The Arabidopsis NF-YA3 and NF-YA8 Genes Are Functionally Redundant and Are Required in Early Embryogenesis

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Abstract

Nuclear factor Y (NF-Y) is a trimeric transcription factor composed of three distinct subunits called NF-YA, NF-YB and NF-YC. In Arabidopsis thaliana, NF-Y subunits are known to play roles in many processes, such as gametogenesis, embryogenesis, seed development, drought resistance, ABA signaling, flowering time, primary root elongation, Endoplasmic Reticulum (ER) stress response and blue light responses. Here, we report that the closely related NF-YA3 and NF-YA8 genes control early embryogenesis. Detailed GUS and in situ analyses showed that NF-YA3 and NF-YA8 are expressed in vegetative and reproductive tissues with the highest expression during embryo development from the globular to the torpedo embryo stage. Plants from the nf-ya3 and nf-ya8 single mutants do not display any obvious phenotypic alteration, whereas nf-ya3 nf-ya8 double mutants are embryo lethal. Morphological analyses showed that the nf-ya3 nf-ya8 embryos fail to undergo the heart stage and develop into abnormal globular embryos with both proembryo and suspensor characterized by a disordered cell cluster with an irregular shape, suggesting defects in embryo development. The suppression of both NF-YA3 and NF-YA8 gene expression by RNAi experiments resulted in defective embryos that phenocopied the nf-ya3 nf-ya8 double mutants, whereas complementation experiments partially rescued the abnormal globular nf-ya3 nf-ya8 embryos, confirming that NF-YA3 and NF-YA8 are required in early embryogenesis. Finally, the lack of GFP expression of the auxin responsive DR5rev::GFP marker line in double mutant embryos suggested that mutations in both NF-YA3 and NF-YA8 affect auxin response in early developing embryos. Our findings indicate that NF-YA3 and NF-YA8 are functionally redundant genes required in early embryogenesis of Arabidopsis thaliana.

Introduction

In plants and animals, developmental processes are controlled by complex networks of transcription factors, often arranged in multiprotein DNA-binding complexes, whose gene regulatory activity derives from intrinsic properties and the properties of their trans-acting partners [1–4].

The NUCLEAR FACTOR Y (NF-Y), also called the CCAAT binding factor (CBF), and the heme-activated protein in yeast (HAP), is a heterotrimeric transcription factor that binds with high affinity and sequence specificity the highly conserved core sequence CCAAT. The CCAAT motif is one of the common cis-elements present in 25% of eukaryotic promoters [5,6]. The NF-Y heterotrimer is composed of distinct subunits: NF-YA (CBF-B, HAP2), NF-YB (CBF-A, HAP3), and NF-YC (CBF-C, HAP5). NF-YB and NF-YC subunits form a tight dimer through protein structures similar to the Histone Fold Motif (HFM), a conserved protein-protein and DNA-binding interaction module [7,8]. This dimer translocates to the nucleus, where it offers a complex surface for the association of the NF-YA subunit [9–12]. The resulting mature NF-Y heterotrimer is then competent to bind with high specificity and affinity the CCAAT motif in promoters [13]. NF-YA and NF-YC subunits contain large domains rich in glutamines and hydrophobic residues with transcriptional activation function [13]. The NF-YA subunit contains a conserved domain (HAP2-homology domain) at the C-terminal of the protein, which is divided into two sub-domains: a Subunit Association Domain (the N-terminal part required for NF-YB-NF-YC association) and a DNA binding domain (the C-terminal part required for DNA binding), separated by a linker region [13,14]. In animals and yeast, each subunit of NF-Y is encoded by a single gene, whereas in the Arabidopsis genome 10 NF-YAs, 10 NF-YBs and 9 NF-YCs are annotated [1,14–16]. Recently, a reclassification of the plant NF-Y genes, based on Arabidopsis NF-Y nomenclature, aimed to avoid confusion with acronyms and related, but functionally distinct, histone fold domain (HFD) protein was proposed [16]. Furthermore, the conserved heterodimerization capacity of At-NF-Y histone-like subunits and the different affinities of At-NF-YAs for the CAAT sequence was determined [17].

Genetic and physiological studies have shown that NF-Ys in plants have several different functions. It has been demonstrated that the Arabidopsis NF-YB and NF-YC genes are involved in gametogenesis, embryogenesis, seed development, drought resistance, ABA signaling, flowering time and primary root elongation [18-27]. NF-YA genes are involved in drought resistance [28], in
Endoplasmic Reticulum (ER) stress response and in blue light and ABA responses with NF-YB and NF-YC [21,29].

Embryogenesis in higher plants is a developmental process divided into three distinct phases: morphogenesis, maturation phase and late embryogenesis [30,31]. During the morphogenesis phase, the basic body plan of the plant is established [32,33]. Following fertilization, the zygotic division generates a smaller apical and a larger basal cell. The apical cell divides vertically and generates a proembryo; the basal cell of the zygote divides transversely to produce the hypophysis and the extraembryonic suspensor [34]. Each cell derived from the apical cell divides periclinally to produce a 16-cell embryo (early globular stage). Then, the cells continue to undergo division producing 32-cell (mid-globular stage) and 64-cell stage embryo (late globular stage); at this time cell division in the protoderm increases producing the cotyledon primordia and leads to the heart stage embryo. Then, after cell divisions and cells elongation, the embryo assumes an elongated shape, typical of the torpedo stage [35].

The embryo now enters into the maturation phase: the torpedo stage embryo elongates into a “walking stick” embryo, then the cotyledons grow and curve over forming the “upturned-U” embryo [35]. Finally, the late embryogenesis phase allows the fully developed embryo to enter a desiccated and metabolically quiescent state.

Developmental pathways are involved in the early events of embryo development and they are linked to apical-basal embryo axis establishment after zygote division. One involves the transcription factors WUSCHEL RELATED HOMEOBOX (WOX) WOX8, WOX9 and WOX2; mutations in the WOX genes determine defects in the zygote stage [36-39]. Another developmental pathway is auxin dependent. Auxin is a signalling hormone implicated in the establishment of the embryonic body axis [40] which undergoes polar transport mediated by asymmetric localization of specific cellular efflux carrier proteins, the PINFORMED (PIN) protein family [41,42]. Immediately after the zygote division, auxin is localized in the apical cell and then in the developing proembryo. At the 32-cell stage it undergoes a basal shift to the hypophysis and finally, at later stages of embryogenesis, is also concentrated in the tips of cotyledons and in the provascular strands [43]. It is known that interfering with auxin transport causes defects in the determination of the apical-basal poles during embryo development [43-45]. Axialization of the embryo requires also AUXIN RESPONSE FACTOR (ARF) genes: MONOPTEROS (MP)/ARF RESPONSE FACTOR 5 (ARF5) and its AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) inhibitor BODENLOS (BDC)/IAA12. It is known that mp and bdc embryos show defects in the embryonic axis [46,47].

