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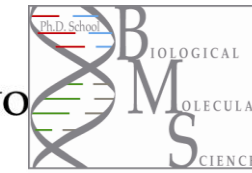

Facoltà di Scienze Matematiche, Fisiche e Naturali
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Corso di Dottorato di Ricerca in Scienze Genetiche e Biomolecolari
Ciclo XXIII

ROLE OF NF-Y TRANSCRIPTION FACTORS IN ARABIDOPSIS EMBRYO DEVELOPMENT

BIO/18

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Ai miei genitori Gianni e Regina,

alla loro forza d'animo nonostante

le difficoltà e alla loro presenza

continua nella mia vita

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INTRODUCTION

In general, organisms must finely regulate gene expression to respond effectively to environmental stimuli. The fundamental idea of gene expression regulation was introduced for the first time from Jacob and Monod: they showed evidence that one gene was associated to one cis-acting sequence, on which trans-acting partners (usually proteins) could act (Jacob and Monod, 1960).

Genes have sequences involved in regulation of their expression; these are localised both upstream and downstream of start codon and they are organised in specific ways for type, number and disposition on DNA; this aspect allows to modulate transcription activity with respect to a simple mechanism on/off.

In eukaryotes, the regulatory sequences are spatially located in three regions with respect to start codon:

- "core", or minimal promoter, that determines the initiation of transcription and it is the shortest sequence on which RNA polymerase can start transcription.

The elements of minimal promoter well characterised are:

- InR (initiator, consensus sequence Py_2CAPy_5), present in almost all promoters, between −3 and +5;
- TATA-box (consensus sequence TATAa/tAa/t), present in about 40% of promoters, in general localised −25 bp from start site;
- DPE (downstream promoter element), present in TATA-less promoters, between +28 and +32.

The minimal promoter functions with low efficiency and other proteins, called activators, are necessary for suitable level of functioning.

- proximal elements, localised between 50 and 200 base pairs from start codon: these elements are recognised and bound by transcriptional

activators. There are essentially three distinct regulatory sequences: CCAAT-box, GC-box and octamer sequences. CCAAT-box is the sequence well characterized, in general located −80/−100 bp from the transcription start site. In TATA-containing promoters the CCAAT-box is preferentially located between -80 and -90 bp, in both orientations and is not found downstream of -50 from the start site. In TATA-less promoters it is usually closer to the +1 signal (at -66 on average) and is sometimes present in proximity to the Cap site (Mantovani, 1998).

GC-box (position -90) is common element of promoters and its sequence is GGGCGG. It is often in more copies and in both orientations.

Finally, octamer sequences (ATTTGCAT sequence) are usually located in a region between -40 and 140 bp from start codon, in variable number and orientation.

- distal elements, called "enhancer" or "silencer", it depends if they are activators or inhibitors of gene expression, respectively. The distal elements can be found at a considerable distance from the transcription start site (thousand base pairs also) and can function independently from orientation, position and distance.

In general, many transcription factors can bind these regulatory elements: they are proteins that recognise a DNA specific consensus sequence.

They play a role as positive or negative transcriptional regulators, recruiting RNA polymerase complex directly, or contacting, by their interaction domains, one or more factors that can operate, a control of the coding region transcription. In this way it is possible to coordinate transcription in each single cell of an organism.

"CCAAT binding proteins" family

The CCAAT-box is one of first regulatory element identified and it is present in promoters of yeast, plants and vertebrates. In a statistical analysis of over 500 promoters, Bucher found that the CCAAT-box is one of the most ubiquitous elements, being present in 30% of eukaryotic promoters (Bucher, 1990), with a strong bias in promoter position. Typically, the CCAAT box is found as a single copy element in the forward or reverse orientation between -80 and -100 of the major start site. The functional importance of CCAAT-boxes has been tested in a variety of diverse experimental systems: there has yet to be study a system in which a CCAAT–box in a canonical position was not shown to play a role in transcriptional regulation. In parallel, specific polypeptides were identified that interact with the pentanucleotidic sequence CCAAT, especially with EMSA and footprinting analyses. The CCAAT motif is present in the promoter of numerous higher eukaryotic genes: constitutive and inducible, tissue-specific or ubiquitously-expressed genes, for example those coding for γ-globulins, major histocompatibility complex (MHC) class II, albumin and regulator genes of sterol biosinthesys (Maity and de Crombrugghe, 1998; Mantovani, 1998, 1999). CCAAT-boxes are extremely conserved within the same gene across species, in terms of position and orientation as well as in terms of nucleotides flanking the central CCAAT pentanucleotide. Considering, for example, the highly conserved *HSP70* genes, CCAAT boxes are found in mammals, *Xenopus laevis*, chicken, maize, *Clamydomonas reinhardti*, but not in *Drosophila melanogaster* (Mantovani, 1999).

Many DNA-binding proteins, containing CCAAT in their acronym, have been characterized: CTF/NF1 (CCAAT Trascription Factor/Nuclear Factor1), C/EBP (CCAAT Enhancer Binding Protein), CDP (CCAAT Displacement Protein). They recognize palindromic sequences, characterized by site-selection analysis,

which are rather different from the widespread consensus sequence which shows no symmetry axis. The NF-Y complex, composed of three distinct subunits NF-YA, NF-YB and NF-YC (also called CBF B/A/C in mouse and HAP 2/3/5 in yeast) strictly requires and specifically binds to the canonical CCAAT sequence, and it is the major, if not the only, CCAAT-box recognizing protein (Mantovani, 1998).

