

A miR169 isoform regulates specific NF-YA targets and root architecture in Arabidopsis

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Summary

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- In plants, roots are essential for water and nutrient acquisition. MicroRNAs (miRNAs) regulate their target mRNAs by transcript cleavage and/or inhibition of protein translation and are known as major post-transcriptional regulators of various developmental pathways and stress responses. In *Arabidopsis thaliana*, four isoforms of miR169 are encoded by 14 different genes and target diverse mRNAs, encoding subunits A of the NF-Y transcription factor complex. These miRNA isoforms and their targets have previously been linked to nutrient signalling in plants.
- By using mimicry constructs against different isoforms of miR169 and miR-resistant versions of NF-YA genes we analysed the role of specific miR169 isoforms in root growth and branching.
- We identified a regulatory node involving the particular miR169defg isoform and NF-YA2 and NF-YA10 genes that acts in the control of primary root growth. The specific expression of MIM169defg constructs altered specific cell type numbers and dimensions in the root meristem. Preventing miR169defg-regulation of NF-YA2 indirectly affected lateral root initiation. We also showed that the miR169defg isoform affects NF-YA2 transcripts both at mRNA stability and translation levels.
- We propose that a specific miR169 isoform and the NF-YA2 target control root architecture in Arabidopsis.

Introduction

MicroRNAs (miR) are small noncoding RNAs that regulate gene expression post-transcriptionally and therefore control development, stress responses and hormone signalling or metabolism in both animals and plants (Bartel, 2009; Voinnet, 2009). In Arabidopsis, miRNAs are mainly processed by Dicer-like 1 (DCL1) in 21 nt double-strand RNAs from long stem-loop precursor RNAs. Generally, the mature miR is incorporated into a RNA-induced silencing complex (RISC) which contains a specific pool of ARGONAUTE 1 (AGO 1) proteins (Schott *et al.*, 2012). These loaded RISCs are subsequently able to guide post-transcriptional gene silencing (PTGS) of complementary or partially complementary mRNA by cleavage and/or inhibition of translation. In plants, miR-dependent translation inhibition could be separated from transcript cleavage activity (Brodersen & Voinnet, 2009).

Amongst plant miRNA genes, 22 families are conserved between monocots and dicots. These conserved miRs are usually

produced by several loci and highly expressed (Rajagopalan *et al.*, 2006), whereas recent or young genes are in single or low copy-number, species specific and expressed at low levels (Axtell, 2008). MiR169, the appearance of which in evolutionary time coincided with that of Angiosperms, has been identified in more than 40 species (Sunkar & Jagadeeswaran, 2008), where it often makes up the largest miR family. The Arabidopsis *miR169* family contains 14 genes. However, only four mature miR isoforms (a, bc, defg and hijklmn), differing by one or two nucleotides at the 5' or 3' ends (Supporting Information Fig. S1), are produced (Li *et al.*, 2010). The miR169 isoforms present distinct expression patterns during development (Gonzalez-Ibeas *et al.*, 2011), in response to biotic (Singh *et al.*, 2012) or abiotic stresses (Licausi *et al.*, 2011; Zhao *et al.*, 2011), suggesting a functional specialization.

In plants, the main targets of miR169 are genes that encode the subunit A of Nuclear Factor Y (Rhoades *et al.*, 2002). This transcription factor (TF), also named heme-activated protein (HAP) or CCAAT-box binding factor (CBF), is a heterotrimeric TF composed of NF-YA (HAP2), NF-YB (HAP3/CBF-A) and NF-YC (HAP5/CBF-C) subunits. NF-Y TF recognizes the

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CCAAT motif frequently observed in eukaryotic promoters at position $-60/-100$ from the transcriptional start site (Mantovani, 1999). NF-YB and NF-YC subunits contain a histone fold domain very similar to H2A and H2B core histones (Baxeianis *et al.*, 1995; Zemzoumi *et al.*, 1999) and these two subunits must form a heterodimer for stable interaction with NF-YA. The NF-YA subunit confers the sequence specificity to the TF complex by an unknown mechanism (Mantovani *et al.*, 1994; Dolfini *et al.*, 2012). In animals, its fixation on DNA could affect specific post-translational modifications of histones and thus modify the chromatin status (Donati *et al.*, 2008; Gatta & Mantovani, 2011). The NF-Y TFs have mainly been considered as activators of transcription; however, recent evidence suggests their involvement in gene repression (Ceribelli *et al.*, 2008; Leyva-González *et al.*, 2012). In contrast to animals where *NF-Y* are generally single genes, in all plant species examined so far, the three NF-Y subunits are encoded by multigenic families (Edwards *et al.*, 1998). In *Arabidopsis* there are 10, 13 and 13 genes for the A, B and C subunits, respectively, and Cao *et al.* (2011) found 36 NF-Y genes in *Brachypodium distachyon*. The *NF-Y* genes present differential expression patterns during development (Gusmaroli *et al.*, 2001, 2002; Siefers *et al.*, 2009; Cao *et al.*, 2011), or in response to environmental conditions (Pant *et al.*, 2009; Des Marais *et al.*, 2012), suggesting that, in different organs or under certain stimuli, only some combinations of subunits can be assembled to form the trimeric functional NF-Y factor. In plants, NF-Y TFs have been linked to development (Lotan *et al.*, 1998; Combiér *et al.*, 2006; Wenkel *et al.*, 2006), signalization (Warpeha *et al.*, 2007) and responses to stresses (Nelson *et al.*, 2007; Li *et al.*, 2008; Liu & Howell, 2010; Zhao *et al.*, 2011). Overexpression of one subunit may be sufficient to affect NF-Y function when the other NF-Y partners are also present in the same tissues, as several phenotypes were observed after individual NF-Y subunit overexpression. However, it could not be ruled out that the results observed were consequences of interactions with member(s) that normally do not interact with this specific subunit. Recently, two reviews have been published concerning plant NF-Y complexes (Petroni *et al.*, 2012; Laloum *et al.*, 2013).

Both miR169 and their *NF-YA* target genes present complex spatio-temporal expression patterns in response to abiotic stresses. *Arabidopsis* seedlings under cold stress show an over-accumulation of miR169 correlated with a reduction of some *NF-YA* target transcripts (Zhou *et al.*, 2008; Lee *et al.*, 2010). MiR169 may also play a role in long-distance signalling, as its abundance in phloem sap decreases during nitrogen (N) and phosphorus (P) limitation (Pant *et al.*, 2009; Buhtz *et al.*, 2010) and several miR169 *NF-YA* targets are induced (Pant *et al.*, 2009; Zhao *et al.*, 2011). Moreover, overexpression of *miR169a* resulted in both a reduction of N accumulation and an increased sensitivity to N starvation (Zhao *et al.*, 2011).

Recent data clearly showed that small noncoding RNAs, including miRs, involved in response to environmental stimuli, play a role in root growth or lateral root (LR) organogenesis and therefore on root architecture (Khan *et al.*, 2011). The root apical meristem (RAM) is established during embryogenesis and is

composed of a quiescent center (QC) surrounded by initial cells. These stem cells subsequently supply different root cell files through divisions and differentiation. They also produce the columella and lateral root cap (Petricka *et al.*, 2012). The RAM size determines primary root growth and mutants affected in the division/differentiation of the initials produce short roots. In *Arabidopsis*, at a certain distance from the root apex, LR are initiated from specific pericycle cells in front of the xylem poles (Dubrovsky *et al.*, 2000, 2001). These pericycle cells, blocked in a specific phase of the cell cycle (Himanen *et al.*, 2002), are primed in the elongation zone (EZ) by oscillations of auxin amounts in adjacent xylem cells (De Smet *et al.*, 2007). Auxin is a major determinant of LR initiation. However, recently, it has also been proposed that oscillating gene expression patterns are involved in priming of pericycle cells and thereby specify the pre-branch sites (Moreno-Risueno *et al.*, 2010). In these sites, LR initiation starts with the asymmetric division of the two primed pericycle cells (Fukaki *et al.*, 2002; Vanneste *et al.*, 2005). Subsequently, both periclinal and anticlinal divisions allow the formation of the LR primordium (LRP). This primordium will emerge and the apical meristem is activated to allow growth of the novel LR. In *Medicago truncatula*, we have recently shown that root architecture is affected by miR396 activities. Indeed, miR396 inactivation by mimicry construct, which induced an over-expression of the miRNA targets, resulted in a clear increase of root biomass (Bazin *et al.*, 2013).

In this work, we have analysed the role of specific miR169 isoforms in root growth and branching. By blocking miR169 action with different mimicry constructs, we showed that a regulatory node involving the miR169defg isoform and its *NF-YA2* and *NF-YA10* targets acts to control RAM length and LR density. Furthermore, perturbing the regulation of *NF-YA2* by miR169defg indirectly affects LR initiation, suggesting a role for this miR169 isoform in LR organogenesis and root architecture.

Materials and Methods

Plant material and growth conditions

All lines used are in the *Arabidopsis thaliana* Columbia (Col0) ecotype. Mimicry lines *MIM169abc* and *defg* have been described previously (Todesco *et al.*, 2010). Seedlings were grown under long photoperiod (16 h : 8 h, light : dark) unless stated otherwise in half strength Murashige & Skoog (1962) medium (Duchefa) supplemented with 1% (w/v) sucrose.

Constructs

For *pNF-YA2:GUS*, *pNF-YA10:GUS*, a 2000-pb region upstream of the start codon was amplified from genomic DNA (all primers sequences used for cloning are in Table S1), to generate Gateway pENTR-D vectors which were recombined in the Gateway binary vector pKGWFS7 (Karimi *et al.*, 2007). The same procedure was used for the *pmiR169d:GUS* and *pmiR169e:GUS* reporter lines; a 1800-pb region upstream of the miRNA stem loop was amplified from genomic DNA. For the *p35S:*

miR169a and *d* lines, 300–400-bp fragments surrounding the miRNA sequence were amplified from genomic DNA, cloned into pENTR-D and transferred in Gateway binary vectors pH7WG2 or pK7WG2D, respectively.

For the transient experiments, the 3'UTR of *NF-YA2* or *NF-YA1* was fused to the GFP coding region and cloned into the pENTR-D. *p35S:GFP-NF-YA2wt* and the *p35S:GFP-NF-YA1wt* were generated by recombination in a homemade Gateway-compatible vector (Marin *et al.*, 2010) or the binary vector pH7WG2, respectively. The miR resistant versions *p35S:GFP-NF-YA1mut* and *p35S:GFP-NF-YA2mut* were obtained by deleting the miR recognition site as shown. To overexpress miR169a the *p35S:miR169a* was used. For *p35S:miR169d* and *p35S:miR169b* a 200–400-bp fragment surrounding the miRNA sequences was amplified from genomic DNA, cloned into pENTR-D and recombined in the binary vector pH7WG2.

Lines expressing the GFP under the *NF-YA2* promoter were transformed by plasmids that resulted from a double recombination of pDONR221 containing GFP-NF-YA2 (wild-type (WT) or mutated) and the *NF-YA2* promoter into the binary vector pK7m24GW,3.

For *NF-YA2* overexpressing lines, the HA tag was fused in frame to the NF-YA2 cDNA including the 3'UTR. A double recombination of this construct and the *NF-YA2* promoter were cloned into the vector pK7m24GW,3. The miR resistant version was generated as previously described for GFP constructs.

Col0 transformation was performed by the floral dip method (Clough & Bent, 1998) and independent stable transgenic lines were selected.

Histochemical and histological analyses

The GUS assays were performed as described (Beeckman & Engler, 1994). To analyse expression in the whole organ, roots were cleared in chloral hydrate solution. Transversal and longitudinal sections were obtained after GUS staining and technovit 7100 resin embedding, using the protocol described by Vanneste *et al.* (2005) and De Smet *et al.* (2004).