In this paper, we present the functional characterization of At-NF-YA3 and At-NF-YA8, two members of the Arabidopsis NF-YA family, very closely related in the phylogenetic tree [1,15]. We show that NF-YA3 and NF-YA8 are expressed in vegetative and reproductive tissues, in particular during embryo development from globular to torpedo stage. Plants from single nf-yA3 and nf-yA8 mutants are similar to wild-type plants, whereas nf-yA3 nf-yA8 double mutants are embryo lethal. Nomarski microscopy analysis show that nf-yA3 nf-yA8 fail to undergo to the heart stage and develop into abnormal globular embryos with both proembryo and suspensor characterized by a disordered cell cluster with an irregular shape. Our data indicate that NF-YA3 and NF-YA8 are required in early embryogenesis.

**Materials and Methods**

**Plant materials and growth conditions**

Wild-type Arabidopsis thaliana seeds (Col-0) and DR5rev:GFP [48] seeds were obtained from the Nottingham Arabidopsis Stock Centre, UK (NASC). The nf-yA3 (SAIL_138_E04 line) and nf-yA8 (SAIL_759_B07 line) T-DNA insertion mutants were found in the SAIL collection (http://signal.salk.edu/cgi-bin/tdnaexpress). Plants were grown in a 22°C±2°C temperature growth room under long-day conditions (14 h light/10 h dark) at a fluence rate of 100 μE m⁻² s⁻¹.

**In situ hybridization**

Flowers and silicles of wild-type Arabidopsis plants were collected at various developmental stages and were fixed and embedded in Paraflast Plus embedding media as described by Cañas et al. (1994). Digoxigenin-labeled antisense and sense RNA probes were generated by in vitro transcription according to the instructions of the DIG RNA Labeling Kit (SP6/T7; Roche, http://www.roche.com). cDNA used for probe transcription was synthesized by PCR with the following primers: a forward primer A3N017a (5'-GTTAGCCAGAGCGCTTAATC-3') and a reverse primer containing a T7 promoter adapter A3+17a (5'-TAATACGACTCACTATAGGGTAAAGCTGAGAAGAAAGG-3') for NF-YA3; a forward primer A8N017T-A2 (5'-GCTATTTGCGCTCTTGAG-3') and a reverse primer containing a T7 promoter adapter A8+17a (5'-TAATACGACTCACTATAGGGTAAAGCTGAGAAGAAAGG-3') for NF-YA8. CDNA used for sense probe transcription was synthesized by PCR with the following primers: a forward primer containing a T7 promoter adapter A5T7S (5'-TAATACGACTCTAGAGGTTAGCGAGCGCTTAATTC-3') and a reverse primer A3noT7S (5'-GTAATTCGATGAAGCGAAAGA-3') for NF-YA3; a forward primer containing a T7 promoter adapter A8T7-S2 (5'-TAATACGACTCTAGAGGTTAGCGAGCGCTTAATTC-3') and a reverse primer A8noT7S (5'-GTAATTCGATGAAGCGAAAGA-3') for NF-YA8. Hybridization and immunological detection were performed as described previously [49]. The sections were mounted with a coverslip and subsequently observed using a Zeiss Axiophot D1 microscope equipped with differential interference contrast (DIC) optics. Images were captured on an AxioCam MRc5 camera (Zeiss) using the AXIOVISION program (version 5.0).

**Constructs and generation of transgenic lines**

The pNF-YA3::GUS fusion was constructed from a 1995-bp genomic region upstream of the NF-YA3 start codon including the entire upstream intergenic region amplified by PCR with a forward primer containing a SalI restriction site at the 5' end (5'-GTCGACACGGCTTTTGTAGAG-3') and a reverse primer containing a SalI site preceding the gene in the binary vector pGPTV-Kan [50]. The constructs for 35S::NF-YA3 and 35S::NF-YA8 lines were obtained using the Gateway cloning system. NF-YA3 and NF-YA8 have been crossed into the Landsberg erecta (Ler) and Col background.
cDNAs were amplified by PCR using the following primers: A3TH5 (5'-AATCCAGCTACACGATGATGTAGAG.ps 9-3 RNAi F GATATGGACAGAG-3') and A3TH3 (5'-GATCCCAGGAATTGC-CATGGCAAGAGACAGAG-3') for NF-YA3 and A3TH5 (5'-AATCTGACGAGCCATTGATGATGTAC-3') and A3TH3 (5'-GATCCCAGGAATTGC-CATGGCAAGAGACAGAG-3') for NF-YA8. The PCR products were cloned in the binary vector in the pGDe25 vector under the Cauliflower Mosaic Virus 35S promoter (35S) according to manufacturer instructions (Gateway, Invitrogen).

For the RNA interference (RNAi) construct, a 200-bp specific fragment for NF-YA8 that shared 80% of identity with NF-YA3 was amplified by PCR using the forward primer 8-3 RNAi F (5'-GCCGTTAATCTCTTTGGAC-3') in combination with the reverse primer 8-3 RNAi R (5'-AGTAGCAGCAGGATG-3') in combination with the reverse primer 8-3 RNAi R (5'-AGTAGCAGCAGGATG-3'). The fragment was placed in both orientations into the pPHR destination vector (derived from pFGC5941; NCBI accession number AY310901) under the control of the 35S promoter using the Gateway technology (Invitrogen). All the constructs were introduced into A. tumefaciens GV3101 [51].

The pNF-YA3::GUS and pNF-YA8::GUS constructs were then transferred to wild-type Col and the 35S::NF-YA3 and 35S::NF-YA8 constructs to nf-ya3 nf-ya3 NF-YA8/nf-ya8 plants by the floral dip method [52]. Homozygous T3 independent lines with a single T-DNA insertion were selected by segregation analysis on MS agar plates supplemented with 1% (w/v) sucrose containing 50 μg ml⁻¹ kanamycin.

The RNAi construct was transferred to nf-ya3 and nf-ya8 single mutants and then T1 independent RNAi::A3/A8 lines were selected on MS agar plates supplemented with 1% (w/v) sucrose containing 20 μg ml⁻¹ hygromycin.

