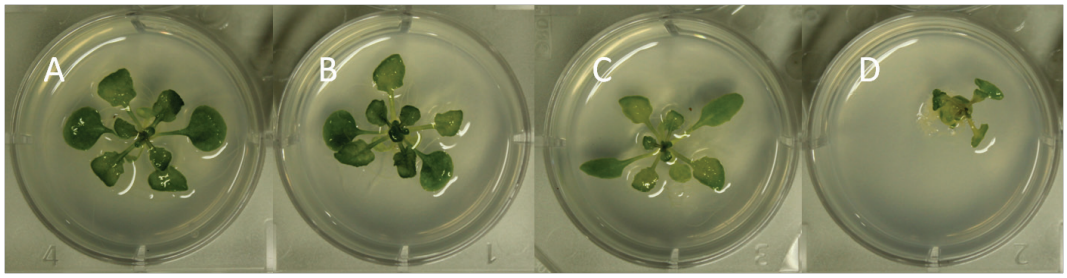
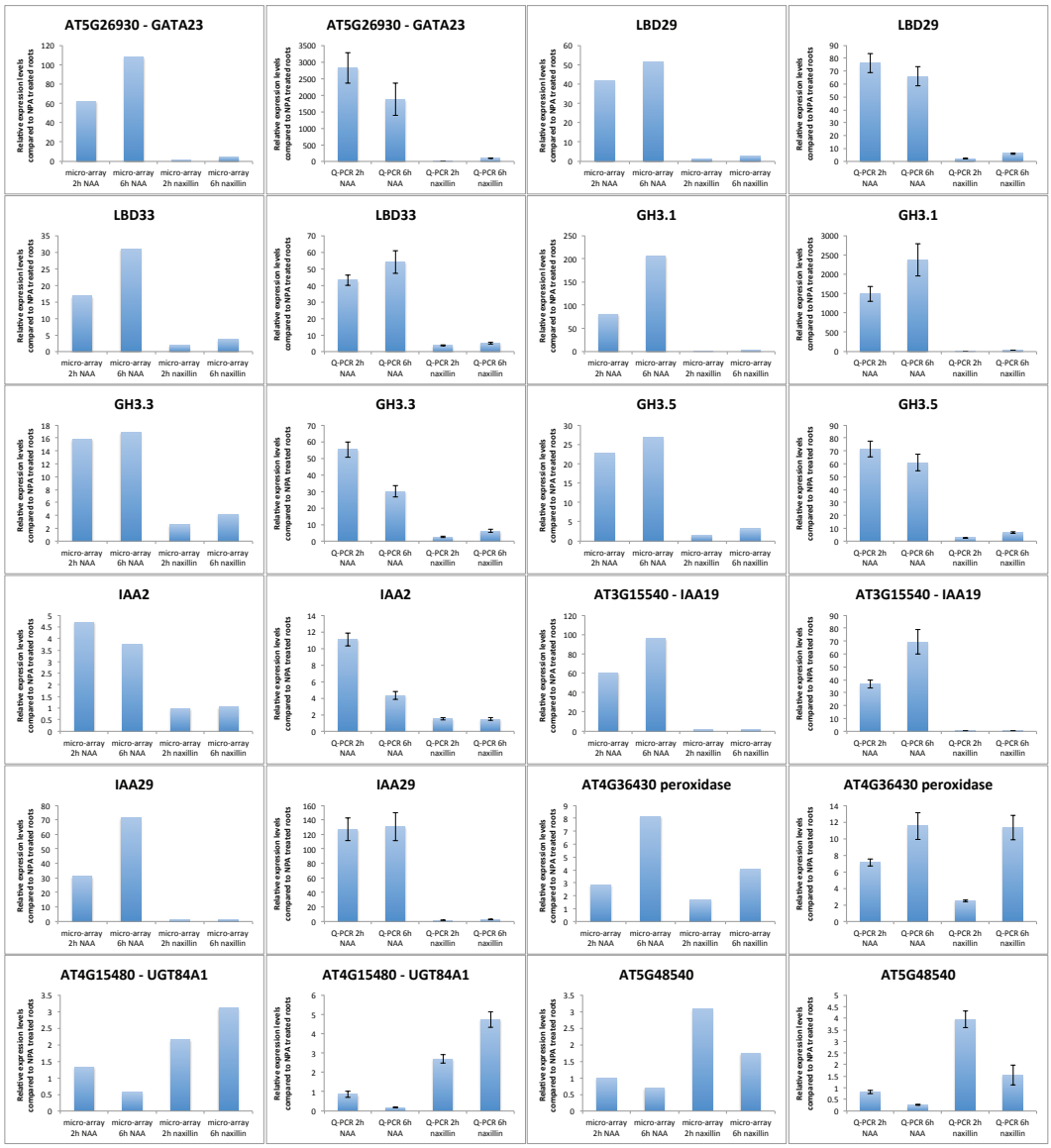
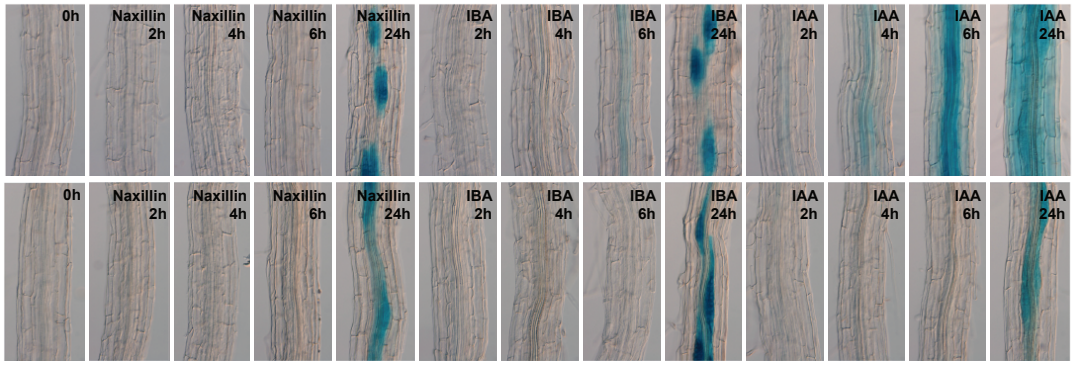