Statistical analysis of over 500 eukaryotic promoters, conducted by Bucher and collaborators (1990), and site-selection experiments showed that nucleotide requirement extend well beyond the central pentanucleotide: the consensus derived from the promoter compilation and from site selection analysis is essentially identical and it is composed by 13 nucleotides: -C,G/A,G/A,C,C,A,A,T,C/G,A/G,C,A/C. In at least three promoters, the sea urchin histone H2B, the human γ-globin and GP91phox-CDP, NF-Y appears to negatively regulate the binding of NF-Y to the CCAAT-box representing a repressor acting on the same target sequence (Barberis et al., 1987; Luo and Skalnik, 1996; Supertifurga et al., 1988)

NF-Y complex

In all species studied, from yeast to echinoderm, from mouse to human, the NF-Y complex is composed of distinct subunits: NF-YA (CBF-B in mouse, HAP2 in yeast), NF-YB (CBF-A, HAP3) and NF-YC (CBF-C, HAP5), all required for DNA binding and coded by a single gene in the genome (Maity and de Crombrugghe, 1998; McNabb et al., 1995).

Comparative analyses of protein sequences showed that each subunit contained an evolutionary conserved part: 56 amino acids at the C-terminal of the human

NF-YA; 90 in the central part of NF-YB and 84 at the N-terminal of NF-YC with identities higher than 70% across species (Fig. 1). Neighbouring regions are not conserved at the primary level (Li et al., 1992; Miyoshi et al., 2003).

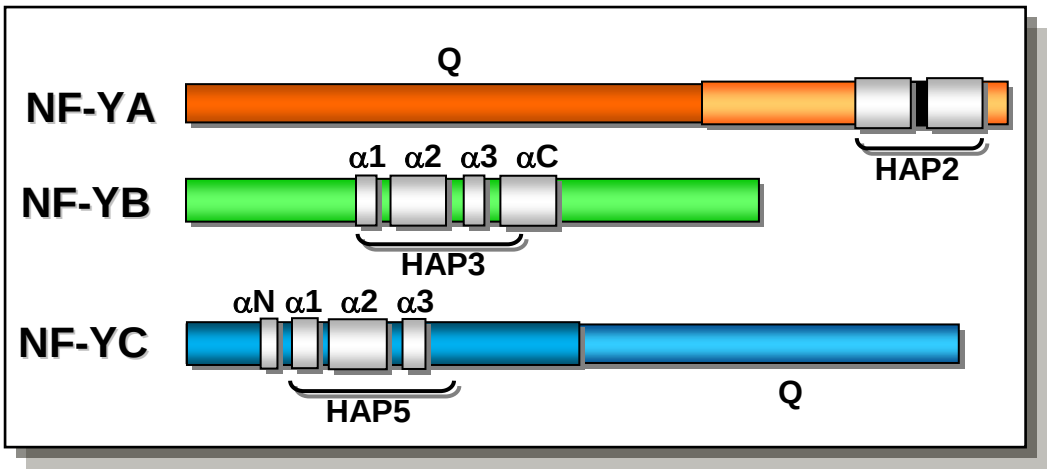

Fig. 1 - Schematic representation of the *NF-Y* genes. The yeast homology domains are indicated by brackets. White boxes in NF-YB and NF-YC indicate the position of the four α-helices of the histone fold domains. Q is a domain rich in glutamines with activation function. (Mantovani, 1999).

In mammals, in sea urchin and in some of the plant *NF-Y* genes, the NF-YA and NF-YC (but not NF-YB) subunits have large domains rich in glutamines and hydrophobic residues. According to a scheme which is classical for transcription factors, the most conserved parts are required for DNA-binding, and whatever protein-protein interactions are necessary for this function, while the less conserved Q-rich domains contain the activation function (Liberati et al., 1999).

The biochemistry of subunits association and the protein domains required have been studied in details in mammals: the NF-YB-NF-YC subunits form in the cytoplasm a tight dimer that translocates to the nucleus where the third subunit, NF-YA, is recruited to generate the mature heterotrimeric NF-Y transcription

factor (Frontini et al., 2004). The resulting trimer can then bind to DNA with high specificity and affinity (Mantovani, 1999) (Fig. 2)

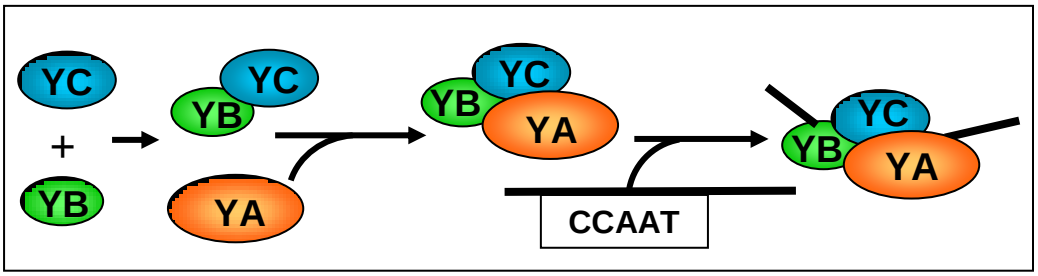

Fig. 2 – Association of NF-Y subunits and binding to DNA, and interaction with various transcriptional activators. NF-YB and NF-YC form a tight dimer, this results in a surface for NF-YA association and the trimer can then bind to CCAAT specific sequence on the promoter (Mantovani, 1999).

The transcriptional activation functions are in the large N-terminal region of NF-YA and in the C-terminal region of NF-YC, as assessed by in vitro transcription studies with recombinant proteins and in transfections of mammalian cells with LexA and GAL4 fusions (Coustry et al., 1998; Li et al., 1992). In the yeast *S. cerevisiae* and *S. pombe*, NF-Y subunits have no Q-rich domains and hydrophobic amino acids, a finding in line with the lack of function of these domains in *S. cerevisiae*. In both these organisms, but apparently not in other species, an additional subunit, HAP4, is present. It associates with the trimer providing an activation surface to the complex, but it is not required for CCAAT-box binding (Forsburg and Guarente, 1989; Stebbins and Triezenberg, 2004). In higher eukaryotes, this function has apparently been incorporated in NF-YA and NF-YC, while DNA-binding and subunits association require the yeast homology domains. In NF-YB and NF-YC conserved domains there is a

characteristic histone-fold structural motif. The NF-YA conserved region, required for the NF-Y/DNA complex, does not resemble a histone-fold motif, other protein-protein interaction motives and any of the known DNA-binding.