To analyse auxin response in nf-ya3 and nf-ya8 embryos, plants nf-ya3/nf-ya3 NF-YA8/nf-ya8 used as females were crossed with the DR5rev::GFP marker line used as male. From the F1 progeny, plants nf-ya3/nf-ya3 NF-YA8/nf-ya8 were selected by PCR genotyping and self-fertilized. The resulting F2 plants were analysed by visual inspection of GFP signal in roots using an Olympus SZX12 stereomicroscope, in order to select those carrying the DR5rev::GFP construct, and then analysed by PCR genotyping to select those with nf-ya3/nf-ya3 NF-YA8/nf-ya8 genotype. Selected F2 plants were then self-fertilized and the resulting F3 plants were analysed by visual inspection of GFP signal in root, in order to identify plants homozygous for the DR5rev::GFP construct.

GUS staining assay

For detection of GUS activity, vegetative tissues and flowers at different stages were incubated for 16 hr at 37°C in the GUS staining buffer (0.5 mg/ml X-galactosidase, 0.1% Triton X-100, and 0.5 mM ferrocyanide in 100 mM phosphate buffer, pH 7). Longitudinally dissected siliques were fixed for 2 hr at ~20°C in 90% acetone and subsequently immersed in GUS staining buffer for 16 hr at 37°C. Vegetative tissues were cleared in 70% ethanol, flowers and siliques were cleared in a solution of chloral hydrate:glycerol:water solution (8:1:2, w:v:v). GUS staining in ovule, pollen and embryos at different stages was observed under Nomarski optics on a Zeiss Axio Imager.D1 microscope with a video camera Axioscam MRc5. GUS staining in other tissues was examined under a stereomicroscope LEICA® MZ6 and the GUS images were taken with LEICA® DC 500 camera.

Genotyping of mutants and transgenic plants by PCR

Genomic DNA was extracted from plants as previously described [53]. Wild-type alleles were amplified using the following primers: atACF3 (5'-CTCTGCAATCGTGTGGTTTG-3') and atACR1 (5'-GGTGGCACCAGTGTAAGAG-3') for the NF-YA3 allele and NF-YA8F2 (5'-CTTTTGCAACACTTGATATGCA-3') and NF-YA8R2 (5'-TGCGTC-AGCTTTGGCTGATTCGAC-3') for the NF-YA8 allele. The nf-ya3 and nf-ya8 mutant alleles were amplified using the following primers: the T-DNA left border-specific primer LB3 (5'-TAGTCATGGCAAGCTGATACCA-3') together with the 35S::NF-YA3-specific primers atACR1 for the nf-ya3 allele or with the 35S::NF-YA8-specific primer NF-YA8R2 for the nf-ya8 allele.

The presence of the complementation constructs was verified by PCR with a forward primer 35S (5'-TCTTAGACAA-GACCCCTTCTGCT-3') in combination with the reverse primer atACR1 for the 35S::NF-YA3 construct or with the reverse primer NF-YA8R2 for the 35S::NF-YA8 construct.

Whole mount preparation and confocal laser scanning

Whole mount embryos were prepared by clearing siliques at different stages of nf-ya3/nf-ya3 NF-YA8/nf-ya8 plants in chloral hydrate:glycerol:water solution (8:1:2, w:v:v). Siliques were dissected under the stereomicroscope and observed under Nomarski optics. Confocal laser scanning microscopy was performed on embryos collected from nf-ya3/nf-ya3 NF-YA8/nf-ya8 plants homozygous for the DR5rev::GFP construct using Leica TCS SP5 AOBS and DMI 6000 CS microscope.

Semi-quantitative RT-PCR analysis

Total RNA was isolated [54] from developing siliques of nf-ya3 and nf-ya8 plants and of 35S::NF-YA3 and 35S::NF-YA8 overexpression lines as previously described [14]. Approximately 5 μg were reverse-transcribed using RT Superscript II (Invitrogen) and an oligo dT, as previously described [55]. After first strand cDNA synthesis, samples were diluted 50 times and used as templates for semi-quantitative RT-PCR, with 35S::NF-YA3-specific primers atACF2 (5'-ATCTTATACTGGAAGTTGCTAGTAC-3') and atACR2 (5'-TTCTGACAGCATATGAGCTTTCA-3') and 35S::NF-YA8-specific primers NF-YA8F2 and NF-YA8R2, ACT2N2 specific primers, ACT2-F (5'-TGTTCTCCTTCCATTTGTTTTGCC-3') and ACT2-R (5'-GGCATCAATCGATCACTCA-3') were used as a control of cDNA concentration. The amplifications were performed on embryos collected from nf-ya3/nf-ya3 NF-YA8/nf-ya8 plants and of 35S::NF-YA3 and 35S::NF-YA8 plants overexpressing NF-YA3 and NF-YA8, respectively.

Quantitative real-time RT-PCR analysis

Total RNA was isolated from siliques of the RNAi::A3/A8 transgenic plants and the cDNA was synthesized as above. Quantitative real-time RT-PCR of NF-YA3 and NF-YA8 was performed using SYBRGreen with the Cfx96TM BioRad® Real Time system in a final volume of 20 μl containing 5 μl of 50-fold diluted cDNA, 0.25–0.4 μM of each primer and 10 μl of 2X SOS Fast™ EVA-Green® Supermix (BioRad Laboratories, Hercules, CA, USA).

Analysis of NF-YA3 and NF-YA8 expression in RNAi::A3/A8 lines was performed using NF-YA3-specific primers NFYA3_RT-F and NF-YA3_RT-R and NF-YA8-specific primers NFYA8_RT-F (5'-CACACAGGAACTCTTGGTCA-3') and NFYA8_RT-R (5'-TTGAAGCTTGAAGATTGGTG-3'). ACT2 and RCE1 were used as the reference genes. Specific primers for ACT2 and RCE1 were amplification of genes, respectively. Specific primers for ACT2 and RCE1 were amplification of genes, respectively.
Relative quantification was analysed using iCycler™ iQ Optical System Software version 3.0a (BioRad Laboratories). The protocol used was as follows: 95°C for 2 min, 55 cycles of 95°C for 15 s, 60°C for 30 s. A melt curve analysis was performed following every run to ensure a single amplified product for every reaction (from 55°C to 95°C, increment 0.5°C for 10 sec).

Relative quantification of the target RNA expression level was performed using the comparative Ct method (User Bulletin 2, ABI PRISM 7700 Sequence Detection System, December 1997; Perkin Elmer Applied Biosystems) in which the differences in the Ct (threshold cycle) for the target RNA and endogenous control RNA, called ΔCt, were calculated to normalise for the differences in the total amount of cDNA present in each reaction and the efficiency of the reverse transcription. Finally, the target RNA expression level was obtained from the equation $2^{-\Delta\Delta Ct}$ and expressed relative to a calibrator (wild-type silique). Standard errors of ΔCt values were obtained from measurements performed in triplicate.