Imaging and image analysis

For epifluorescence imaging, roots (mounted in 50% glycerol) were directly photographed on a widefield microscope (DMI6000B; Leica Microsystems, Wetzlar, Germany) with a $\times 20$ objective. GFP images were made every 0.59 μm to scan the entire root. Deconvolution was done with Huygens software (Scientific Volume Imaging, Hilversum, the Netherlands).

To visualize GUS activity in whole roots we used a microscope equipped with Nomarski optics (DMI6000B; Leica Microsystems).

Phenotypic analyses

Measurements of primary root length and lateral root number were made on scanned images of Petri-dishes containing the seedlings grown on the surface of the agar medium using ImageJ

(<http://rsb.info.nih.gov/ij/>). The number of LRP, in the lateral root formation zone as defined by Dubrovsky & Forde (2012), was counted by using the Nomarski microscope in roots cleared by choral hydrate. The classification of LRP developmental stages was performed according to Malamy & Benfey (1997).

Measurements of meristem size and cell number

The cell wall of the roots was stained according to Truernit *et al.* (2008). A Leica SP8 confocal laser scanning microscope (Leica Microsystems) was used to image root tips. Serial optical sections were reconstituted into 3D image stacks and measurements were made using ImageJ. Cell length and meristem length are averages of two opposite cell files; cell width and thickness are averages of all visible cells in a cross section.

RNA extraction, RNA blot assays, quantitative RT-PCR

Total RNA was extracted as described (Mallory *et al.*, 2001) or by using the Trizol procedure as described by the manufacturer (Life Technologies SAS, Saint Aubin, France). For RNA gel blot analysis, 10 μg of RNA was separated by denaturing (7 M Urea) 15% polyacrylamide gel electrophoresis, blotted to a nylon membrane (Hybond NX; GE Healthcare, Vélizy-Villacoublay, France), and EDC cross-linked as described (Pall *et al.*, 2007). miRNA probes were prepared by end-labelling antisense oligonucleotides with ^{32}P using T4 polynucleotide kinase (Thermo Fischer Scientific Bioscience, Villebon-sur-Yvette, France). RNA gel blots were hybridized (Mallory *et al.*, 2001) with the miR169 probe together with U6 probe. Nonsaturated signals were scanned on a Molecular Dynamics Storm 840, and analysed with ImageJ.

For quantitative RT-PCR analysis (all qPCR primers sequences used can be found in Table S2), total RNA (5 μg) was subjected to DNase I treatment (Thermo Fisher Scientific) and reverse transcribed by using oligodT with Revertaid (Thermo Fisher Scientific). mRNA concentrations were assayed in two to four independent biological replicates and, for each gene, two technical replicates were done. Real time RT-PCR was performed on a Roche LightCycler480 using Roche reagents (<http://www.roche.com>). For the Arabidopsis experiment the At1g13320 and At4g26410 (Czechowski *et al.*, 2005) genes were used as house-keeping genes (HKG) for the normalization of the expression of the target gene (TG). The use of geNORM software (Vandesompele *et al.*, 2002), established that these genes were the most stable whatever the organs, genotypes or hormone treatments. Relative expression levels were calculated following this formula $\Delta\text{Cycle Threshold (Ct)} = \text{Ct}_{\text{TG}} - \text{mean (Ct}_{\text{HKG}})$ and transformed in relative value expression (REV), where $\text{REV} = 2^{-\Delta\text{Ct}}$. The $\Delta\Delta\text{Ct}$ method was used to analyse the relative change in gene expression between two conditions, where $\Delta\Delta\text{Ct} = \Delta\text{Ct}_{\text{mutant/NAA}} - \Delta\text{Ct}_{\text{WT/NPA}}$, and transformed in relative value expression, where $\text{REV} = 2^{-\Delta\Delta\text{Ct}}$ (Livak & Schmittgen, 2001). For the transient experiments in *Nicotiana benthamiana*, *UBI3* (AY912494) and *EF1 α* (D63396) were used as HKG and ΔCt and $\Delta\Delta\text{Ct}$ were used as already described.

The miR169 and miR171bc profiles were obtained by stem-loop RT-PCR as previously described by Varkonyi-Gasic *et al.* (2007) with reverse transcription primers designed according to Chen *et al.* (2005; Table S2) and a pair of qPCR primers (Table S2; Varkonyi-Gasic *et al.*, 2007). qPCR analysis was performed as already described.

Agroinfiltration and transient experiment

Agroinfiltration was performed in 3-wk-old *N. benthamiana* leaves. *p35S:miR169a*, *p35S:miR169b*, *p35S:miR169d*, *p35S:miR390b*, *p35S:GFP-NF-YA1wt*, *p35S:GFP-NF-YA1mut*, *p35S:GFP-NF-YA2wt*, *p35S:GFP-NF-YA2mut* and *p35S:mcherry* were transformed into *Agrobacterium tumefaciens*. For co-infiltrations, individual overnight cultures were harvested and suspended at OD 1 in 10 mM MgCl₂, 10 mM MES, pH 5.8 and 150 µM acetosyringone. After 3 h of incubation at room temperature, various combinations were mixed at a 1 : 1 : 1 ratio; these mixes were used to infiltrate leaves. These leaves were harvested 3 d after the infiltration. Half of them were used immediately for protein extraction and the other half was frozen and used for RNA extraction as described above. The *p35S:mcherry* was always used as an infiltration control and *p35S:miR390b* (Marin *et al.*, 2010) as a control of protein and RNA stability in absence of the targeting miR.

Protein methods

Protein extraction was performed according to Elliott *et al.* (2006). Protein concentration was quantified by Bradford assay. Fluorescence was performed, in duplicate, on 10–20 or 30 µg of total proteins for the transient or stable experiments, respectively, by using the Infinite 200 fluorometer (TECAN Group Ltd, Männedorf, Switzerland). The excitation and emission parameters were 475 nm and 510 nm for GFP and 565 nm and 610 nm for mcherry. For transient experiments, the GFP fluorescence was normalized by the mcherry fluorescence. For the stable experiments, the GFP fluorescence was calculated for 1 µg of proteins.

Statistical analysis and comparison of the agroinfiltration

As the level of expression in the transient experiments could be highly different between each infiltrated leaf, we compared the results obtained from mRNA concentration and fluorescence intensity in leaves transformed with the mutated form of the NF-YA 3'UTR against those obtained with the WT form of the NF-YA 3'UTR. All pair ratios were calculated. For mRNA comparison, the formula used is $\text{ratio}_{\text{mRNA}} = 2^{-(\Delta\text{Ct}_{\text{GFP}} - \Delta\text{Ct}_{\text{mcherry}})_{\text{mut}}/2^{-(\Delta\text{Ct}_{\text{GFP}} - \Delta\text{Ct}_{\text{mcherry}})_{\text{WT}}}}$, where $\Delta\text{Ct}_{\text{GFP}} = \text{Ct}_{\text{GFP}} - \text{Ct}_{\text{HK}}_{\text{G}}$ and $\Delta\text{Ct}_{\text{mcherry}} = \text{Ct}_{\text{mcherry}} - \text{Ct}_{\text{HK}}_{\text{G}}$. For the GFP fluorescence, the formula is $\text{ratio}_{\text{fluorescence}} = (\text{GFP fluorescence/mcherry fluorescence})_{\text{mut}}/(\text{GFP fluorescence/mcherry fluorescence})_{\text{WT}}$. Statistical analysis were performed on R 2.14.2 (R Development Core Team, 2012).

Results

Root architecture is affected in *MIM169defg* plants

In order to study the potential role of miR169 in root architecture, we analysed mimicry plants generated by Todesco *et al.* (2010) for their root phenotype. These transgenic plants expressed specific constructs that sequester either both miR169abc and miR169hijklmn isoforms or only the miR169defg isoform, driven by the cauliflower mosaic virus 35S promoter. These lines named *MIM169abc* or *MIM169defg* by Todesco *et al.* (2010) were reported to present smaller rosettes. In contrast to *MIM169abc* (Fig. S2), *MIM169defg* plants exhibited shorter primary roots when compared with wild-type (WT) plants (Fig. 1a,b). This root phenotype was linked with slower root growth kinetics after seedling germination (Fig. S3). As PR were shorter and the LR number remained unchanged, LR density was enhanced in the *MIM169defg* lines (Fig. 1b,c). *MIM169defg* LR length was also smaller (Fig. 1d). For PR, smaller root length correlated with a shorter meristem length (Fig. 1e,f) at 5 but also at 7 or 12 d-after-germination (DAG). Interestingly, as shown in Fig. 1(f–g), global root thickness was also reduced in these plants. Indeed, the *MIM169defg* root diameter was smaller than in WT (Fig. S4). A reduction in the number of endodermal cells was observed ($n = 10$) (Fig. 2a). Moreover, as shown in Fig. 2(b), significant differences in the thickness of nearly all cell types were detected in 7-DAG roots ($n = 15$). Interestingly, only the width of the two cells which arise from the division of a same initial cell was significantly affected ($n = 15$, Fig. 2c). Therefore, expression of *MIM169defg* constructs (but not *MIM169abc*) altered specific cell type numbers and dimensions, root meristem size and, consequently, primary root growth.

NF-YA2 and NF-YA10 levels are strongly altered in *MIM169defg* lines

We analysed the levels of NF-YA transcripts in *MIM* lines. All NF-YA transcripts except NF-YA4 and NF-YA7 contained putative miR169 binding sites in their 3'UTR, even NF-YA6 which was not previously reported as a putative miR169 target (Fig. S1c). In contrast to *NF-YA4* and *NF-YA7*, all miR169 *NF-YA* targets were faintly expressed in the tissues tested (Fig. S5a), suggesting that miR169-mediated regulation acts in the global regulation of this gene family. The detected *NF-YA2*, *NF-YA10* and *miR169* expression patterns were somehow distinct from those of Zhao *et al.* (2011) for roots and shoots, likely due to changes in growth conditions or seedling ages.