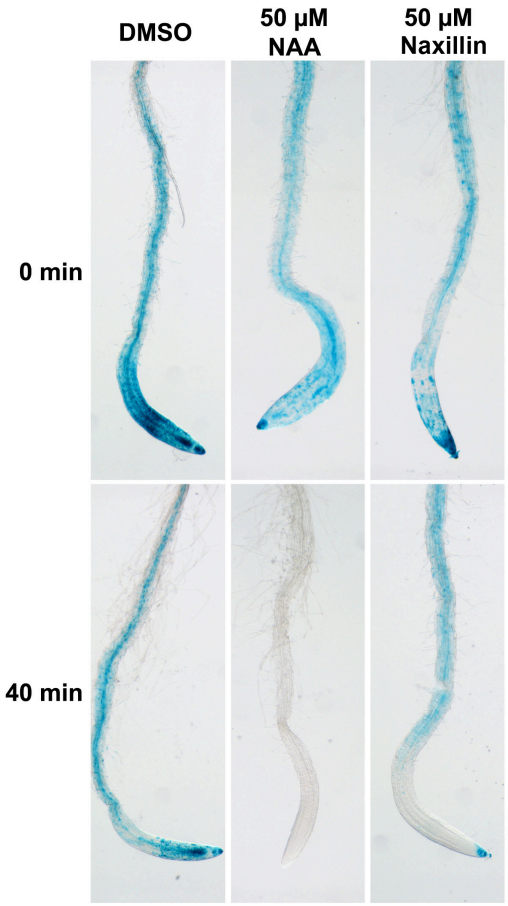
Supplementary Figure 5 Aerial phenotype of 7-day-old seedlings treated with DMSO (mock, A), naxillin (10 µM, B), IBA (10 µM, C) and NAA (10 µM, D) for five additional days.