Results

The duplicated genes NF-YA3 and NF-YA8 have similar expression patterns

Previous expression analyses showed that the NF-YA3 and NF-YA8 genes are both expressed in vegetative and reproductive tissues during flower and seed development [14,15]. Furthermore, NF-YA3 and NF-YA8 share a high sequence similarity. Overall, they are 62% identical at the amino acid level, with 93% identity shared between their conserved domain (HAP2-homology domain) at the C-terminal of the protein. Analysis of their chromosomal locations revealed that they lie within a relatively main) at the C-terminal of the protein. Analysis of their expression pattern in different organs and developmental stages. To this purpose, we obtained transgenic plants carrying a 1995 bp genomic region upstream of NF-YA3 start codon fused to the GUS reporter gene (pNF-YA3::GUS) and plants carrying a 1000 bp genomic region upstream of NF-YA8 start codon fused to the GUS reporter gene (pNF-YA8::GUS). In total, 7 independent transgenic lines for the pNF-YA3::GUS construct were analysed. For both constructs, five repeats. RT-PCR analysis showed a strong reduction of transcript was detected in rosette leaves, which showed high signal in midrib, lateral veins, and trichomes (Fig. 1 A,a). GUS staining was also observed in cauline leaves (Fig. 1 B,b), in roots (Fig. 1 D,d), in all flower organs (Fig. 1 E,e) and in siliques (Fig. 1 F,f). In axes of stem branches, NF-YA8 was more expressed than NF-YA3 (Fig. 1 C,c). Detailed analyses of GUS expression in reproductive organs revealed similar expression of NF-YA3 and NF-YA8 in anthers and in pollen grains at floral stage 8 according to Bowman, 1994 (Fig. 1 G,g), whereas in subsequent stages the signal was in filaments of anthers, but not in mature pollen grains (data not shown). GUS staining was detected in ovules at all developmental stages (Fig. 1 H–K and h–k). After fertilization, there was a low GUS signal level in mature pollen grains (Fig. 2 D,I). The NF-YA3 and NF-YA8 transcripts were both expressed in the placenta (Fig. 2 A,F), in tetrads of microspores (Fig. 2 B,G), in developing pollen grains and in tapetum (Fig. 2 C,H). However, no expression was observed in mature pollen grains (Fig. 2 D,I). The NF-YA3 and NF-YA8 genes are both expressed in vegetative and reproductive tissues (Fig. 1). In particular, a similar GUS expression pattern was observed both in embryo and in endosperm cells. In the cotyledon stage (Fig. 2 P,V), the expression decreased and was restricted to the embryo. Similar hybridization experiments performed with pNF-YA3 and pNF-YA8 sense probes on developing flowers and seeds gave no signal (Figure S1).

Overall, the in situ analyses essentially confirmed the results obtained by the GUS expression analysis and showed that NF-YA3 and NF-YA8 have an overlapping expression profile, with high levels of transcript in the embryo from globular to torpedo stages. Furthermore, these expression analyses make them strong candidates for being functionally redundant during embryo development.

Isolation and characterization of T-DNA insertion mutants in the NF-YA3 and NF-YA8 genes

To determine the function of the Arabidopsis NF-YA3 and NF-YA8 genes, we undertook a reverse genetic approach and isolated T-DNA mutant alleles for both NF-YA3 and NF-YA8.

We identified one T-DNA insertion line in NF-YA3 in the SAIL collection. Sequencing of PCR products obtained from the T-DNA/gene junctions revealed that nfyα3 carries a T-DNA element located 107 bp upstream of the start codon (SAIL_138_E04) corresponding to the intron of the 5' untranslated region (5' UTR) (Fig. 3 A). Both the T-DNA/gene junctions could be amplified with the left border primer, implying that two or more T-DNAs had inserted into this locus as tandem inverted repeats. RT-PCR analysis showed a strong reduction of NF-YA3 transcript levels in nfyα3 developing siliques compared to wild-type (Fig. 3 B). However, there were no obvious morphological alterations in nfyα3 mutants and genotypic analysis of 247 progeny plants from NF-YA3/nfyα3 heterozygotes revealed that the genotypic ratios were not statistically different from the 1:2:1 expected ratios (69:110:68, $\chi^2 = 2.96$, 0.25>P value>0.1).

One insertional mutant in NF-YA8 was found in the SAIL collection (SAIL_759_B07). Genomic PCR and sequencing of T-DNA/gene junctions revealed that a T-DNA had inserted 1049 bp from the start codon (SAIL_759_B07). The NF-YA8 transcript was absent in nfyα8 homozygous developing siliques (Fig. 3 B). However, the knock-out mutation of NF-YA8 did not cause any obvious morphological defect and the segregation analysis of 158 progeny plants from NF-YA8/nfyα8 heterozygotes showed a normal genotypic 1:2:1 ratio (51:69:38, $\chi^2 = 4.67$, P value<0.1).
Double mutants nf-ya3 nf-ya8 are embryo-defective

Since characterization of nf-ya3 and nf-ya8 single mutants did not show any phenotypic defect, in order to verify whether NF-YA3 and NF-YA8 were redundant genes, we aimed to generate a nf-ya3 nf-ya8 double mutant. Therefore, a cross was made between nf-ya3 and nf-ya8 homozygous plants. The resulting double heterozygous F1 plants were self-fertilized and the F2 progeny plants analysed in order to select the double mutants expected at 1/16 frequency. PCR genotyping of 81 F2 progeny plants, however, did not identify any nf-ya3 nf-ya8 double mutants. To increase the expected frequency of recovering plants with the double mutant genotype, F3 progeny from plants that were homozygous for the nf-ya3 mutation and heterozygous for the nf-ya8 mutation were analyzed. One quarter of the progeny from these plants was expected to be double mutants. However, genotyping of 344 F3 individuals derived from a nf-ya3/nf-ya3 NF-YA8/nf-ya8 plant failed to identify any double mutant (Table 1). In addition, the observed ratio between the other genotypic classes (217:127, Table 1) was consistent with a 2:1 ratio ($\chi^2 = 1.906, 0.2 > P > 0.1$). These results suggest that double mutants are embryo lethal and the presence of only one copy of NF-YA3 or NF-YA8 is sufficient to result in normal embryo development.