Siefers *et al.* (2009) reported that *NF-YA* genes can be separated into three different clades group 1 (*NF-YA3*, *NF-YA5*, *NF-YA8* and *NF-YA6*), group 2 (*NF-YA1* and *NF-YA9*) and group 3 (*NF-YA2* and *NF-YA10*). Bioinformatics analyses (http://mpss.udel.edu/at_sRNA/), indicated that NF-YA transcripts might potentially be recognized by different miR169 isoforms (Fig. S1b). Indeed, group 1 is targeted by three isoforms (miR169a-bc-hijklmn), group 2 by all four isoforms (miR169a-bc-defg-hijklmn)

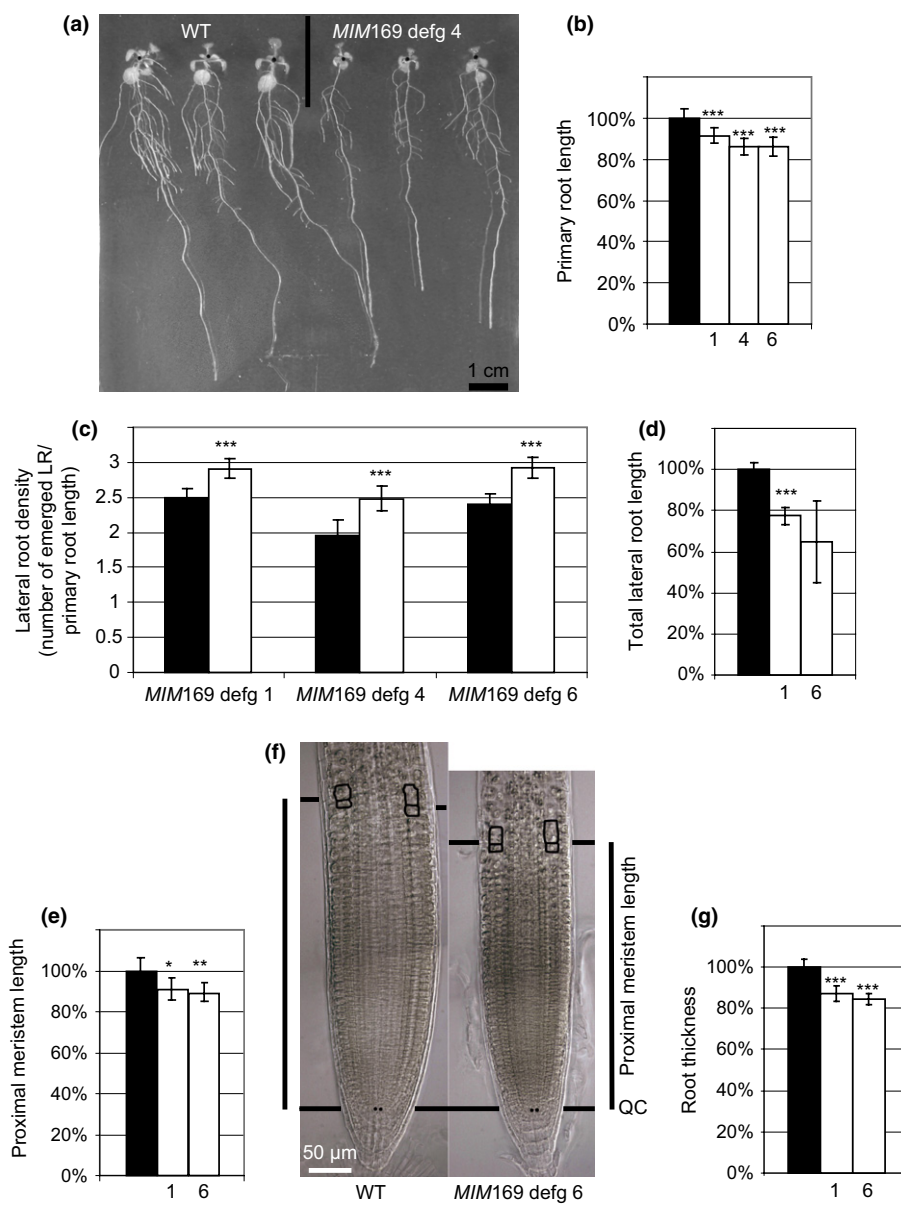


Fig. 1 The sequestration of miR169defg isoform affects root architecture and meristem size. (a) Root architecture of 8 d-after-germination (DAG) Arabidopsis seedling of wild-type (WT) and *MIM169defg*. Bar, 1 cm. In these different panels WT (closed bars) seedlings were compared with independent transgenic lines of *MIM169defg* (open bars). Mean $\pm$ SD, $20 < n < 30$, significant difference with WT are indicated by: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; Student's *t*-test. Percentages are given with 100% for the WT line. (b) Relative primary root length of 8-DAG seedlings. (c) Lateral root density of 8-DAG seedlings. (d) Relative total length of lateral roots of 8-DAG seedlings. (e) Relative length of root proximal meristem of 5-DAG seedlings. The length is determined from the quiescent centre (QC) to the transition zone (TZ) (Perilli & Sabatini, 2010). These distances are indicated by closed bars in (f). (f) Microscopy image of root apical meristem of 5-DAG seedlings. First elongated cortex cells are framed by using black lines. Bar, 50 μ m. (g) Relative root width.

and group 3 by three isoforms (miR169bc-defg-hijklmn) (Fig. S1b). We thus explored the degree of specificity of the *MIM* constructs on the action of miR169 isoforms. Expression of the 10 *NF-YA* genes in 5-d-old roots of *MIM169abc* and *MIM169defg* seedlings showed an over-expression of all the miR169 targeted transcripts in both types of *MIM* roots when compared with WT. However, major differences between the *MIM* lines were detected for *NF-YA2* and *NF-YA10* which are induced more than 20-fold in *MIM169defg* plants (Fig. 3a).

By using stem-loop RT-qPCR with primers similar to those used by Liang *et al.* (2012), we were able to observe a significant and homogeneous decrease of all miR169 isoforms in roots of *MIM169defg* (Fig. S6b). Although a similar variation was observed for *MIM169abc* roots; we always detected higher amounts of the miR169defg isoform in these roots (Fig. S6a), in agreement with Todesco *et al.* (2010). The differences observed for miR169defg between *MIM* lines and the highest

over-accumulation of *NF-YA2* and *NF-YA10* transcripts only in *MIM169defg* lines, suggest that the miR169defg isoform is responsible for this regulation. The over-expression of corresponding proteins (eventually together with other specific *NF-YA* proteins) could explain the root apex phenotype which is not observed in *MIM169abc* plants although we cannot exclude an effect of other miR169 targets. Indeed, analysis of transcript degradation products in degradome analyses of Arabidopsis inflorescences (German *et al.*, 2008; Meng *et al.*, 2012) suggest the potential existence of non-*NF-YA* targets for miR169. Finally, as our results were identical for the four isoforms in *MIM169defg* lines, an alternative possibility is that, in our RT-qPCR conditions, the primers used were not able to discriminate strictly between the miR169 isoforms, which differ only by two nucleotides at the 3' or 5' end of mature miRs. Hence, the *NF-YA2* and *NF-YA10* transcription factors are specifically overexpressed in *MIM169defg* lines.

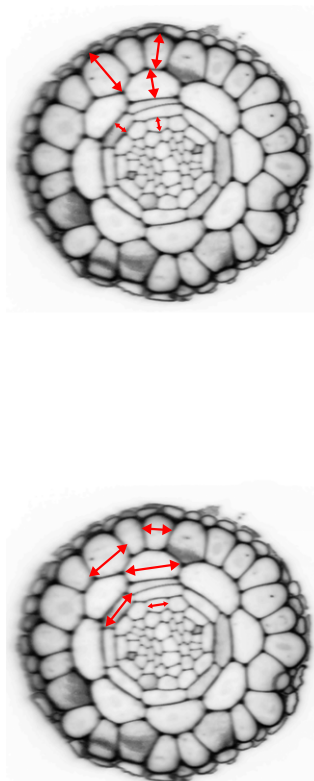
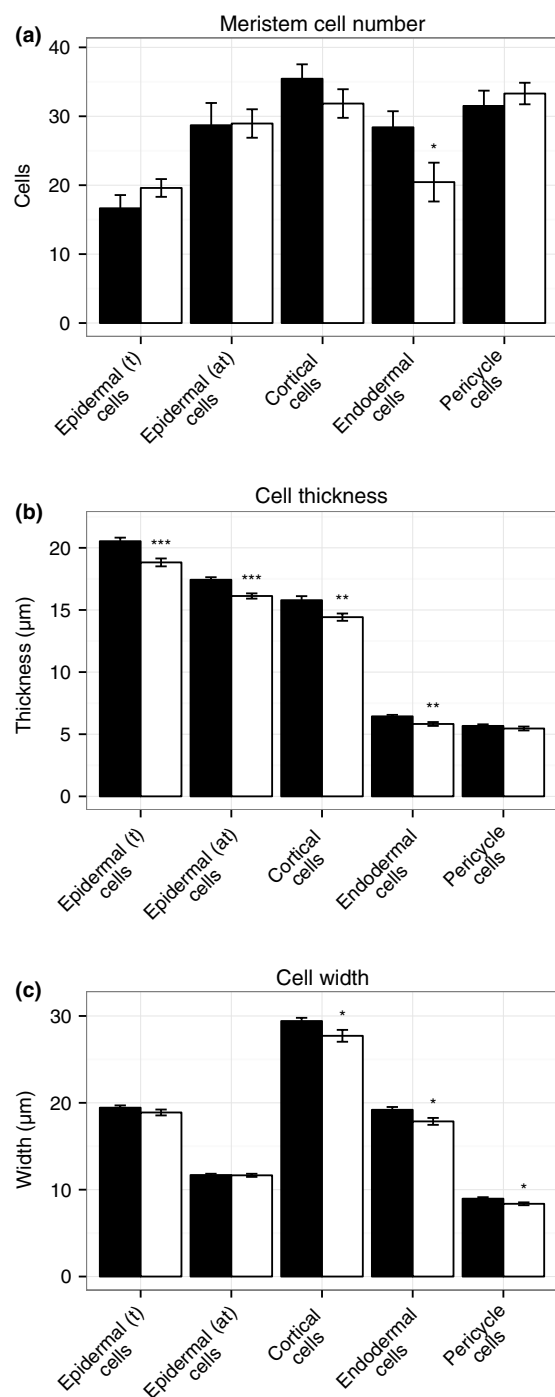


Fig. 2 Cellular modifications observed in 7-DAG *MIM169defg* (open bars) primary root files compared with wild-type (WT; closed bars). (a) Average number of cells in each indicated cell file of the Arabidopsis primary root were determined on serial sections of *MIM169defg* and WT. Two opposite cell files were measured on 10 independent roots per genotype. (b) Average thickness of indicated cells across serial sections of *MIM169defg* and WT roots at 7-DAG at the end of the meristem. All the cells on the cross-section were measured on 15 independent roots per genotype. (c) Average width of indicated cells across serial sections of *MIM169defg* and WT roots at 7-DAG at the end of the meristem. All the cells on the cross-section were measured on 15 independent roots per genotype. Red arrows indicate an example for each cell type of the measured cell thickness in (b) and the measured cell width in (c). Values are means and the average per root was first computed. Error bars, $\pm$ SEM. Significant difference between the two genotype conditions by a Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Different NF-YA genes are primarily regulated by specific miR169 isoforms

In order to test whether particular miR169 isoforms could target specific NF-YA transcripts, we performed stable transformations of Arabidopsis plants. For that, we overexpressed two miRNA precursors that produce two different isoforms, miR169a or miR169d (Fig. S7a,b). In 5-d-old roots of three independent *35S:miR169a* OE plants, qRT-PCR experiments detected a slight reduction of transcript abundance for all miR169 targeted

NF-YA transcripts but NF-YA2, (Fig. 3b), which was not predicted as a target of this isoform. By contrast, for *NF-YA10*, another gene not predicted as miR169a target, we detected the lowest transcript accumulation (Fig. 3b). Hence, as previously proposed by Zhao *et al.* (2011) NF-YA10 transcripts are also cleaved by miR169a isoform. By the same type of experiment, reductions of NF-YA2 and NF-YA10 transcript abundances were detected in roots of the miR169d OE lines, whereas slight differences were observed for the other NF-YA transcripts (Fig. 3c). However, no root growth or lateral root phenotype has been