The histone fold motif

Analysis of protein sequence alignments suggested that these domains of NF-YB and NF-YC contain a histone-like motif (Baxevanis et al., 1995). Histones are among the most conserved proteins in evolution: they contain a 65 amino acid histone fold motif (HFM) shared with low sequence identity (14/18 %), but high structural resemblance to all histone proteins. The HFM consists of a short α-helix (α1) followed by a loop and a β-strand segment (L1), a long helix (α2), another short loop and β-strand (L2), and a short α-helix (α3); β-strand of two monomers associate (L1-L2) to form a short β-sheet or β-bridge (Maity and de Crombrugghe, 1998; Ramakrishnan, 1997). The high-resolution crystal structures of nucleosomes revealed that the histone-fold motives associate with each other in an antiparallel manner to form homodimers (Arents and Moudrianakis, 1995; Luger et al., 1997; Ramakrishnan, 1997) (Fig. 3).

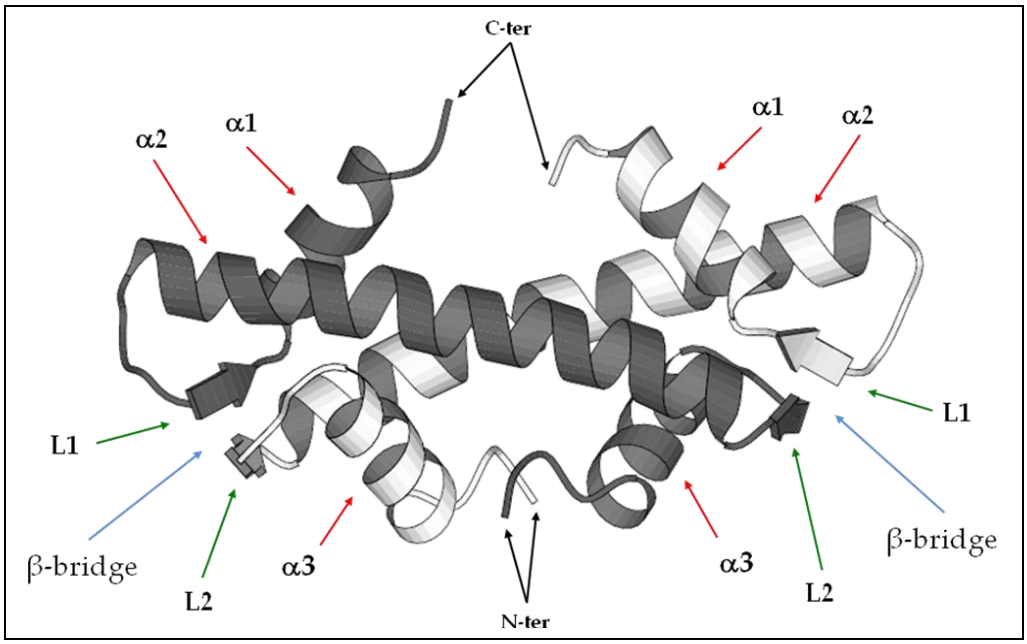

Fig. 3 – The histone homodimer. Antiparallel association of histone-fold motif in an histone homodimer. α1, α2, α3 indicate the three α-helices; L1 and L2 indicate the β-strands (picture modified from Ramakrishnan, 1997).

Comparison of NF-YB and NF-YC conserved parts with H2A, H2B, H3, H4 core histone reveals that NF-YB subunit belongs to the H2B, and NF-YC to the H2A families. Mutagenesis performed in rat and yeast providing evidence that the integrity of the HFMs is absolutely required for dimer formation with NF-YC, that also requires specific amino acids in α2, L2 and α3 (Maity and de Crombrugghe, 1998; Sinha et al., 1995; Xing et al., 1993). NF-YB/NF-YC complex has three distinct domains: one localized in α2 of NF-YB and the others two in α1 and L1 of NF-YC, necessary for interaction with NF-YA. Neither NF-YB/NF-YC nor single NF-Y subunits are able to bind DNA; only the heterodimer can form NF-Y/DNA complex.

NF-YA

It is quite clear that most of the sequence-specific interactions of the trimer are made by NF-YA subunit (Zemzoumi et al., 1999). Unlike NF-YB-NF-YC, the NF-YA homology domain does not resemble any of the known DNA-binding motifs. The ratio of absolutely conserved amino acids in the HAP2-homology domains of NF-YAs is higher, 20 out 56 residues: all these residues were shown to be important either for protein-protein interaction or for CCAAT binding (Xing et al., 1993; Xing et al., 1994). Contrary to the HFM subunits, the NF-YA conserved domain can be sharply divided in two distinct halves separated by linker regions, each 20 amino acids long, and they are predicted to form amphipathic α-helices: the N-terminal part is required for NF-YB-NF-YC association and the C-terminal for DNA-binding. Mutations in region required for DNA-binding are dominant negative, the NF-YA mutant is capable of forming a trimeric complex, but rendering it incapable to bind CCAAT-boxes because of mutations in key DNA-contacting residues (Mantovani et al., 1994).
Interestingly, the linker regions between the two sub-domains are considerably more variable both in composition and in length. Although the known function of this stretch is apparently confined to connecting subunits-interactions and DNA-binding, it may help interact with additional transcription factors (Gusmaroli et al., 2001; Mantovani, 1999).