Supplementary Figure 6 Q-RT-PCR validation (second and fourth columns) of the micro-array results (first and third columns) showing identical expression trends for all genes analyzed.



Supplementary Figure 7 Time-course experiment on three-day-old p*DR5*::GUS (top row) and p*CYCB1;1*::GUS (bottom row) seedlings germinated on 10 µM NPA and subsequently treated with 20 µM naxillin, 10 µM IBA or 10 µM IAA for 0, 2, 4, 6 or 24 hours and subsequently stained for β-glucuronidase activity to assess the rate of lateral root development.



Supplementary Figure 8 Five-day-old pHS::*AXR3*-NT-GUS plants grown on solid medium plates were heat-shocked for 2 hours at 37°C in liquid medium and then transferred to liquid medium at room temperature for 0 minutes or 40 minutes in the presence of 10 µM NAA or 50 µM Naxillin and subsequently stained for β-glucuronidase activity.

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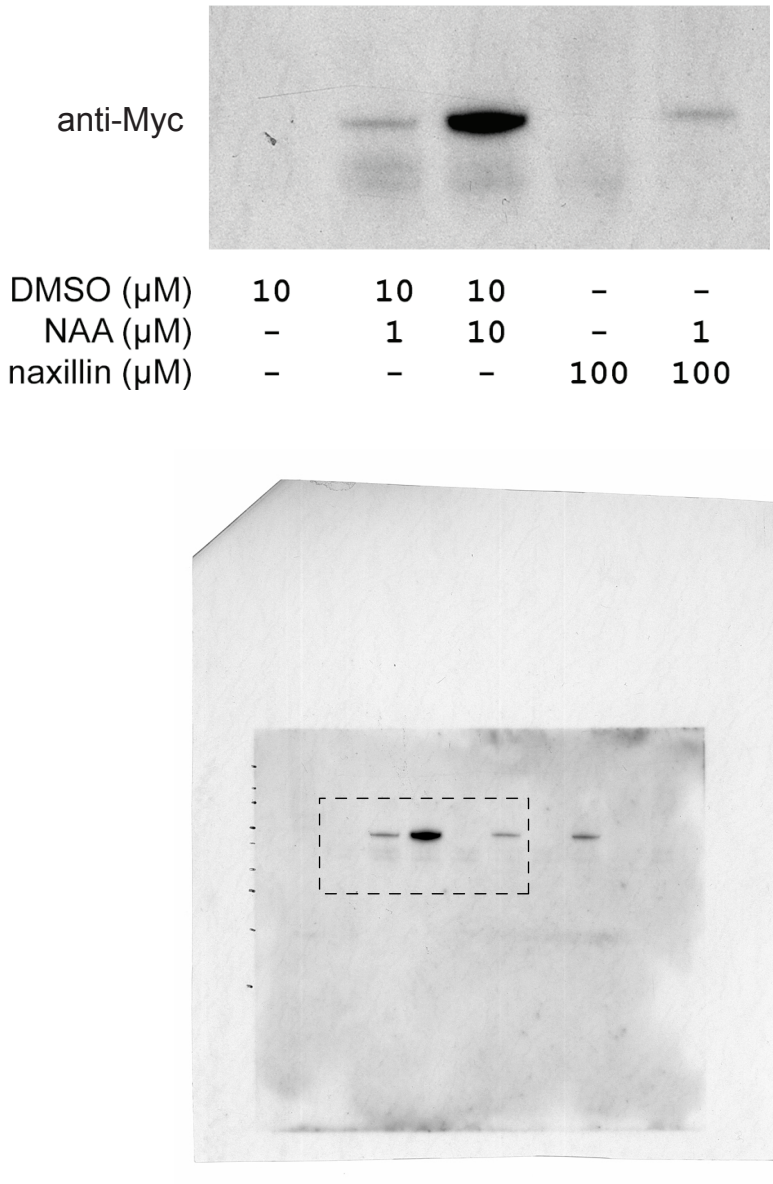

Supplementary Figure 9 *In vitro* pull down experiment using anti-Myc antibodies with TIR1-myc protein and Aux/IAA domain II peptides in the presence of 10 μM NAA or 100 μM naxillin compared to DMSO treatment. Each lane is from identical aliquots of the same batch of TIR1-myc extract and the Aux/IAA peptide is pipetted in and then captured on beads (full gel is in lower panel).