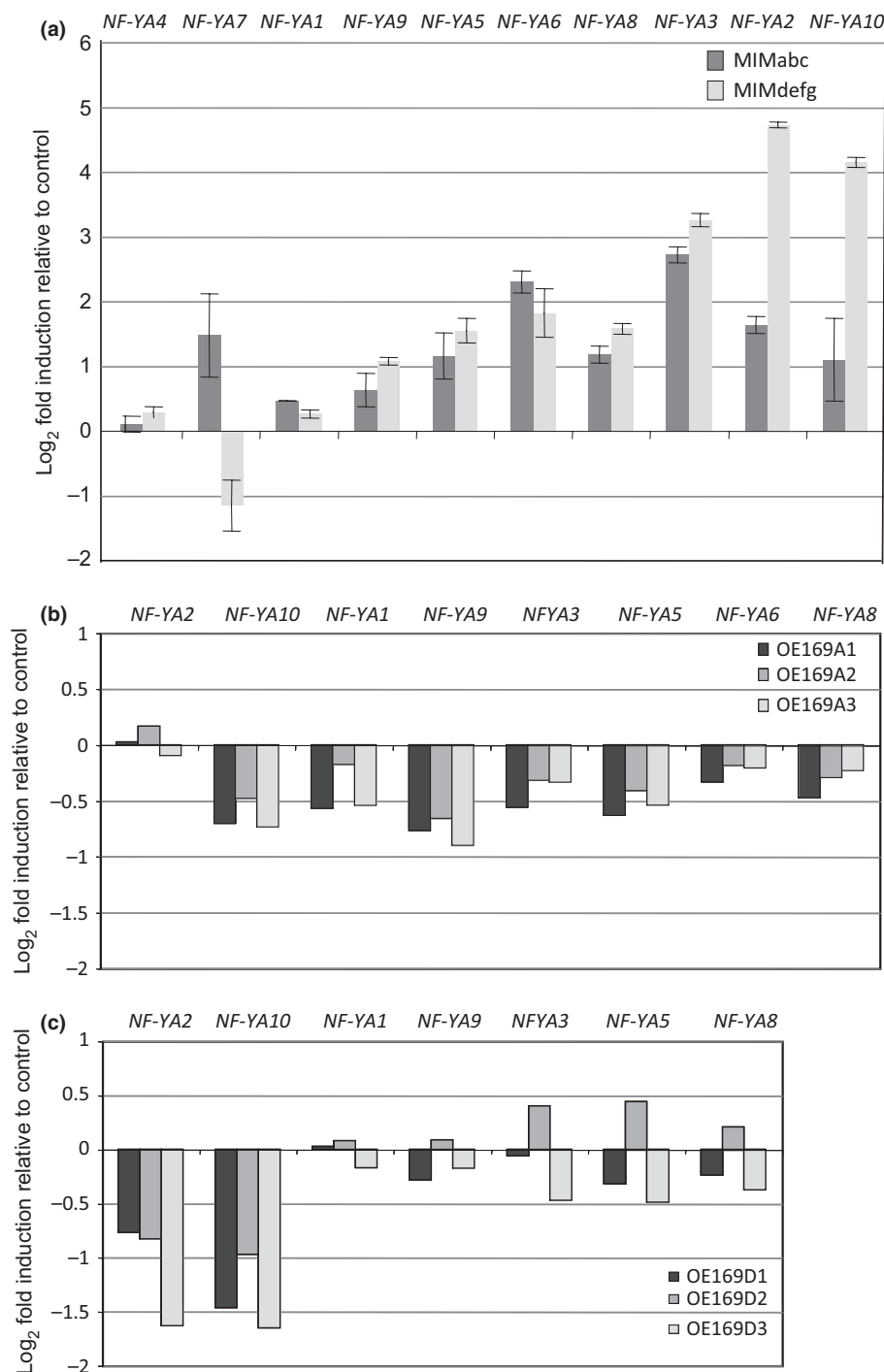


Fig. 3 *MIM169abc*, *MIM169defg*, *35SmiR169a* and *35SmiR169d* affect *NF-YA* transcripts accumulation in roots. (a) RT-qPCR analysis of the accumulation of the 10 *NF-YA* transcripts in 5-DAG roots of *MIM169abc* and *MIM169defg* Arabidopsis lines. Data are presented as log₂ of fold induction relative to control where induction is the ratio of transcript levels in *MIM169* roots to those in wild-type (WT) roots. Error bars, $\pm$ SE of four repeats. (b) RT-qPCR analysis of the accumulation of transcripts of various *NF-YA* genes in 5-DAG roots of three independent *miR169a* over expressing (OE) lines (b) or *miR169d* OE lines (c). Data are presented as log₂ of fold induction relative to control, where induction is the ratio of transcript levels in *MIM169* roots to those in WT roots.

observed for these *miR169* OE lines (Fig. S8). Thus, a 50% reduction in *NF-YA2* and *NF-YA10* transcript accumulation seems insufficient to affect root growth. In this respect, in *MIM169defg* an increase of at least $\times 4$ or 5 (log₂ fold changes) of transcript accumulation has been detected for these two genes. Moreover, we cannot exclude the idea that the amounts of other *NF-YA* proteins (and/or other potential targets) could be involved in the phenotype. Taken together, opposite regulation of *NF-YA2* and *NF-YA10* in *MIM169defg* and *miR169d* OE lines confirmed the specificity of targeting of these two transcripts

by this isoform. In roots or seedlings of *miR169* OE lines, a partial reduction of *NF-YA* transcripts was observed (our work and Li *et al.*, 2008; Zhao *et al.*, 2011). This suggests that mRNA cleavage is only part of *miR169* action on its targets.

miR169 isoforms could affect the translation of *NF-YA2* gene

As *miR169*-recognition sites are in the 3'UTR of *NF-YA* genes, this miRNA may act at two levels: RNA stability and translation

inhibition. To analyse the mode of action of miR169, we used transient *N. benthamiana* transformations with different *p35S::GFP-NF-YA* constructs. For that, we expressed, under the control of the *35S* promoter, the *GFP* reporter gene fused to the 3'UTR of *NF-YA1* (*p35S::GFP-NF-YA1wt*) or *NF-YA2* (*p35S::GFP-NF-YA2wt*) genes. In addition, we constructed a miR-resistant version (*p35S::GFP-NF-YA1mut*) and (*p35S::GFP-NF-YA2mut*) in which the miR169 binding site was deleted from the *NF-YA1* or *NF-YA2* 3'UTR (Fig. S9). These constructs were used for co-infiltrations with strains that overexpress miR169a, miR169bc, miR169defg isoforms or miR390b (as a negative control) and *p35S::mcherry* (as a transfection control). GFP protein and mRNA concentrations were measured 72 h post-infiltration and normalized by quantification of mCherry fluorescence or mRNA concentrations (see the Materials and Methods). For all

these samples, the quantification of miRNA accumulation was done by northern blots (Fig. S10). For each miRNA tested against *NF-YA1* or *NF-YA2* constructs, we analysed different independent experiments for mRNA or protein (fluorescence) ratio (*p35S::GFP-NF-YA1mut/p35S::GFP-NF-YA1wt*) and (*p35S::GFP-NF-YA2mut/p35S::GFP-NF-YA2wt*). This data presentation is well adapted when results show large dispersion in absolute values among experiments. Figure 4(a) left and right corresponds to the ratio of *NF-YA1* mRNA and protein, respectively. In this system, as miR390b cannot target *NF-YA1* 3'UTR, co-infiltrations with miR390b had no effect on the ratio of GFP (mut)/GFP (wt) mRNA (Fig. 4a left) or fluorescence (Fig. 4a right). Our results for miR169a and miR169d were very similar to this control; thus, we concluded that these two miRNAs have no effect on *NF-YA1* transcripts or protein concentrations, whereas,

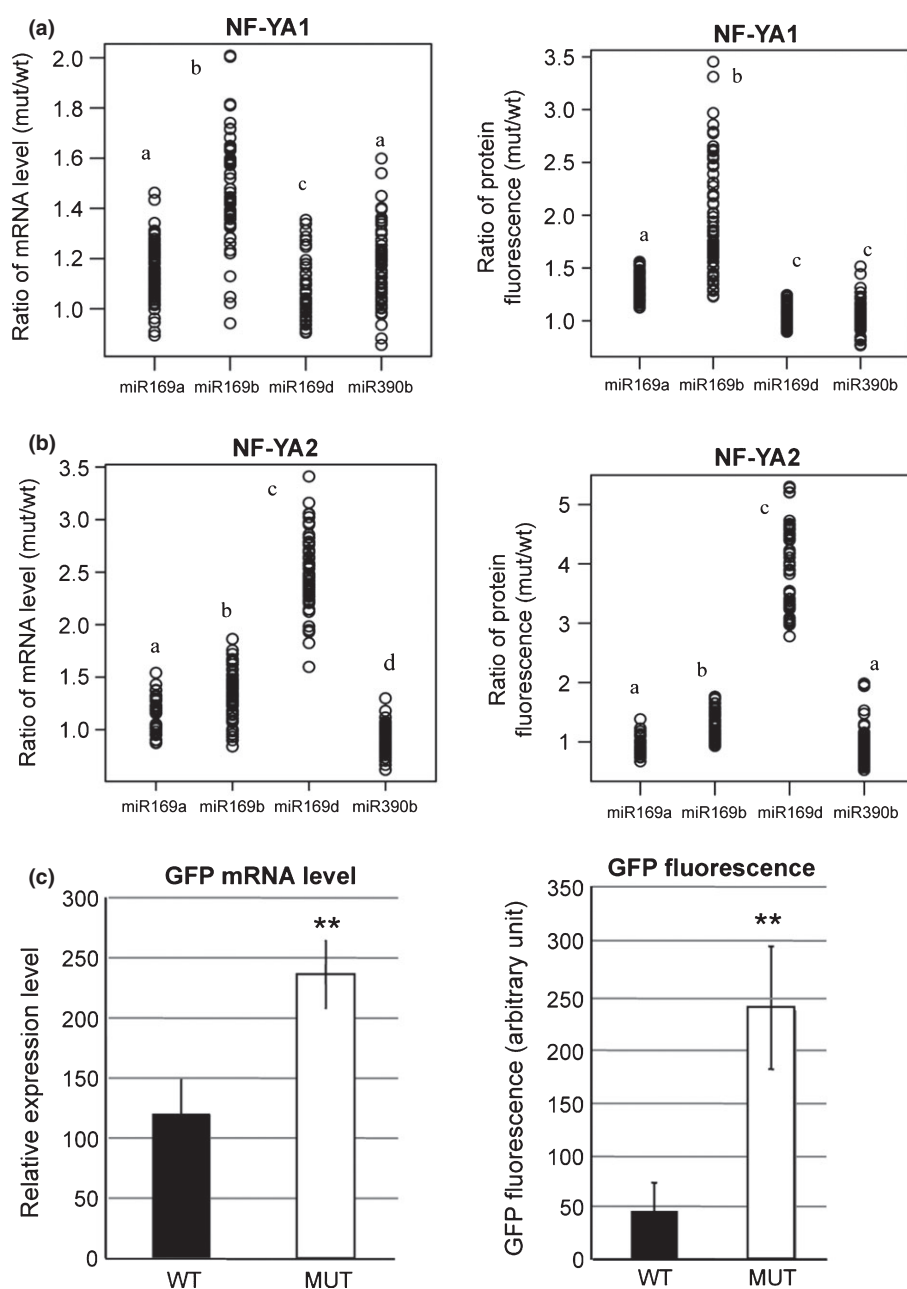


Fig. 4 Some miR169 isoforms act at both RNA stability and inhibition of translation levels on *NF-YA* regulation. Comparison of the expression of the GFP fused to 3'-UTR miR-sensitive (*p35S::GFP-NF-YAwt*) and miR-resistant (*p35S::GFP-NF-YAmut*) form of *NF-YA1* (a) and *NF-YA2* (b) genes (at the mRNA and protein levels) co-infiltrated with different miR169 isoforms. *p35S::mcherry* was co-infiltrated as control of transformation and expression in *Nicotiana benthamiana* leaves. miR390b was used as a negative control for both target genes. Two biological experiments with 5–7 agroinfiltrated plants for each constructs were used. A Kruskal–Wallis one-way ANOVA test and a post-hoc Tukey's test were performed and statistically identical values are attributed identical letters. (c) RT-qPCR analysis of GFP mRNAs in roots of three independent *p35S::GFP-NF-YA2wt* (WT; closed bars) and *p35S::GFP-NF-YA2mut* (Mut; open bars) transgenic Arabidopsis lines. Error bars, $\pm$ SD of two repeats of three independent lines. GFP mRNA levels and GFP fluorescence quantifications were performed twice in roots of three independent WT and Mut lines. Data are presented as expression ratios relative to the At1g13320 and At4g26410 reference genes ($2^{-\Delta CT}$). Statistically significant differences between *p35S::GFP-NF-YA2wt* and *p35S::GFP-NF-YA2mut* were determined by the Mann and Whitney test: **, $P < 0.01$.

for miR169b, we observed a slight variation. Figure 4(b) left and right correspond to the ratio of NF-YA2 mRNA and protein, respectively. In this system, miR390b cannot target *NF-YA2* 3'UTR, thus co-infiltrations with miR390b had no effect on the ratio of GFP (mut)/GFP (wt) mRNA (Fig. 4b left) or fluorescence (Fig. 4b right). Again miR169a and miR169b gave similar results as the control, suggesting no effect on *NF-YA2* expression. Interestingly, we observed robust differences for miR169d at both the NF-YA2 transcript and protein levels (Fig. 4b left and right). Therefore, the miR169defg isoform likely targets NF-YA2 transcripts both at the mRNA stability and translation levels. This double modality of action was also observed in roots of seedlings that stably overexpressed *p35S:GFP-NF-YA2wt* or *p35S:GFP-NF-YA2mut* (Fig. 4c). Indeed, in these transgenic plants, the impact of miR169 regulation on NF-YA2 was 2.5× and 5× at the transcript and protein levels, respectively. Taken together, these results show that the miR169defg isoform acts on *NF-YA2* gene expression at both the transcript stability and translational levels in Arabidopsis roots as well as in *N. benthamiana* leaves.