Silicas of 17 plants nf-ya3/nf-ya3 NF-YA8/nf-ya8 and silicas of 10 plants nf-ya3/nf-ya3 NF-YA8/NF-YA8, among F3 progeny, were
analysed phenotypically. For each plant, 10 siliques were collected and observed. Siliques obtained from the nf-ya3/nf-ya3 NF-YA8/nf-ya8 plants contained white-colored arrested seeds (Fig. 4). We found 546 arrested seeds out of 2272 seeds analysed, corresponding to a percentage of 24.03%, that is not statistically different from the expected 25% corresponding to the nf-ya3/nf-ya3 nf-ya8/nf-ya8 genotypic class ($\chi^2 = 1.136, 0.5 > P > 0.2$) (Table 2). On the contrary, in siliques obtained from nf-ya3/nf-ya3 NF-YA8/NF-YA8 plants, we found a percentage of arrested seeds (0.095%, n = 3163), which is comparable to those observed in wild-type siliques (0.099%, n = 1008) (Fig. 4). Therefore, these data indicate that there is a cosegregation between the genotypic class nf-ya3/nf-ya3 nf-ya8/nf-ya8 and the phenotype of arrested seeds.

NF-YA3 and NF-YA8 control the suspensor and embryo proper development

To determine the developmental origin of the embryo lethality, we analyzed whole-mount preparations of developing siliques after self-fertilization of nf-ya3/nf-ya3 NF-YA8/nf-ya8 mutant plants. In particular, our aim was to identify the precise stage of embryo development which is impaired in nf-ya3 nf-ya8 double mutants. Morphological characterization of developing siliques confirmed the presence of abnormal embryos (Fig. 5). Until 4-cell stage, the mutant embryos were normal (Fig. 5 A,B), whereas phenotypic deviations from the wild-type were first identified in embryos at the 8-cell stage, when the double mutant embryos presented a delay in cell divisions (Fig. 5 C,D). Embryos at 32-cell stage showed a hypophysis cell (uppermost region of the suspensor) with a longitudinal, instead of a transversal, division plan as found in wild-type and an abnormal number of proembryo cells caused by defects in cell division plans (Fig. 5 E,F). Afterwards, double mutant failed to undergo to the heart stage; in particular, the embryos proceeded extremely slowly and abnormally through the other stages of development (Fig. 5 G–N), and only very late in development they became an abnormal globular mass with proembryos and suspensors characterized by a disordered cell cluster with an irregular shape (Fig. 5 P,R). These data suggest that mutations in NF-YA3 and NF-YA8 compromise embryo development pattern and, in particular, they are required for both suspensor and embryo proper development and embryo survival.

RNAi suppression of NF-YA3 and NF-YA8 causes embryonic defects

A post-transcriptional silencing approach mediated by RNA interference (RNAi) was carried out to verify whether the embryo lethal phenotype is due to the mutations in both NF-YA3 and NF-YA8 genes. A 200-bp specific fragment for NF-YA8 that shared 80% of identity only with NF-YA3 was used to suppress the NF-YA3 and NF-YA8 gene expression at the same time. Single mutants nf-ya3 and nf-ya8 were transformed with the RNAi construct specific for NF-YA3 and NF-YA8 under the control of the 35S promoter and T1 primary transformants were recovered on hygromycin selection. Four out of 51 T1 nf-ya3 RNAi::A3/A8 transgenic lines and four out of 49 T1 nf-ya8 RNAi::A3/A8
transgenic lines produced defective T2 seeds. More specifically, T1 plants from *nf-ya3 RNAi::A3/A8* lines segregated 5.36%, 18.18%, 17% and 24.78% arrested T2 seeds and T1 plants from *nf-ya8 RNAi::A3/A8* lines segregated 6.9%, 17.13%, 24% and 26% arrested T2 seeds. Although the RNAi::A3/A8 construct was incompletely penetrant, these results suggest that *NF-YA3* and *NF-YA8* are required for embryo development. In particular, the analysis of 10 cleared siliques collected at 10 DAP (Days After Pollination) of *nf-ya3 RNAi::A3/A8* line (24.78% of arrested seeds) and *nf-ya8 RNAi::A3/A8* line (26% of arrested seeds) showed that in the defective seeds the embryos phenocopied the *nf-ya3 nf-ya8* double mutants: the embryos proper and the suspensors were characterized by aberrant division patterns resulting in defects in the apical and basal domains (Fig. 6 A). To confirm that abnormal embryo development resulted from silencing of both *NF-YA3* and *NF-YA8* genes, we analyzed *NF-YA3* and *NF-YA8* RNA levels in the transgenic siliques by quantitative real-time RT-PCR using specific primers for the *NF-YA3* and *NF-YA8* genes. Our results indicated that siliques of *nf-ya8 RNAi::A3/A8* lines displaying embryos with the mutant phenotype possessed undetectable levels of *NF-YA3* and *NF-YA8* and siliques of *nf-ya3 RNAi::A3/A8* line possessed a low level of *NF-YA8* and an undetectable level of *NF-YA3* (Fig. 6 B). Despite both *NF-YA3* and *NF-YA8* were essentially undetectable in seeds of these RNAi lines, no post-embryonic phenotypes, such as aberrant seedlings, or other phenotypic alterations in plant development were observed in T2 plants. Together, these results suggest that concurrent suppression and reduction of *NF-YA3* and *NF-YA8* gene expression induced defects in the embryo development.

**Partial rescue of nf-ya3 nf-ya8 mutant phenotype by complementation**

To further demonstrate that the embryo-lethal phenotype is determined by the loss of activity of *NF-YA3* and *NF-YA8*, a complementation experiment was performed. Plants *nf-ya3/nf-ya3 NF-YA8/nf-ya8* were transformed with a construct carrying the coding sequence (CDS) of *NF-YA3* or with a construct with the coding sequence of *NF-YA8* driven by a 35S promoter (35S::

![Figure 3. Structure of the T-DNA insertional mutants and semi-quantitative RT-PCR analyses.](image)

**Table 1. Segregation analysis of F3 progeny from nf-ya3/nf-ya3 NF-YA8/nf-ya8 plants.**

| Progeny genotype | Observed/expected (%) | Observed/expected |
|------------------|-----------------------|-------------------|
| nf-ya3/nf-ya3 NF-YA8/NF-YA8 | 127/86.25 | 36.92/25 |
| nf-ya3/nf-ya3 NF-YA8/nf-ya8 | 217/172.5 | 63.08/50 |
| nf-ya3/nf-ya3 nf-ya8/nf-ya8 | 0/86.25 | 0/25 |
| Total | 344/344 | 100/100 |

^aChi-square test for a 2:1 segregation hypothesis.