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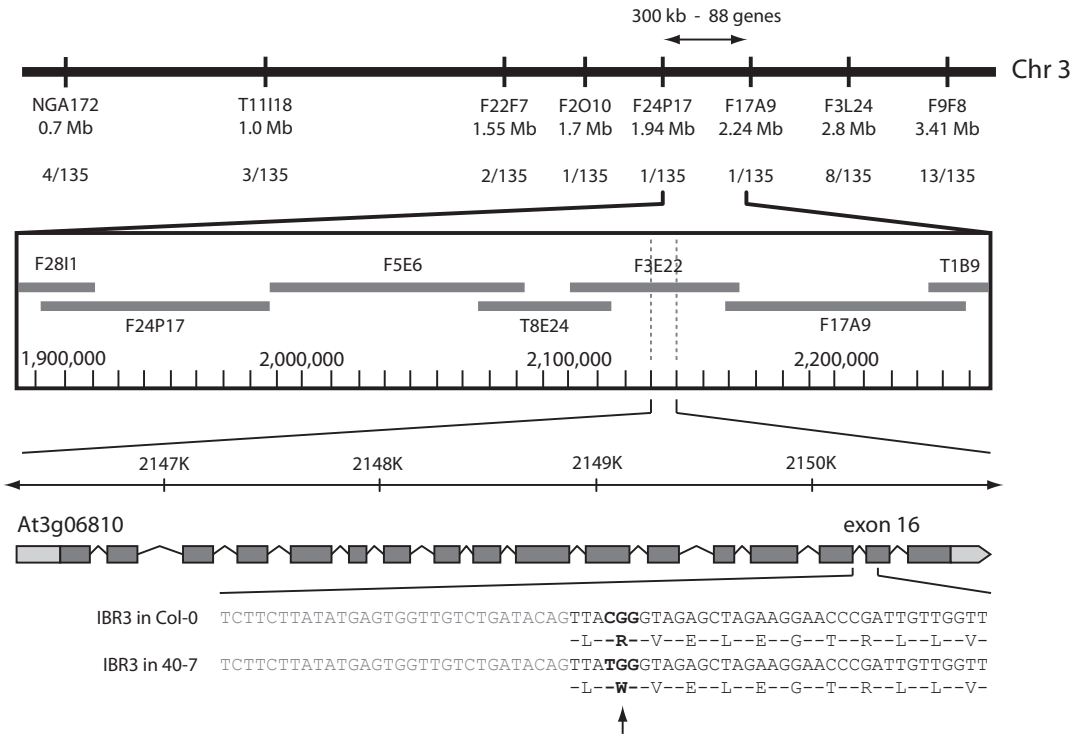
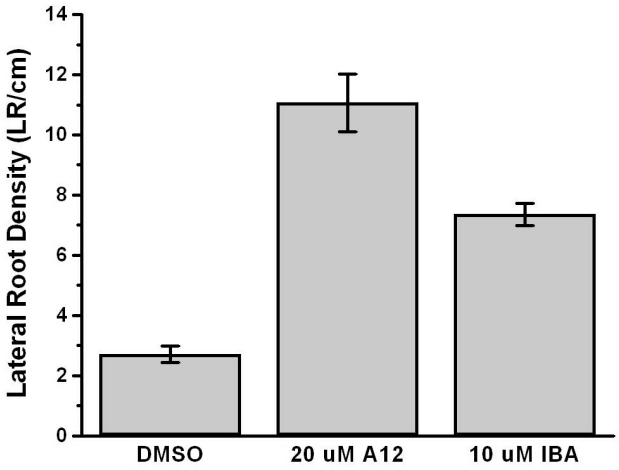


Supplementary Figure 10 Physical map of the chromosomal region in which the mutation that causes naxillin resistance in *nar1* is located. The illustration shows the analyzed polymorphic markers, recombination frequency, BAC map of the 300 kb candidate region, genomic structure of *IBR3* and the location of the mutation.