Role of NF-Y factors in transcriptional regulation

The NF-Y complex binds the CCAAT promoter element and this can determine positive as well negative transcriptional regulation (Ceribelli et al., 2008; Peng

and Jahroudi, 2002, 2003). Peng and Jahroudi demonstrated that the NF-Y complex has a dual function in transcriptional regulation of VWF (human Von Willebrand factor): it is an activator in endothelial cells and a repressor in non-endothelial cells. NF-Y is an activator when interacts with CCAAT sequence, but it functions as a repressor when interacts with another NF-Y binding sequence located in the first exon and not conform to the consensus NFY-binding sequence CCAAT. Repression is mediated by NF-Y association with DNA-binding factors and cofactors. GATA6 is the GATA family member that interacts with the VWF promoter and is associated with NF-Y specifically in non-endothelial cells. NF-Y recruits histone deacetylases (HDACs) to the VWF promoter, which may result in deacetylation of GATA6 factor as well as of histones in non- endothelial cells, thus leading to promoter inactivation.

In the promoter region, the CCAAT-box is invariably flanked by at leat one functionally important promoter element. It has been shown that the distance between the two elements is important and variations by half helical turns result in dramatical negative effects on transcription (Kusnetsov et al., 1999; Wright et al., 1995). This implies that the reciprocal interplay of NF-Y and the given transcription factor is essential for the function of the unit. In some case, NF-Y considerably increases the affinity of the neighbouring factor for DNA making the two complexes much more stable on the DNA (Jackson et al., 1998). In human, for example, the connections of NF-Y with sterol regulatory element binding proteins (SREBPs) regulate expression of genes involved in sterol metabolism. SREBP binding to the SRE sequence target is enhanced by NF-Y complex; the mechanism is not completely clear, but direct protein-protein contacts on the DNA cannot be excluded (Dooley et al., 1998).

In addition to promoting and/or stabilizing DNA binding of nearby activators, at least one other mechanism of NF-Y-mediated synergism has been documented. In the albumin promoter, a high affinity CCAAT is juxtaposed to a C/EBP

(CCAAT/Enhancer Binding Protein) site and the two synergize functionally. So that transcription function, the two proteins must interact strictly and if two site are artificially moved away, proteins DNA-binding occurs but not the transcription (Milos and Zaret, 1992). Thus, precise protein-protein connections, indipendent of cooperative DNA-binding, are essential for some transcriptional unit.

Further studies demonstrated that TBP (TATA-binding protein) has affinity for NF-YB and NF-YC (Bellorini et al., 1997); TBP recognises TATA-box and, in association with TAFIIs, formes transcriptional complex TFIID that is necessary for RNA polymerase recruiting.

A few studies have started to address the chromatin structure of NF-Y dependent regulatory regions in vivo: in particular in the *Xenopus laevis HSP70* promoter, the CCAAT-box facilitates the activity of Heat Shock Factor and of the general machinery by preventing deposition of nucleosomes on the core promoter (Landsberger and Wolffe, 1995).

It was also observed that NF-Y complex substitutes H2A and H2B histones on active cell-cycle promoters and recruits CoREST, nucleosome binding factor. In this way, NF-Y participates of H3 methylation modulation (Gatta and Mantovani, 2008).

Interestingly, recent studies demonstrated the interplay between NF-Y and p53, a master transcription factor controlling the response to stress signal endangering genome integrity, often mutated in human cancer. The connection between p53 and NF-Y is crucial in determing cell survival or death. In particular, the NF-Y/p53 -and p63, p73- interaction results in transcriptional repression, under DNA-damage conditions, of a subset of genes activated by NF-Y in normal condition. Moreover, recent data shows that p53 activates important pro-apoptotic genes by associating to the NF-Y/CCAAT complex. NF-Y is also involved indirectly in pro-apoptotic activities by being targeted by

a p53-induced non coding RNA involved in apoptosis suppression. In addition, TopBP1, a protein involved in DNA repair and replication, targets NF-Y in tumor cell with mutant p53 (Imbriano et al., 2012).

The CCAAT box-binding transcription factor NF-Y seems to have a role in the regulation of human proteasome genes and may be a potential target for cancer therapy. The basal expression of CCAAT box-containing proteasome genes is regulated by NF-Y complex and is known that protein degradation by the proteasome plays an important role in all major cellular pathways; in fact, the aberrant proteasome activity is associated with numerous human diseases including cancer and neurogical disorders (Xu et al., 2012).

NF-Y genes in *Arabidopsis thaliana*

Compared to the remarkable amount of biochemical information obtained in yeast and in mammals, our knowledge of the biology of NF-Y genes in plants is still rudimentary.

Redundancy of regulatory genes is far from unusual in plants, where many transcription factors are duplicated to amazing numbers: in mammals, MYB transcription factors comprise a small family of three proteins involved in the control of cell proliferation, while in *Arabidopis thaliana*, more than 100 such proteins have been reported, believed to determine a full range of developmental and adaptive responses (Kranz et al., 1998). In particular, *R2R3-MYB* genes control various aspects of plant secondary metabolism, such as cells identity and fate (Stracke et al., 2001).

EMSA assay (Electrophoretic Mobility Shift Assay) on protein extracts from several tissues or organs of *Arabidopis thaliana* with a short Y-box

oligonucleotide containing a canonical NF-Y binding site, showed several retarded bands obtained with every organ, suggesting that multiple NF-Y complexes are indeed present (Gusmaroli et al., 2001).

TBLASTN analyses on the GenBank ESTs and Genomic Database using entire protein sequences of the human NF-YA, NF-YB and NF-YC subunits (Gusmaroli et al., 2002), and following BLAST analyses using amino acid sequences from highly conserved (across genera) regions (Siefers et al., 2009), allowed to identify 36 Arabidopis sequences: 10 NF-YA, 13 NF-YB and 13 NF-YC that can theoretically combine to form 1690 unique complexes. All sequences obtained encode genes present in not uniform way on five chromosomes: ten on chromosome I, six on II, six on III, one on IV and thirteen on V. All sequences were aligned and all phylogenetic trees were determined and constructed using MEGA4 program (Siefers et al., 2009). (Fig. 4).