**Figure 4. Phenotypic analysis of nf-ya3/nf-ya3 NF-YA8/nf-ya8 siliques.**

A) Siliques of wild-type plants with normal seeds. B) Siliques of *nf-ya3/nf-ya3 NF-YA8/nf-ya8* mutant plants with arrested seeds (arrow). Bar = 500 μm.
and 35S::CDS-NF-YA8. Primary T1 transformants were identified by kanamycin (KAN) selection, whereas the genotype and the presence of the complementation construct were determined by PCR.

We identified twenty-five nf-ya3/nf-ya3 NF-YA8/nf-ya8 T1 plants that contained the complementation construct 35S::CDS-NF-YA3. These plants were grown to maturity and siliques analysed to identify complemented lines. In particular, if only one copy of the complementation construct had inserted, fully complemented lines should show 6.25% (1/16) of arrested seeds, whereas partially complemented lines should show a percentage of arrested seeds between 6.25% and 25%. In the case of two copies of the construct, the arrested seeds of fully complemented lines should be 1.56% (1/64), whereas in partially complemented lines the percentage of arrested seeds should be between 1.56% and 25%. Non-complemented lines would show 25% of arrested seeds. We identified four lines that showed various levels of partial complementation (Table 3). Segregation analysis for kanamycin resistance indicated that three lines had two copies of the 35S::CDS-NF-YA3 construct (KANR:KANS, 15:1) and showed 7.5%, 13.71%, 17.53% of arrested seeds, respectively. One line, with one copy of the construct (KANR:KANS, 3:1), showed 14.29% of arrested seeds.

For all four lines, we then isolated by kanamycin selection nf-ya3/nf-ya3 NF-YA8/nf-ya8 T2 plants homozygous for the complementation construct. Siliques of nf-ya3/nf-ya3 NF-YA8/nf-ya8 T3 plants showed a percentage of arrested seeds lower than 25%, confirming a partial complementation. Green seedlings of these four lines (T4 generation) were analysed by PCR, which showed that some of them were indeed nf-ya3 nf-ya8 double mutants. These results suggest that the introduced construct 35S::CDS-NF-YA3 can partially complement the embryo arrested phenotype.

The same complementation experiment was performed using the 35S::CDS-NF-YA8 construct to transform nf-ya3/nf-ya3 NF-YA8/nf-ya8 plants. Sixty independent KAN-resistant T1 lines were isolated and four of them showed various levels of partial complementation (Table 4). Lines 16 and 21 carried one copy of the 35S::CDS-NF-YA8 construct and showed 18.38% and 18.86% arrested seeds, respectively.

Table 2. Percentage of normal and arrested seeds of nf-ya3/nf-ya3 NF-YA8/nf-ya8 mutant plants.

| Seeds          | Observed/expected | Observed/expected (%) |
|----------------|-------------------|-----------------------|
| normal         | 1726/1704         | 75.97/75              |
| arrested       | 546/568           | 24.03/25              |
| Total          | 2272/2272         | 100/100               |

*Chi-square test for a 3:1 phenotypic segregation hypothesis.

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Figure 5. Defects in embryogenesis in nf-ya3 nf-ya8 double mutants. Nomarski images of nf-ya3/nf-ya3 NF-YA8/nf-ya8 siliques collected at different stages. Cleared wild-type or nf-ya3/nf-ya3 NF-YA8/nf-ya8 seeds (A,C,E,G,I,K,M,O,Q) and nf-ya3/nf-ya3 NF-YA8/nf-ya8 mutant seeds (B,D,F,H,J,L,N,P,R). (A,B) 4-cell stage, (C,D) 8-cell stage, (E,F) 32-cell stage, (G,H) globular stage, (I–J) mid-heart stage, (K–L) late-heart stage, (M,N) torpedo stage, (O,P) late-torpedo stage, (Q,R) cotyledon stage. Arrow indicates cells that have undergone abnormal division. Bars = 10 μm.

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NF-YA3 and NF-YA8 Are Required in Embryogenesis
NF-YA3 and NF-YA8 Are Required in Embryogenesis

In parallel, transgenic plants overexpressing NF-YA3 or NF-YA8 were obtained by transforming the wild-type plants with the same constructs used for the complementation. RT-PCR showed that the NF-YA3 or NF-YA8 transcript levels in overexpression lines were higher than in wild-type (Fig. 7). Phenotypic analysis and detailed morphological analysis of these overexpression lines carried out by Nomarski microscopy did not reveal morphological defects in seedlings or plant development in and ovule, pollen and embryo development (data not shown).

Auxin response is altered in the nf-ya3 nf-ya8 embryos

Defective cell division plans in nf-ya3 nf-ya8 embryos raised the question whether auxin response was affected in nf-ya3 nf-ya8 embryos. To address this question, we crossed the auxin response marker line DR5rev::GFP to nf-ya3/nf-ya3 NF-YA8/nf-ya8 plants. The DR5 synthetic auxin-responsive promoter is used to visualize the spatial pattern of auxin response and indirectly the auxin distribution and it is specifically active from the early embryo stages [43]. Our analysis revealed that in wild-type embryos GFP signal was restricted to the developing proembryo (Fig. 8A), whereas from the 32-cell globular stage onwards it was restricted to the hypophyseal region (Fig. 8B). No GFP signal was detected in nf-ya3 nf-ya8 defective embryos (Fig. 8E), whereas normal GFP signal was detected in earlier stages (Fig. 8D). Furthermore, in wild-type embryos at later stages GFP signal was highly detected in the tips of the developing cotyledons and in the provascular strands (Fig. 8C), whereas again no GFP signal was observed in nf-ya3 nf-ya8 embryos at later stages showing aberrant cell divisions and development (Fig. 8F). We can conclude that defects in the nf-ya3 nf-ya8 embryos are associated to a defective auxin response.