NF-YA2 and NF-YA10 are expressed in vascular tissues

In order to explore the expression patterns of *NF-YA2* and *NF-YA10*, the two miR169defg targets, we transformed WT plants with promoter:GUS-GFP constructs that contained the 2-kb genomic sequence upstream from their initiation codons (Fig. 5). The expression patterns in shoots were identical to those of Siefers *et al.* (2009), with a high level of expression in the cotyledon vasculature and hypocotyl for *pNF-YA2:GUS* and a faint expression at the apical zone for *pNF-YA10:GUS* (Fig. 5a). In roots, high staining of *pNF-YA2:GUS* was visible in vascular tissues (Fig. 5a,b,d) whereas a weaker signal was observed for *pNF-YA10:GUS* (Fig. 5a,c,e). For these two genes, expression in vascular tissues started at the EZ (Fig. 5b–e). In 5-d-old seedlings, a strong *pNF-YA2:GUS* signal was transiently detected in the columella (Fig. 5a,b). Moreover, in 5-DAG roots, a clear expression of *NF-YA10* was discerned in xylem cells at the transition zone (Fig. 5c), whereas the expression of *NF-YA2* was muted in pericycle and very faint in endodermal cells (Fig. 5b). Transversal sections and longitudinal views revealed low staining in pericycle and vasculature (Fig. 5d,e). These patterns are in agreement with those of Siefers *et al.* (2009). GUS staining was also observed in vascular tissues of emerged LR (Fig. 5f,g) and at the LR tip for *pNF-YA2:GUS*.

Deregulation of NF-YA2 increases LR initiation

Our attempts to identify knockdown plants of *NF-YA2* and *NF-YA10* in SALK lines and/or to prepare RNAi lines using a constitutive promoter to downregulate either one or the two genes were unsuccessful. Pagnussat *et al.* (2005) previously showed that a T-DNA insertion in the *NF-YA2* gene resulted in defect of fertility (mutant line *une8*) and our lines overexpressing *NF-YA2* under the 35S constitutive promoter showed strong sterility and retarded growth (Fig. S11). As shown above, *35S:mir169a* and

35S:mir169d did not induce very strong reductions of *NF-YA2* transcripts in roots and hence cannot be considered as mutant KO lines (Fig. 3b,c).

In order to further characterize the function of *NF-YA2*, we generated transgenic plants which expressed *NF-YA2* gene under its own promoter. Again two constructs, *pNF-YA2:HA-NF-YA2wt* having the intact 3'UTR region of the gene or *pNF-YA2:HA-NF-YA2mut* (with miR site deletion in the 3'UTR) were used. Expression of the intact *NF-YA2* gene, sensitive to miR169 repression, had no effect on root architecture (Fig. S12). For *pNF-YA2:HA-NF-YA2mut* lines, the size of all aerial organs of adult plants (3 wk old) was always smaller in the glasshouse. By contrast, *in vitro*, during the first 10 d of culture, we never saw a difference between the rosette leaves size of *pNF-YA2:HA-NF-YA2mut* lines and WT. Nine-day-old plants grown *in vitro* produced more LR than the WT (Fig. 6a,c) without affecting the PR size (Fig. 6a,b). Hence, the emerged lateral root density was higher in these plants (Fig. 6c). Statistic analysis of LR densities obtained for three independent lines strengthened the fact that the *NF-YA2* gene plays an important role in LR development. We further analysed the effects of *NF-YA2* OE on LR formation by counting the numbers of LR primordia from stages I to VII as described by Dubrovsky & Forde (2012). Increase in stages I to IV primordia could suggest a role of *NF-YA2* on LR initiation (Fig. 6d). LR initiation requires auxin responses, mediated by several AUX/IAA modules (Goh *et al.*, 2012). In order to discriminate between direct or indirect action on NF-Y complex in LR development, we used the lateral root induction system described by Himanen *et al.* (2002). Under these conditions, we were not able to observe any differences in the transcript levels of genes involved in LR initiation (*IAA2*, *IAA11*, *GATA23*, *E2FA* and *SHY2/IAA3* genes) in *pNF-YA2:HA-NF-YA2mut* lines when compared with WT (Fig. S13). These latter results clearly suggest that NF-YA2 and/or the NF-Y complex act indirectly on LR density and root architecture.

The regulatory miR169defg/NF-YA2 node acts at least in pericycle cells

In order to explore *miR169defg* expression patterns, we transformed WT plants with constructs that contained the 2-kb genomic sequences upstream of the miR169d or miR169e stem-loop structures fused to GUS-GFP reporter genes. Indeed, in our culture conditions, we were never able to detect miR169 f and g precursors. Faint histological GUS staining was detected for *miR169d* and *miR169e* promoters in several independent transgenic lines (Fig. 7a). In our conditions, we never observed a strong signal in the stele for miR169e, as previously described by Li *et al.* (2010). Root transversal sections showed GUS staining at phloem poles for *miR169d* and *miR169e* promoters (with an additional expression in pericycle cells for miR169e), whereas *NF-YA2* and *NF-YA10* were expressed in the whole pericycle (Fig. 7b) and faintly in the vasculature. These results are in agreement with data derived from the analysis of small RNAs libraries which detected miR169 in phloem cells at least in Arabidopsis and *Brassica napus* (Buhtz *et al.*, 2010).

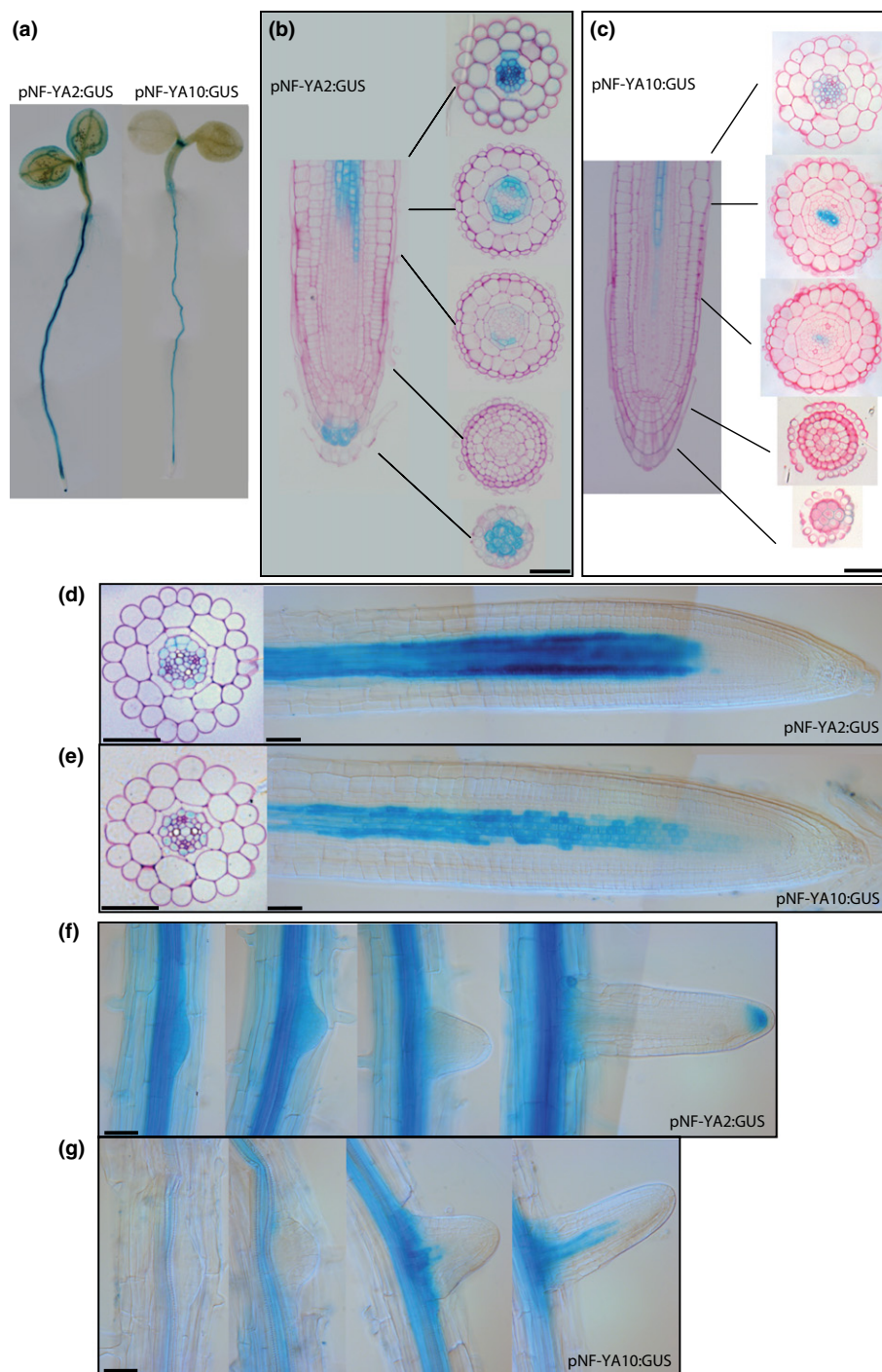


Fig. 5 *NF-YA2* and *NF-YA10* are expressed root vasculature. (a–c) GUS localization in Arabidopsis seedlings 24 h after germination (HAG). (b) Root longitudinal and transversal sections localized *NF-YA2* expression in vascular tissues and pericycle cells in the elongation zone and in columella. (c) *NF-YA10* is expressed in the same tissues except for the columella. (d–g) GUS expression in 5-DAG roots. *NF-YA2* (d) and *NF-YA10* (e) are expressed from the transition zone throughout the stele. Sections in upper part of the root indicate an expression in pericycle cells. (f, g) GUS expression during lateral root (LR) development and emergence for *NF-YA2* and *NF-YA10*, respectively. Bars, 50 μ m.