Note that NF-YB12 and NF-YB13 are sometimes included in a separate DR1-related, two-member protein family (CCAAT-DR1 transcription factor family) (Riechmann et al., 2000; Siefers et al., 2009). Nevertheless, NF-YB12 and NF-YB13 clearly show much stronger homology to the larger NF-B family than to any other proteins, and there is currently no functional evidence to differentiate them as unique. Thus, we consider them to be divergent, but related, NF-YB family members (Siefers et al., 2009).

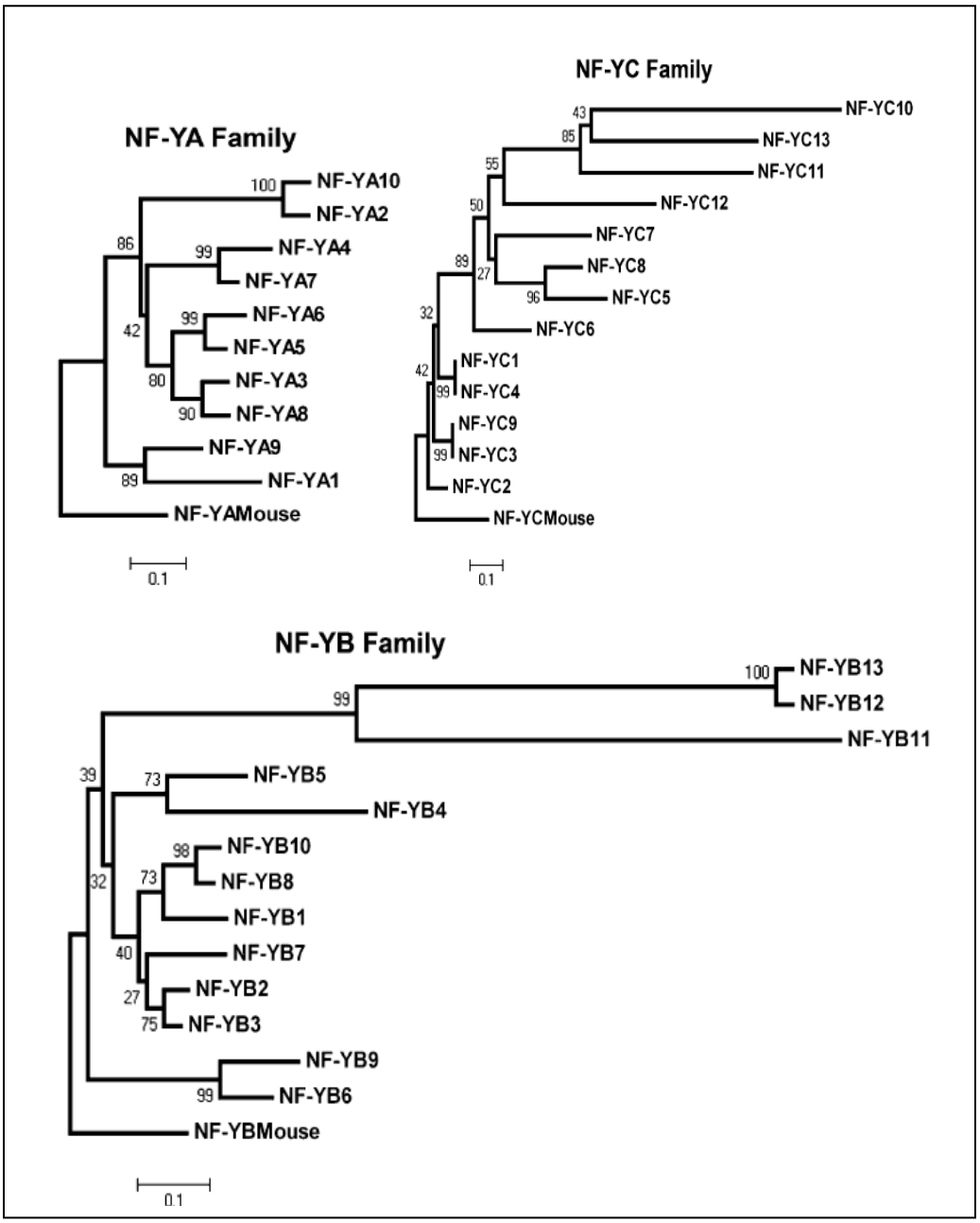

Fig. 4 - NF-Y family phylogenies. Phylogenetic trees for each family were constructed by neighbor joining using conserved regions. Reliability values at each branch represent bootstrap samples (2000 replicates). All trees were determined and constructed using MEGA4 (Siefers et al., 2009).

NF-YB and NF-YC families have well-described subunit interaction and DNA-binding domain. For the majority of NF-YB and NF-YC proteins, required amino acids are well conserved; phylogenetically distant Arabidopsis family members, such as NF-YB11 to -YB13 and NF-YC10 to -YC13 are much more likely to have undergone changes in required amino acids. Because the required amino acids are so highly conserved across evolutionary time and space, it is likely that nonconservative changes will significantly alter protein function (Siefers et al., 2009). Between subunits sub-families can be traced: NF-YA5 and NF-YA6 have very similar C-terminal ends, as it is the case for NF-YB6 and NF-YB9; NF-YC6, NF-YC7, NF-YC5 and NF-YC8 are very similar at the N-terminal. It should be noted that N-terminal of all NF-YAs and N- and C-terminals of NF-YCs are particularly rich in glutamines and hydrophobic residues; this feature is suggestive of the presence of transcriptional activation domains: the corresponding regions in mammals and *Xenopus* indeed incorporate this function. This would be a strong indication that, unlike in fungi where a fourth subunit (HAP4) is required, *Arabidopsis* lacks an HAP4-like equivalent and the transcriptional activation function is incorporated in the trimer (Gusmaroli et al., 2001).