Discussion

NF-YA3 and NF-YA8 are functionally redundant regulators of embryo development

In this study, using a reverse genetic approach, we have shown that the Arabidopsis NF-YA3 and NF-YA8 control embryo development. The GUS expression analysis of the pNF-YA3::GUS and pNF-YA8::GUS transgenic lines revealed that both genes are expressed in vegetative and reproductive tissues. In particular, GUS staining was low during pollen and ovule development, whereas it was high during embryo development from the globular embryo stage to the torpedo stage and then decreased in the mature embryo. In situ hybridization essentially confirmed these GUS expression results, except for endosperm, where a clear GUS signal was missing. Since GUS results were obtained by cloning putative NF-YA3 and NF-YA8 promoter regions upstream of the GUS reporter gene, they may not exactly reflect the real expression pattern of genes, because lacking endosperm-specific regulatory elements possibly located in 3' untranslated regions (3'UTR) or introns not included in the constructs. Interestingly, previous GUS expression analyses from Siebers and colleagues [11] of NF-YA3 and NF-YA8 revealed that GUS staining was undetectable in flowers, in root tips and low in rosette leaves. We suggest that these differences in GUS expression results are due to differences in

Figure 6. Phenotype of RNAi::A3/A8 embryos and quantitative real-time RT-PCR analyses. (A) Cleared seeds viewed with Nomarski optics. Wild-type embryo at cotyledon stage and defective arrested embryos present in the same siliques at 10 DAP from nf-ya3 RNAi::A3/A8 and nf-ya8 RNAi::A3/A8 lines. Bars = 50 μm. (B) Expression levels of NF-YA3 and NF-YA8 genes in RNAi::A3/A8 lines. Total RNAs were extracted from siliques of wild-type and of nf-ya3 RNAi::A3/A8 and nf-ya8 RNAi::A3/A8 plants and analyzed by real time RT-PCR. Wild type siliques were used as calibrator for relative expression levels for each gene analyzed. Bars represent standard deviations of measurements performed in triplicate in three biological replicates.

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of arrested seeds, respectively. Two copies were inserted in lines 34 and 52, that showed a reduction to 18.72% and 18.93% arrested seeds, respectively. Also in this case, for all four lines we then isolated by kanamycin selection nf-ya3/nf-ya3 NF-YA8/nf-ya8 T3 plants homozygous for the complementation construct. The analysis of siliques from nf-ya3/nf-ya3 NF-YA8/nf-ya8 T3 plants indicated that lines 16, 21 and 52 were partially complemented. Also in this case, PCR analysis confirmed that some of T3 plants were nf-ya3 nf-ya8 double mutants. Interestingly, in line 34 which carried two copies of the complementation construct, siliques showed 100% normal seeds. No post-embryonic phenotypes, such as aberrant seedlings, or other phenotypic alterations in plant development were observed in complemented plants. These data indicate that the complementation construct was able to partially rescue the arrested embryos and that in only one line the 35S::CDS-NF-YA8 construct was sufficient to fully complement the embryo lethality.

Auxin response is altered in the nf-ya3 nf-ya8 embryos

Defective cell division plans in nf-ya3 nf-ya8 embryos raised the question whether auxin response was affected in nf-ya3 nf-ya8 embryos. To address this question, we crossed the auxin response marker line DR5rev::GFP to nf-ya3/nf-ya3 NF-YA8/nf-ya8 plants. The DR5 synthetic auxin-responsive promoter is used to visualize the spatial pattern of auxin response and indirectly the auxin distribution and it is specifically active from the early embryo stages [43]. Our analysis revealed that in wild-type embryos GFP signal was restricted to the developing proembryo (Fig. 8A), whereas from the 32-cell globular stage onwards it was restricted to the hypophyseal region (Fig. 8B). No GFP signal was detected in nf-ya3 nf-ya8 defective embryos (Fig. 8E), whereas normal GFP signal was detected in earlier stages (Fig. 8D). Furthermore, in wild-type embryos at later stages GFP signal was highly detected in the tips of the developing cotyledons and in the provascular strands (Fig. 8C), whereas again no GFP signal was observed in nf-ya3 nf-ya8 embryos at later stages showing aberrant cell divisions and development (Fig. 8F). We can conclude that defects in the nf-ya3 nf-ya8 embryos are associated to a defective auxin response.

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NF-YA3 and NF-YA8 are required in embryogenesis

The morphological analysis performed by Nomarski microscopy on siliques produced by nf-ya3/nf-ya3 NF-YA8/nf-ya8 plants revealed the specific role of NF-YA3 and NF-YA8 during early embryogenesis. In particular all the arrested seeds analysed contained abnormal embryos, in which the division plan of cells and number of cells in proembryo and suspensor were incorrect. Therefore, the mutant embryo showed an abnormal division of the uppermost region of the suspensor close to the proembryo (i.e., the hypophysis cell). These morphological defects suggests that NF-YA3 and NF-YA8 have a role during early embryogenesis.

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In plants, axis formation involves the signaling molecule auxin, a plant hormone that has a gradient of accumulation and distribution during the time of development and that is responsible for structural plant organization and for correct plan determination [61]. PINFORMED-dependent asymmetric auxin efflux and the maintenance of normal auxin gradients within the embryo are necessary for the correct apical-basal axis determination in the embryo [39]. PIN1 and PIN7 localization correlated with the apical-basal auxin gradients during early embryogenesis. After zygotic division, auxin flux is initially directed up into the apical cell by PIN, that localizes to the apical end of the basal daughter cell; moreover, PIN1 marks the apical cell and the proembryo cell boundaries in a non-polar fashion. At the 32-cell stage, auxin undergoes apical-to-basal transport; it is transported into the

Table 3. Complementation of nf-ya3/nf-ya3 nf-ya8/nf-ya8 double mutants with 35S:CDS-NF-YA3 construct.

| T1, line | KAN\(^b\)/KAN\(^d\) | Normal seeds | Arrested seeds | Total | P value\(^c\) | Observed arrested seeds (%) |
|---------|-----------------|--------------|---------------|-------|--------------|---------------------------|
| 11      | 15:1            | 74/78.75     | 6/1.25        | 80/80 | P<0.0001     | 7.5                       |
| 13      | 15:1            | 258/294.33   | 41/4.67       | 299/299 | P<0.0001     | 13.71                     |
| 16      | 15:1            | 160/190.97   | 34/3.03       | 194/194 | P<0.0001     | 17.53                     |
| 48      | 3:1             | 126/137.81   | 21/9.19       | 147/147 | P<0.0001     | 14.29                     |

\(^{a}\)nf-ya3/nf-ya3 NF-YA8/nf-ya8 T1 plants carrying the 35S:CDS-NF-YA3 complementation construct.

\(^{b}\)Arrested seeds expected in case of full complementation.

\(^{c}\)Chi-square test for 1/16 (one copy of construct inserted) and 1/64 (two copies of construct inserted) segregation hypothesis.

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Table 4. Complementation of nf-ya3/nf-ya3 nf-ya8/nf-ya8 double mutants with 35S:CDS-NF-YA8 construct.

| T1, line | KAN\(^b\)/KAN\(^d\) | Normal seeds | Arrested seeds | Total | P value\(^c\) | Observed arrested seeds (%) |
|---------|-----------------|--------------|---------------|-------|--------------|---------------------------|
| 34      | 15:1            | 178/215.58   | 41/3.42       | 219/219 | P<0.0001     | 18.72                     |
| 52      | 15:1            | 167/202.78   | 39/3.22       | 206/206 | P<0.0001     | 18.93                     |
| 16      | 3:1             | 182/209.06   | 41/13.94      | 223/223 | P<0.0001     | 18.38                     |
| 21      | 3:1             | 185/213.75   | 43/14.25      | 228/228 | P<0.0001     | 18.86                     |

\(^{a}\)nf-ya3/nf-ya3 NF-YA8/nf-ya8 T1 plants carrying the 35S:CDS-NF-YA8 complementation construct.