In order to visualize root cells where miR169 can post-transcriptionally regulate *NF-YA2*, we analysed crosses between *MIM169defg* or WT and *pNF-YA2:GFP-NF-YA2wt* plants. In WT background, we were not able to detect any GFP signal (Fig. 8a). By contrast, in *MIM169defg* background (Fig. 8b) where miR169 were sequestered, or in *pNF-YA2:GFP-NF-YA2mut* lines, where *NF-YA2* was overexpressed (Fig. 8c), a clear GFP signal was observed in specific cell files, likely pericycle cells although the faint fluorescence did not allowed us to do a 3D deconvolution. A faint expression of *NF-YA2* gene has also been observed in the stele that may be linked to *miR169d* and

miR169e expression in phloem. Notwithstanding this, it has been shown – at least for miR165/166 – that miRNAs can circulate through plasmodesma from pericycle to stele (Carlsbecker *et al.*, 2010) in an opposite manner to certain TF or target transcripts. One alternative could also be that miR169 moves from the phloem to pericycle cells to inhibit its targets.

Discussion

Modulation of root architecture, by PR root growth and LR development, allows plants to cope with abiotic stresses such as

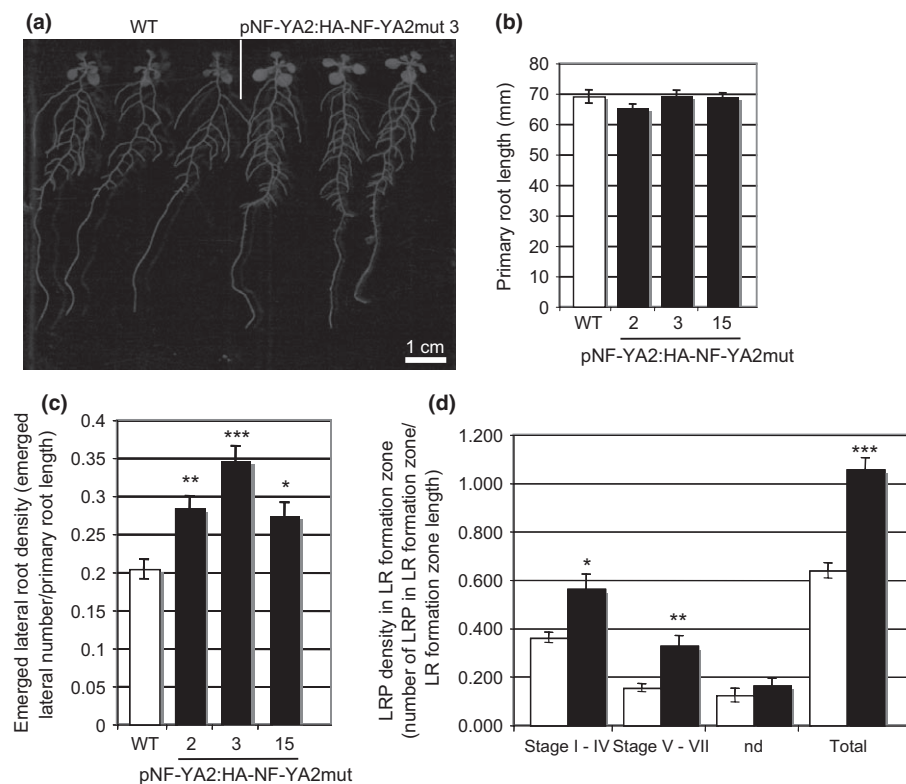


Fig. 6 *NF-YA2* overexpression affects lateral root development. (a) Overview showing 9-d-old Arabidopsis seedlings from wild-type (WT) and three plants that over-express *NF-YA2mut* (insensitive to miR169 regulation) under its own promoter (*pNF-YA2:HA-NF-YA2mut*). (b–d) Phenotypic characterization of WT, and *NF-YA2* over-expressing plants (WT, open bars; *pNF-YA2:HA-NF-YA2mut*, closed bars). (b) Primary root length ($n = 13–18$). (c) Emerged lateral root (LR) density ($n = 13–18$). (d) Distribution of lateral root stages of 5-d-old seedlings. The number of LRP at each developmental stage was counted in 9–10 roots per line. nd, not determined. Statistically significant differences determined using Student's *t*-test: ***, $P < 0.0001$; **, $P < 0.001$; *, $P < 0.01$. Error bars, $\pm$ SE.

drought or nutrient availability. Both miRNAs and transcription factors are known to play crucial roles in plant development processes and in response to environmental conditions but few have been linked to root growth and architecture (Khan *et al.*, 2011). Here, by using a mimicry approach and deregulation of the miR169 targets *NF-YA2* and *NF-YA10*, we have shown that genes regulated by the miR169defg isoform were involved in primary root growth and/or LR initiation. In Arabidopsis, miR169 has previously been involved in drought and nitrate responses. Indeed, overexpression of the miR169a isoform, which principally targets *NF-YA5*, induced drought sensitivity or altered nitrogen responses (Li *et al.*, 2008; Zhao *et al.*, 2011) and miR169defg isoform is induced by nitrate starvation in Arabidopsis roots (Liang *et al.*, 2012). Moreover, in roots, a clear upregulation of *NF-YA2* and *NF-YA10* has been observed in response to phosphate starvation (Woo *et al.*, 2012). Recently, functional and transcriptional analysis of Arabidopsis *NF-YA* gene family also revealed a clear impact of NF-Y complexes on carbohydrate metabolism and cell wall modifying enzymes (Leyva-González *et al.*, 2012). These results, all together, are consistent with the fact that miR169 target genes, the *NF-YA* family, could act directly or indirectly in developmental responses to environmental signals such as the modification of root architecture induced by abiotic stresses. Indeed, all of these stresses can affect, at different levels, auxin and sucrose homeostasis and transport (Kazan, 2013; Lemoine *et al.*, 2013) two well-known operators of growth and development in plants. The impact of the miR169/NF-Y complex on root architecture identified here and more particularly the regulation of LR density could be linked to the

regulation observed in *M. truncatula* – nodule development by miR169 (Combiér *et al.*, 2006). The formation of nodules is actually a reprogramming of root cells into a new organ to allow legume plants to cope with nitrogen starvation.

In Arabidopsis, promoter:GUS reporter lines (Li *et al.*, 2010) have previously shown that many *miR169* genes are mostly expressed in vascular tissues, and some genes have presented distinct patterns in these tissues. The maximum level of GUS activity in roots has been observed for *miR169e*. However, in roots or seedlings all miR169 precursors have been detected in similar amounts (Li *et al.*, 2008; Zhao *et al.*, 2011). Using cell-sorting technology on cell types of three root regions followed by deep sequencing, Breakfield *et al.* (2012) have observed that the *miR169* gene family was moderately expressed and that miR169 isoforms presented the lowest variance in quantity across root tissues. We have limited our investigation to the *miR169d* and *miR169e* genes, which produce the miR169defg isoform and whose expression was detected in phloem pole cells in transversal root sections. This is consistent with both Li *et al.* (2010) and the existence of an abundant quantity of mature miR169 in small libraries from phloem cells (Buhtz *et al.*, 2010). Taken together, these data suggest that *miR169* genes could be principally expressed in the stele. The homogenous accumulation of miR169 in different cell files in the root reported by Breakfield *et al.* (2012) may thus be explained by different mechanisms such as changes in miR169 stability in certain cell types or even movement between cell files. Alternatively, the 2-kb promoter length used in this study may not accurately reflect the expression of *miR169d* and *miR169e* genes.

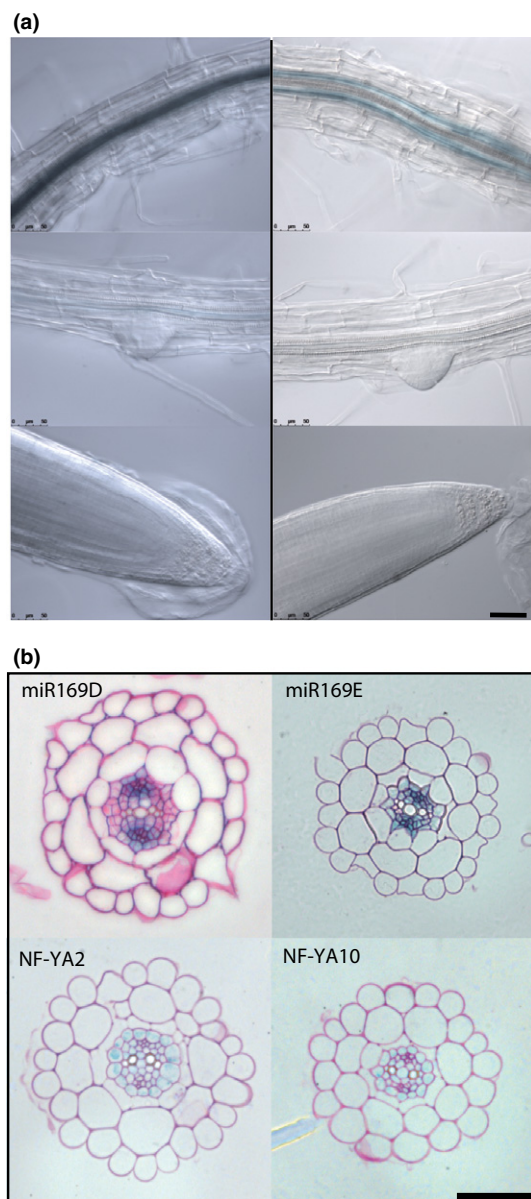


Fig. 7 *MiR169d* and *miR169e* genes are expressed in cells at the root phloem poles. (a, b) *pmir169d:GUS* and *pmir169e:GUS* staining in 5-DAG Arabidopsis roots. (a) The two miR are expressed in root vasculature (left panel: *miR169d*; right panel: *miR169e*). (b) In root transversal sections, very faint signals are observed at the phloem poles and in pericycle cells. Bars, 50 μ m.

The *miR169defg* genes and their related *NF-YA2* and *NF-YA10* targets are both expressed in root stele and vascular tissues. In *MIM169defg* lines, high over-expression of the two latter genes (eventually together with minor changes in other *NF-YA* genes) was responsible for a reduction of root length and RAM size which correlated with a decrease in cell numbers in the RAM. Thus, sequestration of the *miR169defg* isoform could reduce cell division or increase cell recruitment at the meristem transition zone. However, *NF-YA2* and *NF-YA10* expression patterns, over the transition zone, suggested that these genes do not act directly on cell cycle. Transcriptomic analyses of PXVE:*NF-YA* lines

(inducible OE lines) by Leyva-González *et al.* (2012) showed that many strongly downregulated genes encoded enzymes involved in cell wall modifications. Cell wall composition changes could affect cell shape or size and may disturb the function or transport of vascular tissues or affect apoplastic pathways of nutrients, and consequently impact the primary root meristem. This could partly explain the phenotype observed for *MIM169defg* lines at the cellular level.

Our promoter-GUS studies showed *NF-YA2* and *NF-YA10* expression in pericycle cells, whereas *miR169d* and *miR169e* are expressed in phloem poles. As LR arise from pericycle cells close to the xylem poles, we can speculate first that *miR169* could migrate from phloem to specific pericycle cells in front of these poles to strongly inhibit *NF-YA2* expression or secondly that, in these specific cells, only *miR169f* and *g* genes are expressed.