Supplementary Figure 11 Quantification of the lateral root density of three-day-old *nar1* mutant plants complemented with p*35S*::IBR3 when transferred to 20 µM naxillin or 10 µM IBA for five more days. Error-bars are means ± standard errors.

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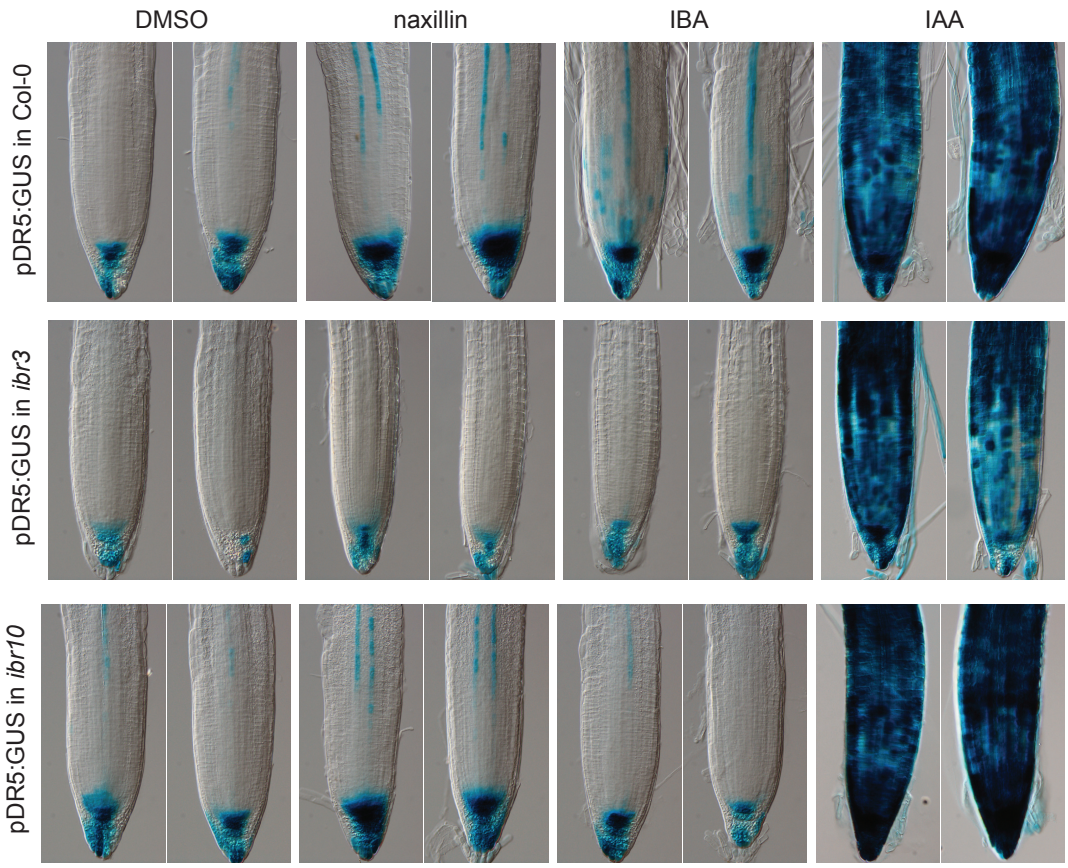
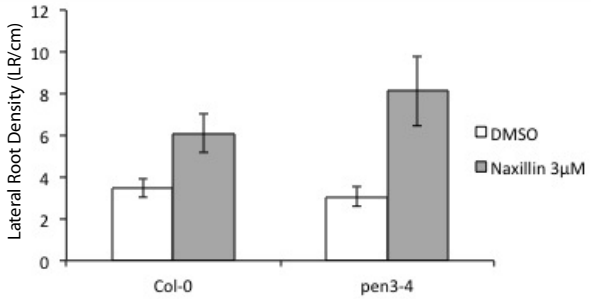