A precondition for formation of functional NF-Y complexes is the translocation of the subunits into the nucleus. In Arabidopsis, recent GFP fusion assay demonstrated that NF-YA and NF-YC were exclusively detected in the nucleus, while NF-YB are found to be localized in the cytoplasm. In contrast to NF-YA and NF-YC, all NF-YBs lack nuclear localization signal (NLS) and is solely imported into the nucleus after necessary interaction with NF-YC, as a NF-YC associated heterodimer, while NF-YA and NF-YC are imported into the nucleus without auxillary assistance (Hackenberg et al., 2011). Yeast two-hybrid experiments demonstrated that 10 NF-YBs (NF-YB1 to NF-YB10) and seven NF-YCs (NF-YC1 to C4, C5, C9, and C12) interact in high number of

combinations to form different heterodimers and they form an interactomic network among Arabidopsis NF-YB and NF-YC subunits. Moreover, these experiments indicated that, as already previously shown for the assembly of the trimeric human complex NF-Y *in vitro* (Mantovani et al., 1999), the initial formation of the NF-YB/NF-YC heterodimer is predicted to be obligatory for a stable interaction of Arabidopis NF-YA with the other two subunits. In accordance with a stepwise assembly mechanism described for NF-Y assembly of filamentous fungus *Aspergillus Nidulans*, *Saccharomyces cerevisiae*, and *Homo sapiens* (Kahle et al., 2005; McNabb and Pinto, 2005; Steidl et al., 2004), the plant NF-YA apparently requires the combinated protein surface of the NF-YB/NF-YC dimer. In addition, the nuclear translocation of NF-Y in Arabidopsis is not regulated by cellular redox; in fact based on structure model of the HFM, disulfide bonding among intramolecular conserved cysteine residues of NF-YB, which is responsible for the redox-regulated assembly of NF-YB and NF-YC in human and *Aspergillus Nidulans*, can be excluded for Arabidopsis NF-YB (Hackenberg et al., 2011).

Reproductive processes in *Arabidopsis thaliana*

Arabidopsis (Fig. 5) is a model plant with separate vegetative and reproductive growth phases. Following germination, the apical meristem produces leaves with little elongation between successive leaves forming a rosette. When the plant becomes florally induced, the apical meristem switches to producing flowers. Subsequent to the production of the first few flower primordia, the plant bolts due to increased internode elongation between the uppermost leaves and between flowers. Thus, the basal positions on the primary inflorescence shoot are occupied by a small number of cauline (stem) leaves and the apical

positions by a potentially indeterminate number of flowers. Secondary inflorescence meristems that reiterate the pattern of development of the primary inflorescence shoot develop in the axil of each of the cauline leaves of the primary inflorescence shoot and tertiary inflorescence shoots arise in the axils of the cauline leaves on the secondary inflorescence shoots. In addition, further inflorescence meristems develop in the axils of the rosette leaves (Bowman, 1994).

The mature flower of *Arabidopis thaliana* is perfect and hermaphrodite; it is composed by four sepals, four petals, six anthers and a gynoecium (Fig. 5).

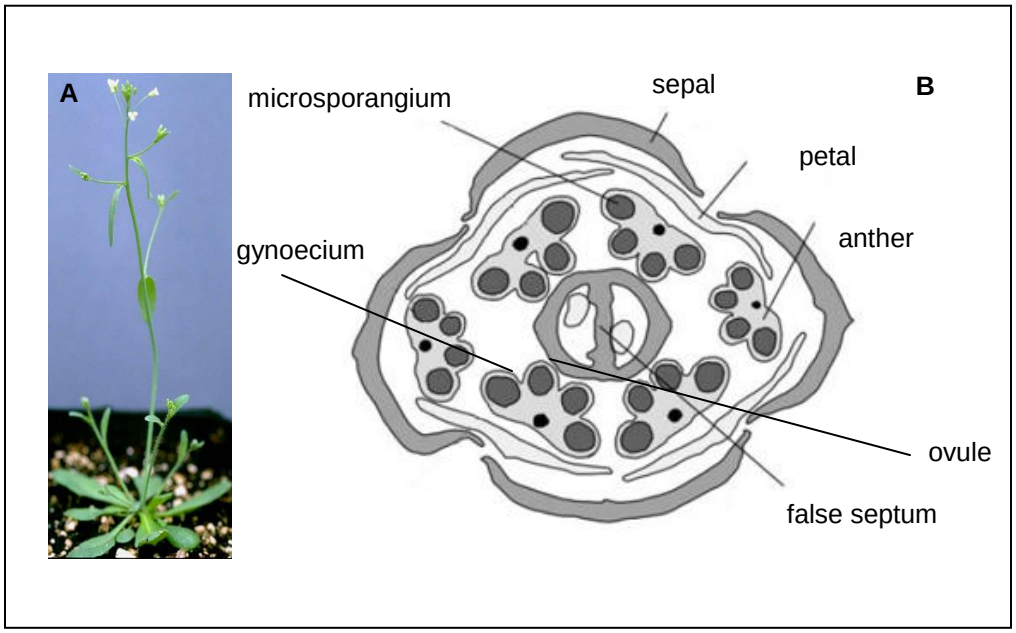

Fig. 5 – *Arabidopsis thaliana* (A) and scheme of a transverse section through an Arabidopis floral bud (B).
(B) The figure show the number, position and orientation of the floral organs (figure modified from Scott et al., 2004).

Ovule development

The gynoecium (or ovary) of Arabidopis consists of two congenitally fused carpels that develop as a single cylinder. The ovary is divided into two locules by a false septum (Fig. 5) and contains the female gametes, the ovules. The ovule primordial arise from parietal placental tissue at the margins of fusion of the carpels, where the septum merges with the ovary wall (Bowman, 1994).