\(^{b}\)Arrested seeds expected in case of full complementation.

\(^{c}\)Chi-square test for 1/16 (one copy of construct inserted) and 1/64 (two copies of construct inserted) segregation hypothesis.

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future hypophysis and this event is marked by the shift of PIN1 localization in the basal membranes of the provascular cells facing the hypophysis and by the reversal of PIN7 polarity in the basal ends of suspensor cells. Milder defects at the basal embryo pole were observed in the pin7 mutant. In pin7 mutants the embryos fail to establish the apical-basal auxin gradient and the specification of the apical daughter cell of the zygote is compromised because there is a horizontal instead of vertical division. In most cases, the defects are confined to the lower region of the proembryo and in some cases two proembryos developed on top of each other; the lower maintained the morphology of the suspensor and the limit between apical and basal structures was not defined. At the globular stage pin7 mutant shows a recovery of normal phenotype; then, the defects are confined to the basal part of the embryo. Other PIN genes are expressed during embryogenesis; PIN3 in the basal pole of the heart stage embryo and PIN4 in the basal pole of the globular stage embryo after reversal of the gradient, that is in the progeny cells of the hypophysis and in the provascular initial cells of the root meristem. These genes are redundant and the recovery of pin7 corresponds with PIN1 basal localization, PIN4 expression and reversal of auxin gradient. Interestingly, pin1 pin3 pin4 pin7 mutants failed to be recovered and showed serious defects in apical-basal embryo axis determination producing aberrant filamentous globular embryos [43].

Promoter-reporter gene studies using DR5rev::GFP revealed that the GFP signal was completely absent in nf-ya3 nf-ya8 defective embryos, except at proembryo stage when they displayed a normal embryo phenotype. These findings suggest that mutations in NF-YA3 and NF-YA8 may interfere with auxin response in the embryos. In particular, a first possibility is that the inhibition and/or alteration of specific cell divisions in the proembryo and in the suspensor may affect the auxin transport by PIN carriers, leading to embryo patterning defects. In order to validate this hypothesis it will be necessary to check the correct localization of PIN proteins in the mutant embryos. A second possibility is that the NF-Y and PIN genes could have a relationship during early stages of embryo development. The NF-YA3 and NF-YA8 redundant subunits may form equivalent NF-Y complexes with NF-YB and NF-YC subunits [17,62], which could directly or indirectly regulate the expression of PIN genes, thus controlling the regulation of directional auxin transport implicated in embryo developmental processes. Further studies will be necessary to understand whether NF-Y and PIN genes may act together in the determination of the auxin distribution in developing embryos.

Complementation and RNAi experiments showed that NF-YA3 and NF-YA8 are required from the globular stage of embryo development

The complementation experiment performed on nf-ya3/nf-ya3 NF-YAB/nf-ya8 plants revealed that the 35S::CDS-NF-YA3 construct can partially rescue the arrested phenotype of nf-ya3 nf-ya8 mutant embryos. Similarly, when we used the 35S::CDS-NF-YA8 construct to complement nf-ya3 nf-ya8 double mutants, we found three partially complemented lines and one line fully complemented, showing siliques with totally normal seeds. The partial complementation may be due to a low efficient expression of the CaMV 35S promoter during the early stages of embryo development. The 35S promoter has been previously used to complement embryo lethal mutants in Arabidopsis [63–65]. However, it was reported that the CaMV 35S promoter is not active in globular stage embryos, but it is activated in the heart stage embryo [62,66,67]. Possibly, a better complementation experiment would be achieved by using the endogenous NF-YA3 and NF-YA8 putative promoters or the RPS5A promoter [68]. Nonetheless, in this work we showed that overexpressing NF-YA3/NF-YA8 under the 35S promoter was sufficient to partially rescue nf-ya3 nf-ya8 abnormal globular embryos, thus suggesting that NF-YA3 and NF-YA8 are required from the globular stage of embryo development, since abnormal cell division plans can be already observed at the early globular stage. This hypothesis is supported by the GUS expression experiment and the in situ analyses, showing that NF-YA3 and NF-YA8 are already expressed at low levels in the early-globular stage and at higher levels in the globular-heart stage of embryo development.

Interestingly, the suppression of NF-YA3 and NF-YA8 gene expression by RNAi experiments confirmed the complementation results. The RNAi construct showed incomplete penetrance probably because, also in this case, it is controlled by the 35S promoter, the same used for complementation analyses. The small
fraction of RNAi transgenic lines showing embryo defects likely reflects the weak activity of 3SS promoter in globular embryo stages. However, although not all embryos of RNAi::A3/A8 transgenic plants were arrested, we showed that the suppression of the two NF-YA3 and NF-YA8 genes produced defective embryos with mutant phenotypes similar to those of T-DNA insertional double mutant embryos. Moreover, results obtained from quantitative real-time RT-PCR are in agreement with this conclusion. Analysis of RNAi::A3/A8 lines showed that, in transgenic siliques, the absence of NF-YA3 and NF-YA8 endogenous transcripts, respectively, are necessary and sufficient to obtain abnormal globular embryos.

Conclusions

Our results show that NF-YA3 and NF-YA8 are functionally redundant genes required for Arabidopsis early embryogenesis. In particular, we have shown that the nf-ya3 nf-ya8 embryos have an irregular globular shape showing defects in cell divisions in both embryo proper and suspensor. We also found that mutations in both NF-YA3 and NF-YA8 affect the auxin response in early developing embryo, known to be involved in embryo axis establishment by its polar transport. Finally, complementation and RNA interference experiments demonstrated that NF-YA3 and NF-YA8 are new functionally redundant genes specifically required from the globular stage of embryo development.

Supporting Information

Figure S1 In situ analyses using NF-YA3 and NF-YA8 sense probes in developing flower and seed. No hybridization signal was detected in developing flowers (A, C) and embryo at 10 DAP (B, D) using NF-YA3 (A, B) and NF-YA8 (C, D) sense probes. Bars = 50 μm. (TIF)

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Author Contributions

Conceived and designed the experiments: CT KP. Performed the experiments: MF VC. Analyzed the data: SM CT KP. Contributed reagents/materials/analysis tools: CT. Wrote the paper: MF KP.

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