Supplementary Figure 12 p*DR5*::GUS expression in root tips of WT, *ibr3* and *ibr10* mutants upon treatment with 20 µM naxillin, 10 µM IBA or 5 µM IAA for 24 hours.



Supplementary Figure 13 Lateral root density of WT and *pen3-4* roots treated with DMSO (mock) or naxillin showing hypersensitivity of the *pen3-4* mutant to naxillin.

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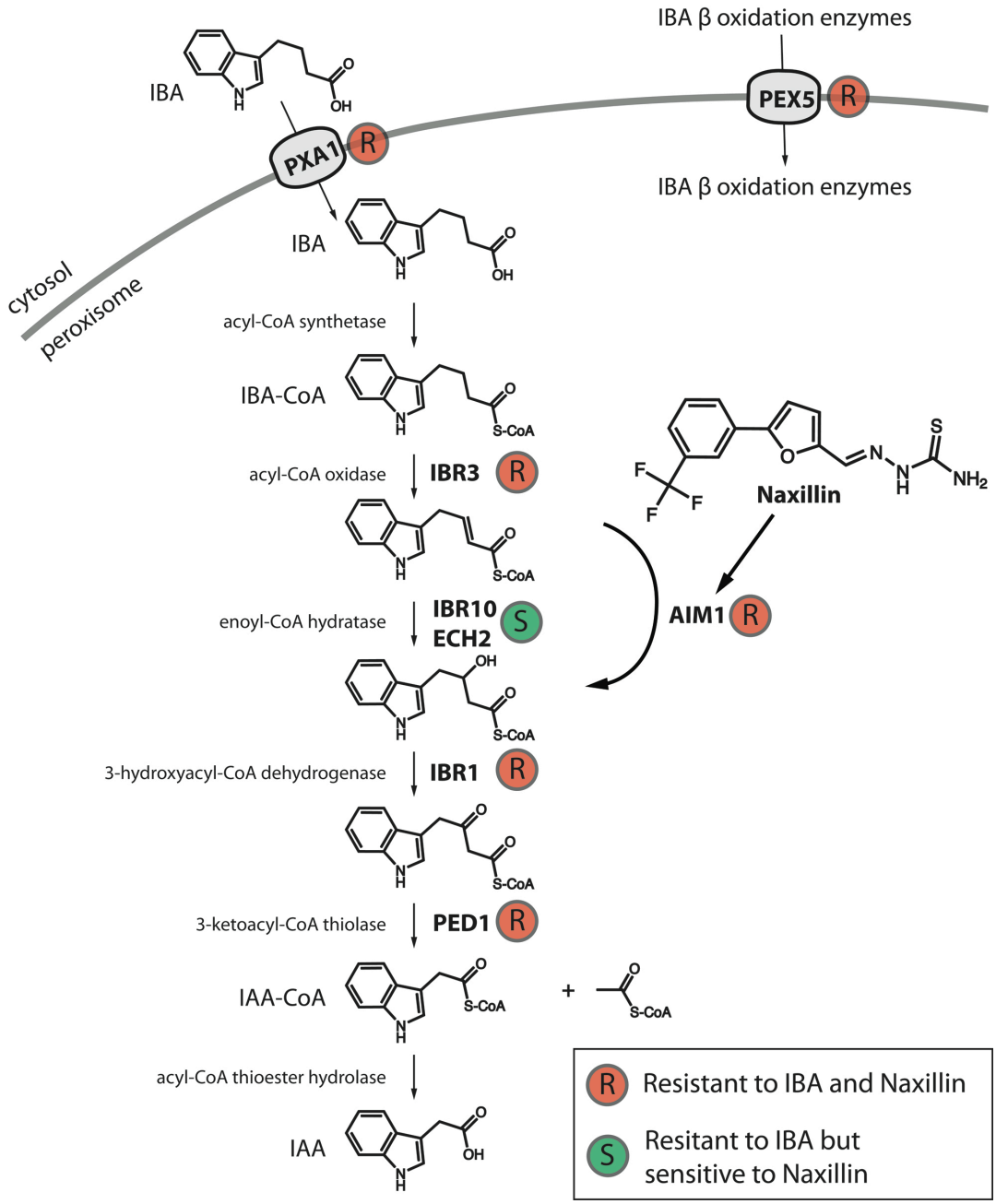