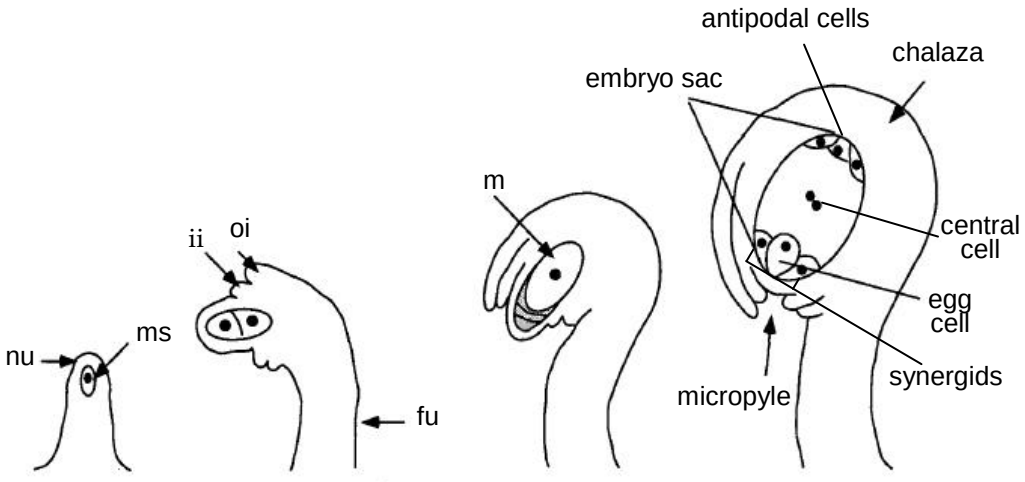

Fig. 6 – Arabidopsis ovule development. Stages of ovule development. **fu**, funiculus; **m,** functional megaspore; **ms**, megaspore mother cell; **nu**, nucellus; **oi**, outer integument; **ii**, inner integument (figure modified from Reiser and Fisher, 1993)

Nucellus (megasporange) arises from apical portion of ovule primordium and contains megaspore mother cell (Fig. 6). Then the funiculus, that connects the ovule at the ovary wall, begin to differentiate; this is followed by integuments initiation (outer and inner) at the base of the nucellus region. The integuments elongate and, at maturity, don't completely overgrow the embryo sac: an aperture remains, called micropyle (Bowman, 1994).

In the meantime, the megaspore mother cell undergoes meiotic divisions to form four megaspores. Three of these degenerate and one (functional megaspore) undergoes four mitotic divisions to form the cells of embryo sac. The embryo sac of Arabidopsis consists of seven cells that contain eight nuclei: the egg, two synergids, a single diploid central cell, and three antipodals (Fig. 6) (Bowman, 1994). The egg and synergids are intimately arranged in the micropylar chamber of the embryo sac, there forming what is known as the egg apparatus. The central cell separates the egg apparatus from the triad of antipodals in the chalazal portion of the embryo sac. A total of 40-60 ovules are produced in four rows, with the two rows of ovules in each locules interdigitating (Bowman, 1994).

Pollen development

Arabidopis androecium consists of six stamens, formed by an anther and a filament which transmits water and nutrients to the anther and connects the anther at the rest of the flower. The archesporial cells, located at each of four corners of immature anther, form pollen sacs (microsporangium) (Fig 7) (Bowman, 1994; Scott et al., 2004).

The archesporial cells undergo periclinal divisions to give rise to the primary parietal and primary sporogenous layers. The primary parietal layer undergoes two more periclinal divisions such that three anther wall layers are eventually formed. The epidermis of anther encloses the three anther wall layers of anthers: the outermost of the three layers is the endothecium, the innermost (adjacent to the sporogenous tissue) is the tapetum, and the middle one is appropriately termed the middle layer (Fig. 7).

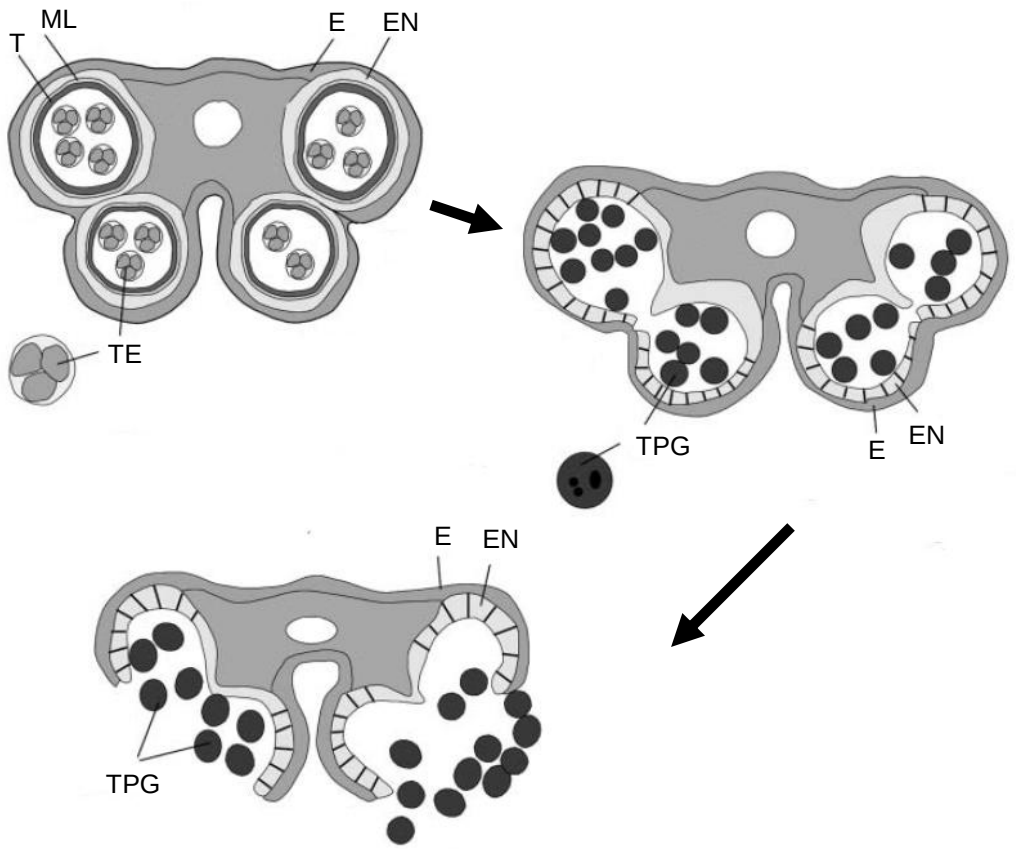

Fig. 7 – Arabidopsis pollen development.
Scheme of a transverse section through an Arabidopis anther at different developmental stages. **E**, epidermis; **EN,** endothecium; **T**, tapetum; **TE**, tetrads of miscrospores; **TPG**, tricellular pollen grains; **ML**, middle layer (figure modified from Scott et al., 2004).