When the post-transcriptional regulation of *NF-YA2* transcripts by *miR169*, was disrupted (*pNF-YA2:HA-NF-YA2mut* lines) the size of all aerial organs was smaller than that of soil-grown WT plants. In contrast to this aerial phenotype observed in older plants, in 9-DAG plants, the number of LR was increased when compared with WT yet there was no impact on the rosette leaves size. As previously mentioned, sugars play an important role in meristem activity and can leave phloem through either an apoplastic or a simplastic pathway (Patrick, 1997). One possibility is that *NF-YA2* overexpression in *pNF-YA2:HA-NF-YA2mut* lines specifically affects transport into sink tissues. Indeed, an increase in sucrose content has been detected in *p35S:NF-YA2* plants by Leyva-González *et al.* (2012). Alternatively, OE of *NF-YA2* alone can induce a stress response known to promote root growth. Curiously, *IAA12*, one key gene of the LR initiation module, has been detected as a direct target of *NF-YA2* by Leyva-González *et al.* (2012). By using the NPA/NAA LR induction system, we showed that the increase of LR in *pNF-YA2:HA-NF-YA2mut* lines did not have a direct effect on LRI but instead an indirect effect likely mediated through the vascular tissues. This indirect effect observed here could be linked to sucrose action as signal molecule or nutrient, as it can increase the number of LR (Roycewicz & Malamy, 2012). Alternatively, an indirect action on auxin homeostasis or transport could be involved as ten *NF-YA2* potential direct targets are involved in the auxin pathway. In any way, the indirect action on root architecture could also result from differential gene expressions induced by modifications or deposition of histones by *NF-Y* complex, which encompass *NF-YA2*, as has been proposed in animals (Gatta & Mantovani, 2011).

MiR169 and their *NF-YA* targets have been proposed to control many plant responses to environmental stresses. Leyva-González *et al.* (2012) constructed a model in which the *NF-Y* complex inhibits an early abiotic stress response to allow the expression of a late response that confers resistance. By monitoring the precise action of *miR169defg* and *NF-YA2* in specific cells, we showed that this regulatory node indirectly connects the *NF-YA2/NF-YA10* TFs to root growth and architecture, a trait very sensitive to the soil environment. This node controls root growth and development and is a new element to be considered for regulating root architecture.

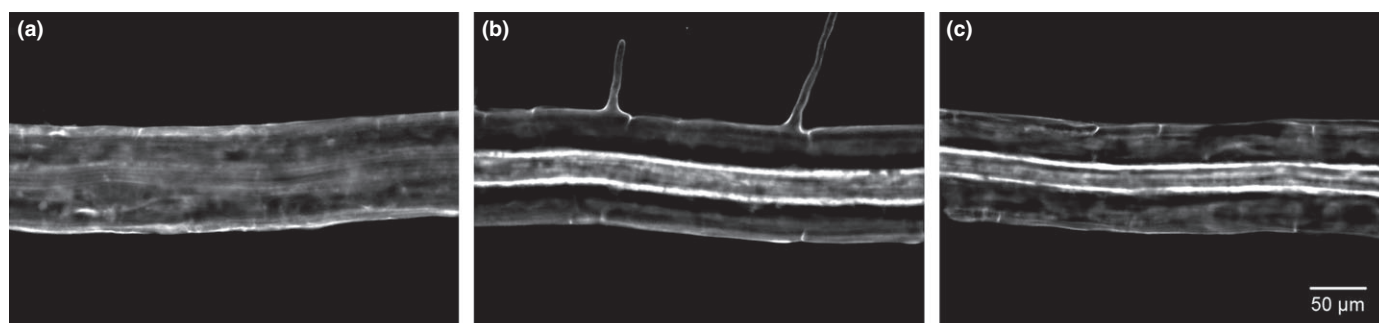


Fig. 8 NF-YA2 is regulated by miR169 in specific cell files. Confocal images of whole Arabidopsis roots of (a) *pNF-YA2:GFP-NF-YA2wt* line, (b) crosses between *MIM169defg* and *pNF-YA2:GFP-NF-YA2wt* line (c) *pNF-YA2:GFP-NF-YA2mut* line.

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References

- Axtell MJ. 2008. Evolution of microRNAs and their targets: are all microRNAs biologically relevant? *Biochimica et Biophysica Acta* 1779: 725–734.
- Bartel DP. 2009. MicroRNAs: target recognition and regulatory functions. *Cell* 136: 215–233.
- Baxeianis AD, Arents G, Moudrianakis EN, Landsman D. 1995. A variety of DNA-binding and multimeric proteins contain the histone fold motif. *Nucleic acids research* 23: 2685–2691.
- Bazin J, Khan G, Combier JP, Bustos-Sanmamed P, Debernardi JM, Rodriguez R, Sorin C, Palatnik J, Hartmann C, Crespi M *et al.* 2013. miR396 affects mycorrhization and root meristem activity in the legume *Medicago truncatula*. *Plant Journal* 74: 920–934.
- Beekman T, Engler G. 1994. An easy technique for the clearing of histochemically stained plant tissue. *Plant Molecular Biology Reporter* 12: 37–42.
- Breakfield NW, Corcoran DL, Petricka JJ, Shen J, Sae-Seaw J, Rubio-Somoza I, Weigel D, Ohler U, Benfey PN. 2012. High-resolution experimental and computational profiling of tissue-specific known and novel miRNAs in Arabidopsis. *Genome Research* 22: 163–176.
- Brodersen P, Voinnet O. 2009. Revisiting the principles of microRNA target recognition and mode of action. *Nature Reviews Molecular Cell Biology* 10: 141–148.
- Buhtz A, Pieritz J, Springer F, Kehr J. 2010. Phloem small RNAs, nutrient stress responses, and systemic mobility. *BMC Plant Biology* 10: 64.
- Cao S, Kumimoto RW, Siriwardana CL, Risinger JR, Holt BF. 2011. Identification and characterization of NF-Y transcription factor families in the monocot model plant *Brachypodium distachyon*. *PLoS ONE* 6: e21805.
- Carlsbecker A, Lee JY, Roberts CJ, Dettmer J, Lehesranta S, Zhou J, Lindgren O, Moreno-Risueno MA, Vatin A, Thitamadee S *et al.* 2010. Cell signalling by microRNA165/6 directs gene dose-dependent root cell fate. *Nature* 465: 316–321.
- Cerbelli M, Dolfini D, Merico D, Gatta R, Viganò AM, Pavesi G, Mantovani R. 2008. The histone-like NF-Y is a bifunctional transcription factor. *Molecular and Cellular Biology* 28: 2047–2058.
- Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR. 2005. Real-time quantification of microRNAs by stem-loop RT-PCR. *Nucleic acids research* 33: e179.
- Clough SJ, Bent AF. 1998. Floral dip: a simplified method for Agrobacterium mediated transformation of *Arabidopsis thaliana*. *Plant Journal* 16: 735–743.
- Combier JP, Frugier F, de Billy F, Boualem A, El-Yahyaoui F, Moreau S, Vernie T, Ott T, Gamas P, Crespi M *et al.* 2006. MtHAP2-1 is a key transcriptional regulator of symbiotic nodule development regulated by microRNA169 in *Medicago truncatula*. *Genes & development* 20: 3084–3088.
- Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR. 2005. Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. *Plant physiology* 139: 5.
- De Smet I, Chaerle P, Vanneste S, De Rycke R, Inzé D, Beeckman T. 2004. An easy and versatile embedding method for transverse sections. *Journal of microscopy* 213: 76–80.
- De Smet I, Tetsumura T, De Rybel B, Frey NF, Laplace L, Casimiro I, Swarup R, Naudts M, Vanneste S, Audenaert D *et al.* 2007. Auxin-dependent regulation of lateral root positioning in the basal meristem of Arabidopsis. *Development* 134: 681–690.
- Des Marais DL, McKay JK, Richards JH, Sen S, Wayne T, Juenger TE. 2012. Physiological genomics of response to soil drying in diverse *Arabidopsis* accessions. *The Plant Cell* 24: 893–914.
- Dolfini D, Gatta R, Mantovani R. 2012. NF-Y and the transcriptional activation of CCAAT promoters. *Critical Reviews in Biochemistry and Molecular Biology* 47: 29–49.
- Donati G, Gatta R, Dolfini D, Fossati A, Cerbelli M, Mantovani R. 2008. An NF-Y-dependent switch of positive and negative histone methyl marks on CCAAT promoters. *PLoS ONE* 3: e2066.
- Dubrovsky JG, Doerner PW, Colón-Carmona A, Rost TL. 2000. Pericycle cell proliferation and lateral root initiation in Arabidopsis. *Plant physiology* 124: 1648–1657.
- Dubrovsky JG, Forde BG. 2012. Quantitative analysis of lateral root development: pitfalls and how to avoid them. *The Plant Cell* 24: 4–14.
- Dubrovsky JG, Rost TL, Colón-Carmona A, Doerner P. 2001. Early primordium morphogenesis during lateral root initiation in *Arabidopsis thaliana*. *Planta* 214: 30–36.
- Edwards D, Murray JAH, Smith AG. 1998. Multiple genes encoding the conserved CCAAT-box transcription factor complex are expressed in Arabidopsis. *Plant physiology* 117: 1015–1022.
- Elliott AR, Schen PM, Grof CPL. 2006. *Quantitative analysis of GFP in plant extracts using The VersaFluor™ Fluorometer System*. BIO-RAD tech note 2434. Brisbane, Australia: CSIRO Tropical Agriculture.
- Fukaki H, Tameda S, Masuda H, Tasaka M. 2002. Lateral root formation is blocked by a gain of function mutation in the *SOLITARY ROOT/IAA14* gene of Arabidopsis. *Plant Journal* 29: 153–168.