Supplementary Figure 14 Schematic representation of the IBA-to-IAA conversion pathway. The resistance or sensitivity to IBA and/or naxillin of the mutants in the conversion enzymes involved are indicated; as well as the proposed site of action of naxillin.

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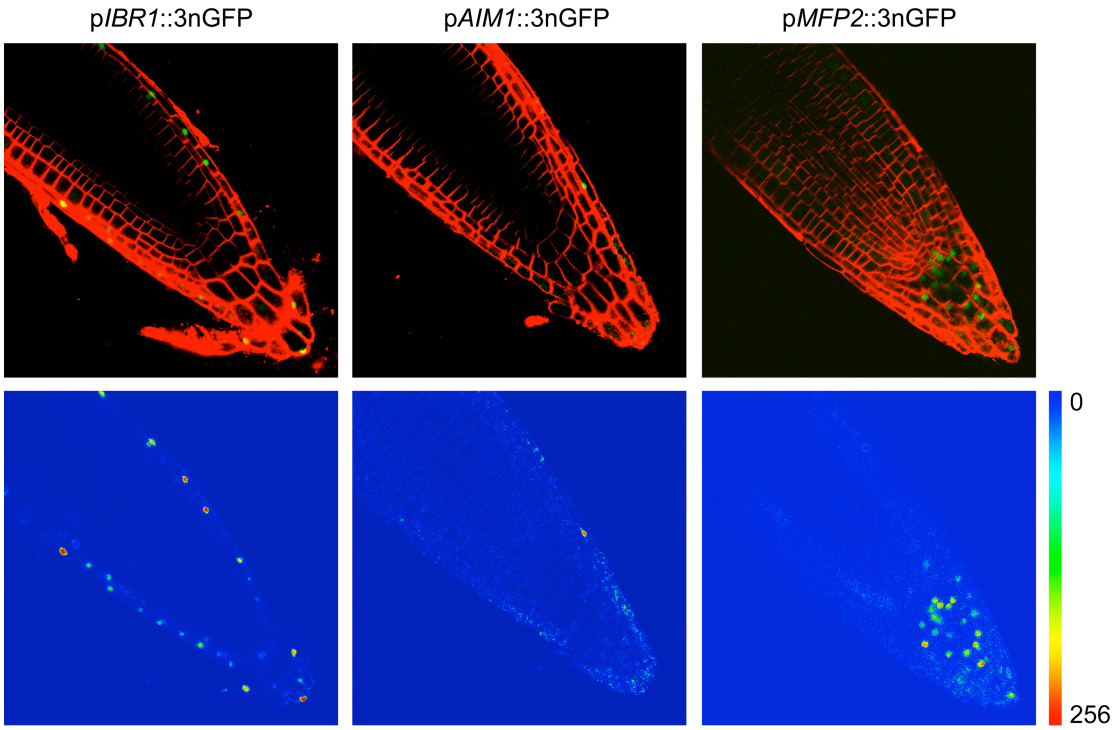

Supplementary Figure 15 Expression patterns of p*IBR1*::n3GFP*;* p*AIM1*::n3GFP *and* p*MFP2*::n3GFP, including a false colour-scale image (below) of the GFP channel showing the high intensity in nuclei in red.

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SUPPLEMENTARY METHODS

Microarray analysis and statistical data processing

Arabidopsis thaliana (L). *Heynh*., Col-0 seeds were germinated vertically on solid medium derived from standard MS medium supplemented with 10 μM NPA. Three days after germination, plants were transferred to 10 μM NAA, 50 μM naxillin or 10 μM NPA (mock) for 2 and 6 hours. RNA isolation was performed on 500 root sections (only root without meristems) for each sample. All sampling points were performed in three independent experiments. RNA was extracted as described for quantitative real-time PCR below. We used 2 μg of total RNA as input for the Affymetrix amplification; 20 μg of amplified RNA (aRNA) was used for Affymetrix fragmentation and 15 μg of fragmented aRNA was used to prepare the hybridization cocktail of 300 μl. From this 300 μl, 200 μl was used for hybridization according to the method used in the Microarray Facility (MAF) of the VIB (http://www.microarrays.be/). The expression values were RMA normalized [1] with R (www.r-project.org) and the Bioconductor package affylmGUI (http://bioinf.wehi.edu.au/affylmGUI/) and a 2-factor Anova analysis was performed in TMEV4.0 [2] on the raw microarray data. Genes that showed a significant (<0.05) p-value (corrected for multiple testing using BH-test) for a difference in effect of the compound (NAA or naxillin) were selected. Next, genes that had a more than 2-fold induction on naxillin for 2h or 6h time-point were selected. The selected 275 genes were clustered according to the tissue specific expression data [3] and those specifically expressed in the xylem pole pericycle cells (indicated by J0121 enhancer trap line) [4] were selected for further analysis (see **Supplementary Dataset1**).

Calculating overrepresentation of genes in longitudinal layers

In order to get the longitudinal tissue expression values of the genes in the seedlings of *Arabidopsis thaliana*, a set of arrays L1SB to L12SB [5] were normalized with the robust multi-array average algorithm [6]. For NAA and A12 treatments, arrays were also normalized with the robust multi-array average algorithm and the fold changes and p-values were determined with the affylmGUI R package [7] with no adjustment methods for 4 independant contrasts; NPA vs NAA and NPA vs A12, 2 and 6 hours after treatment. Affymetrix probe-sets to AGI ID assignment was done using the affy_ATH1_array_elements-2010-12-20.txt file downloaded from TAIR (www.arabidopsis.org). Datasets have been combined using Excel packages and "control", "AGI no_match" and ambiguous probe-sets were subsequently removed. Enrichment was calculated for each selected longitudinal and radial layer by addressing the percentage of experimentally observed genes satisfying a set of conditions compared to the expected number of genes which would have been observed with an independent effect of the tissue specificity and the treatment. A gene was considered as being overrepresented for a layer if its normalized expression value

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in this layer compared with the mean of the normalized expression values in the other layers was above a given threshold. A gene was considered as being regulated upon a treatment if the fold change and the p-value satisfied a given threshold. The formula used to calculate the enrichment can be summarized as Enrichment(%)= LTObs x LTot x 100 / (TTot x LSpec ; LTObs being the observed number of genes both specific for a given layer and significantly regulated upon the treatment; T*Tot* being the total number of genes regulated by the treatment; L*Tot* being the total number of genes presenting a layer specificity; L*Spec* being the number of genes specific for the given layer. Eighteen different combinations of values for the Fold Changes and the p-value were generated to test the robustness and the coherence of the query (respectively 1, 2 or 3 for the Fold Change and 0.05 or 0.01 for the p-value) and were used to generate the histogram. The p-values were generated for each enrichment value using the Fisherexact Excel addin (http://www.obertfamily.com/software /fisherexact.html). The significant enrichment values (p-value > 0.05) for the 18 different combinations were averaged and a standard deviation was calculated to generate the histograms displayed.

References to Supplemental Methods

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