- Gatta R, Mantovani R. 2011. NF-Y affects histone acetylation and H2A. Z deposition in cell cycle promoters. *Epigenetics: Official Journal of the DNA Methylation Society* 6: 526.
- German MA, Pillay M, Jeong DH, Hetawal A, Luo S, Janardhanan P, Kannan V, Rymarquis LA, Nobuta K, German R *et al.* 2008. Global identification of microRNA–target RNA pairs by parallel analysis of RNA ends. *Nature Biotechnology* 26: 941–946.
- Goh T, Kasahara H, Mimura T, Kamiya Y, Fukaki H. 2012. Multiple AUX/IAA–ARF modules regulate lateral root formation: the role of Arabidopsis SHY2/IAA3-mediated auxin signalling. *Philosophical Transactions of the Royal Society of London B* 367: 1461–1468.
- Gonzalez-Ibeas D, Blanca J, Donaire L, Saladié M, Mascarell-Creus A, Cano-Delgado A, Garcia-Mas J, Llave C, Aranda M. 2011. Analysis of the melon (*Cucumis melo*) small RNAome by high-throughput pyrosequencing. *BMC genomics* 12: 393.
- Gusmaroli G, Tonelli C, Mantovani R. 2001. Regulation of the CCAAT-Binding NF-Y subunits in *Arabidopsis thaliana*. *Gene* 264: 173–185.
- Gusmaroli G, Tonelli C, Mantovani R. 2002. Regulation of novel members of the *Arabidopsis thaliana* CCAAT-binding nuclear factor Y subunits. *Gene* 283: 41–48.
- Himanen K, Boucheron E, Vanneste S, de Almeida Engler J, Inzé D, Beeckman T. 2002. Auxin-mediated cell cycle activation during early lateral root initiation. *The Plant Cell* 14: 2339–2351.
- Karimi M, Bleys A, Vanderhaeghen R, Hilson P. 2007. Building blocks for plant gene assembly. *Plant physiology* 145: 1183.
- Kazan K. 2013. Auxin and the integration of environmental signals into plant root development. *Annals of Botany* 112: 1655–1665.
- Khan G, Declerck M, Sorin C, Hartmann C, Crespi M, Lelandaïs-Brière C. 2011. MicroRNAs as regulators of root development and architecture. *Plant Molecular Biology* 77: 47–58.
- Laloum T, De Mita S, Gamas P, Baudin M, Niebel A. 2013. CCAAT-box binding transcription factors in plants: Y so many? *Trends in plant science* 18: 157–166.
- Lee H, Yoo SJ, Lee JH, Kim W, Yoo SK, Fitzgerald H, Carrington JC, Ahn JH. 2010. Genetic framework for flowering-time regulation by ambient temperature-responsive miRNAs in *Arabidopsis*. *Nucleic acids research* 38: 3081–3093.
- Lemoine R, La Camera S, Atanassova R, Dédaldéchamp F, Allario T, Pourtau N, Bonnemain JL, Laloi M, Coutos-Thévenot P, Maurousset L *et al.* 2013. Source-to-sink transport of sugar and regulation by environmental factors. *Frontiers in Plant Science* 24: 272.
- Leyva-González MA, Ibarra-Laclette E, Cruz-Ramírez A, Herrera-Estrella L. 2012. Functional and transcriptome analysis reveals an acclimatization strategy for abiotic stress tolerance mediated by *Arabidopsis* NF-YA family members. *PLoS ONE* 7: e48138.
- Li WX, Oono Y, Zhu J, He XJ, Wu JM, Iida K, Lu XY, Cui X, Jin H, Zhu JK. 2008. The *Arabidopsis* NFYA5 transcription factor is regulated transcriptionally and posttranscriptionally to promote drought resistance. *The Plant cell* 20: 2238–2251.
- Li Y, Fu Y, Ji L, Wu C, Zheng C. 2010. Characterization and expression analysis of the *Arabidopsis* mir169 family. *Plant Science* 178: 271–280.
- Liang G, He H, Yu D. 2012. Identification of nitrogen starvation-responsive microRNAs in *Arabidopsis thaliana*. *PLoS ONE* 7: e48951.
- Licausi F, Weits DA, Pant BD, Scheible WR, Geigenberger P, Van Dongen JT. 2011. Hypoxia responsive gene expression is mediated by various subsets of transcription factors and miRNAs that are determined by the actual oxygen availability. *New phytologist* 190: 442–456.
- Liu JX, Howell SH. 2010. bZIP28 and NF-Y transcription factors are activated by ER stress and assemble into a transcriptional complex to regulate stress response genes in *Arabidopsis*. *The Plant cell* 22: 782–796.
- Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$. *Methods* 25: 402–408.
- Lotan T, Ohto M, Yee KM, West MAL, Lo R, Kwong RW, Yamagishi K, Fischer RL, Goldberg RB, Harada JJ. 1998. *Arabidopsis* LEAFY COTYLEDON1 is sufficient to induce embryo development in vegetative cells. *Cell* 93: 1195–1205.
- Malamy JE, Benfey PN. 1997. Organization and cell differentiation in lateral roots of *Arabidopsis thaliana*. *Development* 124: 33–44.
- Mallory AC, Ely L, Smith TH, Marathe R, Anandalakshmi R, Fagard M, Vaucheret H, Pruss G, Bowman L, Vance VB. 2001. HC-Pro suppression of transgene silencing eliminates the small RNAs but not transgene methylation or the mobile signal. *The Plant Cell* 13: 571–583.
- Mantovani R. 1999. The molecular biology of the CCAAT-binding factor NF-Y. *Gene* 239: 15–27.
- Mantovani R, Li XY, Pessara U, van Huisduijnen RH, Benoist C, Mathis D. 1994. Dominant negative analogs of NF-YA. *Journal of Biological Chemistry* 269: 20 340–20 346.
- Marin E, Jouanet V, Herz A, Lokerse AS, Weijers D, Vaucheret H, Nussaume L, Crespi MD, Maizel A. 2010. miR390, *Arabidopsis* TAS3 tasiRNAs, and their AUXIN RESPONSE FACTOR targets define an autoregulatory network quantitatively regulating lateral root growth. *The Plant cell* 22: 1104–1117.
- Meng Y, Shao C, Ma X, Wang H, Chen M. 2012. Expression-based functional investigation of the organ-specific microRNAs in *Arabidopsis*. *PLoS ONE* 7: e50870.
- Moreno-Risueno MA, Van Norman JM, Moreno A, Zhang J, Ahnert SE, Benfey PN. 2010. Oscillating gene expression determines competence for periodic *Arabidopsis* root branching. *Science* 329: 1306–1311.
- Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473–497.
- Nelson DE, Repetti PP, Adams TR, Creelman RA, Wu J, Warner DC, Anstrom DC, Bensen RJ, Castiglioni PP, Donnarummo MG. 2007. Plant nuclear factor Y (NF-Y) B subunits confer drought tolerance and lead to improved corn yields on water-limited acres. *Proceedings of the National Academy of Sciences, USA* 104: 16 450.
- Pagnussat GC, Yu HJ, Ngo QA, Rajani S, Mayalagu S, Johnson CS, Capron A, Xie LF, Ye D, Sundaresan V. 2005. Genetic and molecular identification of genes required for female gametophyte development and function in *Arabidopsis*. *Development* 132: 603–614.
- Pall GS, Codony-Servat C, Byrne J, Ritchie L, Hamilton A. 2007. Carbodiimide-mediated cross-linking of RNA to nylon membranes improves the detection of siRNA, miRNA and piRNA by northern blot. *Nucleic acids research* 35: e60.
- Pant BD, Musialak-Lange M, Nuc P, May P, Buhtz A, Kehr J, Walther D, Scheible WR. 2009. Identification of nutrient-responsive *Arabidopsis* and rapeseed microRNAs by comprehensive real-time polymerase chain reaction profiling and small RNA sequencing. *Plant physiology* 150: 1541–1555.
- Patrick JW. 1997. PHLOEM UNLOADING: sieve element unloading and post-sieve element transport. *Annual Review of Plant Physiology and Plant Molecular Biology* 48: 191–222.
- Perilli S, Sabatini S. 2010. Analysis of root meristem size development. *Methods in Molecular Biology* 655: 177–187.
- Petracka JJ, Winter CM, Benfey PN. 2012. Control of *Arabidopsis* root development. *Annual review of plant biology* 63: 563–590.
- Petroni K, Kumimoto RW, Gnesutta N, Calvenzani V, Fornari M, Tonelli C, Holt BF, Mantovani R. 2012. The promiscuous life of plant NUCLEAR FACTOR Y transcription factors. *The Plant Cell* 24: 4777–4792.
- R Development Core Team. 2012. *R: a language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing.
- Rajagopalan R, Vaucheret H, Trejo J, Bartel DP. 2006. A diverse and evolutionarily fluid set of microRNAs in *Arabidopsis thaliana*. *Genes & development* 20: 3407–3425.
- Rhoades MW, Reinhart BJ, Lim LP, Burge CB, Bartel B, Bartel DP. 2002. Prediction of plant microRNA targets. *Cell* 110: 513–520.
- Roycewicz P, Malamy JE. 2012. Dissecting the effects of nitrate, sucrose and osmotic potential on *Arabidopsis* root and shoot system growth in laboratory assays. *Philosophical Transactions of the Royal Society of London B* 367: 1489–1500.
- Schott G, Mari-Ordóñez A, Himber C, Alioua A, Voinnet O, Dunoyer P. 2012. Differential effects of viral silencing suppressors on siRNA and miRNA loading support the existence of two distinct cellular pools of ARGONAUTE1. *The EMBO journal* 31: 2553–2565.
- Siefers N, Dang KK, Kumimoto RW, Bynum WE IV, Tayrose G, Holt BF III. 2009. Tissue-specific expression patterns of *Arabidopsis* NF-Y transcription

- factors suggest potential for extensive combinatorial complexity. *Plant physiology* 149: 625–641.
- Singh K, Talla A, Qiu W. 2012. Small RNA profiling of virus-infected grapevines: evidences for virus infection-associated and variety-specific miRNAs. *Functional & integrative genomics* 12: 659–669.
- Sunkar R, Jagadeeswaran G. 2008. *In silico* identification of conserved microRNAs in large number of diverse plant species. *BMC plant biology* 8: 37.
- Todesco M, Rubio-Somoza I, Paz-Ares J, Weigel D. 2010. A collection of target mimics for comprehensive analysis of microRNA function in *Arabidopsis thaliana*. *PLoS genetics* 6: e1001031.
- Truernit E, Bauby H, Dubreucq B, Grandjean O, Runions J, Barthélémy J, Palauqui J-C. 2008. High-resolution whole-mount imaging of three-dimensional tissue organization and gene expression enables the study of phloem development and structure in *Arabidopsis*. *The Plant Cell* 20: 1494–1503.
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome biology* 3: research0034.
- Vanneste S, De Rybel B, Beemster GT, Ljung K, De Smet I, Van Isterdael G, Naudts M, Iida R, Gruissem W, Tasaka M *et al.* 2005. Cell cycle progression in the pericycle is not sufficient for SOLITARY ROOT/IAA14-mediated lateral root initiation in *Arabidopsis thaliana*. *The Plant cell* 17: 3035–3050.
- Varkonyi-Gasic E, Wu R, Wood M, Walton EF, Hellens RP. 2007. Protocol: a highly sensitive RT-PCR method for detection and quantification of microRNAs. *Plant Methods* 3: 12.
- Voinnet O. 2009. Origin, biogenesis, and activity of plant microRNAs. *Cell* 136: 669–687.
- Warpeha KM, Upadhyay S, Yeh J, Adamiak J, Hawkins SI, Lapik YR, Anderson MB, Kaufman LS. 2007. The GCR1, GPA1, PRN1, NF-Y signal chain mediates both blue light and abscisic acid responses in *Arabidopsis*. *Plant physiology* 143: 1590–1600.
- Wenkel S, Turck F, Singer K, Gissot L, Le Gourrierec J, Samach A, Coupland G. 2006. CONSTANS and the CCAAT box binding complex share a functionally important domain and interact to regulate flowering of *Arabidopsis*. *The Plant Cell* 18: 2971–2984.
- Woo J, MacPherson CR, Liu J, Wang H, Kiba T, Hannah M, Wang XJ, Bajic VB, Chua N-H. 2012. The response and recovery of the *Arabidopsis thaliana* transcriptome to phosphate starvation. *BMC plant biology* 12: 62.
- Zemzoumi K, Frontini M, Bellorini M, Mantovani R. 1999. NF-Y histone fold α 1 helices help impart CCAAT specificity1. *Journal of molecular biology* 286: 327–337.
- Zhao M, Ding H, Zhu JK, Zhang F, Li WX. 2011. Involvement of miR169 in the nitrogen-starvation responses in *Arabidopsis*. *New phytologist* 190: 906–915.
- Zhou X, Wang G, Sutoh K, Zhu JK, Zhang W. 2008. Identification of cold-inducible microRNAs in plants by transcriptome analysis. *Biochimica et Biophysica Acta* 1779: 780–788.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 miR169 sequences and NF-YA family tree.

Fig. S2 *MIM169abc* have no particular root phenotype.

Fig. S3 The sequestration of miR169defg isoform affects primary root length.

Fig. S4 *MIM169defg* have smaller cortex cells and fewer cells in other files when compared with WT.

Fig. S5 Expression of *NF-YA* genes and miR169 in different organs.

Fig. S6 miR169 isoforms accumulation in *MIMabc* and *MIM169defg* plants.

Fig. S7 Accumulation of miR169 in *35s:miR169a* and *35S:miR169d* transgenic lines.

Fig. S8 Root phenotype of over expressing lines (OE) for miR169a and miR169d.

Fig. S9 Diagram of *GFP-NF-YA* reporter constructs.

Fig. S10 miR169 and miR390b accumulation in agroinfiltrated *N. benthamiana* leaves.

Fig. S11 *35S:NF-YA2* *Arabidopsis* lines have a dramatic developmental phenotype.

Fig. S12 Root phenotypes of WT and *pNF-YA2:HA-NF-YA2wt* lines.

Fig. S13 *NF-YA2* overexpression does not modify auxin responsive gene expression in a lateral root induction system.

Table S1 Primer sequences for cloning

Table S2 AGI numbers of genes and primers sequences for qPCR and RT

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