The tapetum is composed by nurce cells with a nutritive primary function: they have to assist the pollen grain development. The primary sporogenous cells develop into pollen mother cells, that undergo two meiotic divisions (microsporogenesis) and form haploid tetrads of microspores (Fig. 7) (Bowman, 1994). Each tetrad is surrounding by callose wall that is broken down after meiosis by callase enzyme produced by tapetal cells; in this way the individual microspores are released in the anther locules. Then the microspores round up and their wall thicken due to sporopollenin exine deposition by tapetal cells and

the protoplast produces an inner polysaccharide intine layer that thickens further on the wall of microspores (Bowman, 1994). The protoplast undergoes a first asymmetric mitotic division resulting in a large vegetative cell (that forms the pollen tube) and a small generative cell. Subsequentely, the second mitotic division occurs, the generative cell divides and gives rise two cells with spermatic nucleus; the mature pollen grains are tricellular (Fig. 7). Finally, the anther walls and the pollen grains desiccate and the anthers dehisce, releasing functional pollen (Fig. 7).

Embryogenesis

Embryogenesis in Arabidopsis is a developmental process divided into three distinct phases. The early phase is morphogenesis, during which embryo apical-basal pattern formation occurs; in particular the basic body plan of the plant is established with the specification of the shoot-root axis of polarity and the formation of the embryonic organ and tissue systems. The second phase is maturation, during which macromolecules, particularly storage proteins, lipids and carbohydrates are synthesized in order to support seedlings development during germination phase; finally, late embryogenesis, a preparation phase for seed desiccation, inhibition of precocious germination, and establishment of dormancy (Goldberg et al., 1994; West and Harada, 1993). In this phase, the embryo becomes quiescent metabolically until environmental favourable conditions activate the germination (Parcy et al., 1997; Harada, 2001) (Fig. 8).

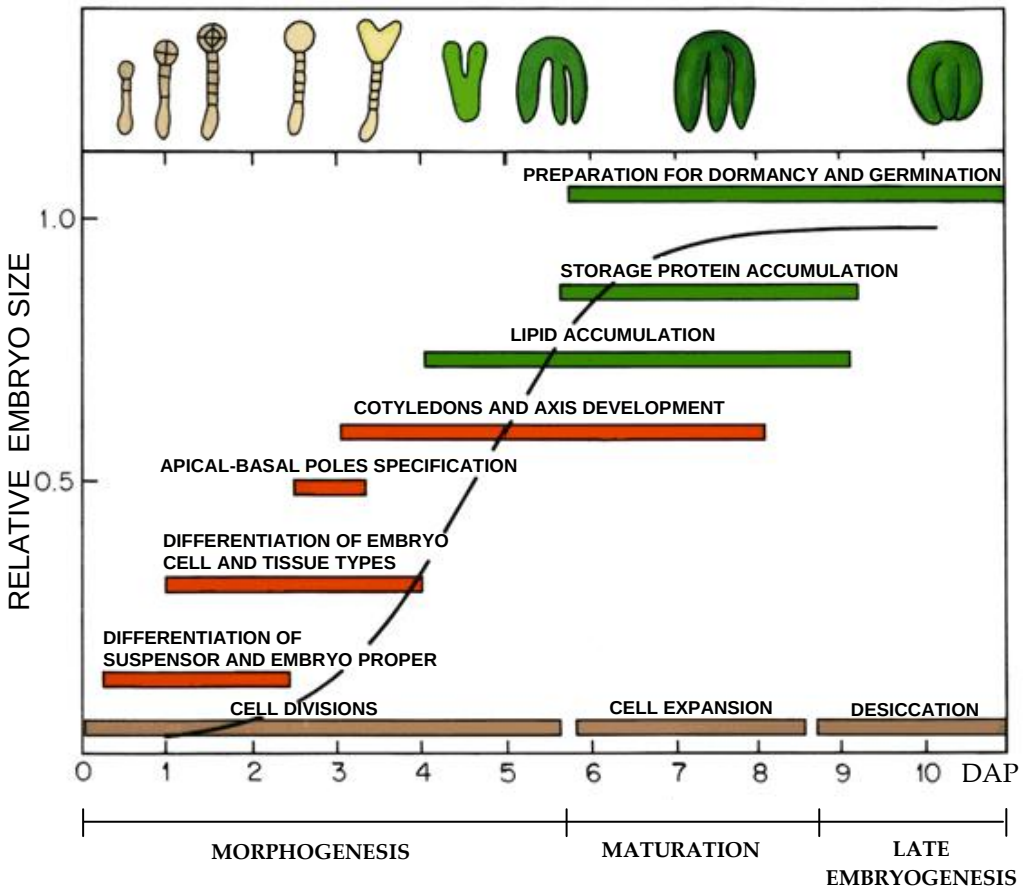

Fig. 8 – Three phases of embryogenesis in Arabidopsis.
Embryonic phases in relation to embryo relative dimension and all the processes during embryo development. DAP indicates Day After Pollination (figure modified from http://www.mcdb.ucla.edu/Research/Goldberg/Images/Arab-seed-development-cart.gif).

The different phases of embryogenesis are moreover divided in embryonic stages, according to the number of embryo cells or the shapes of the developing embryo:

- proembryonic phases: zygote, 2/4/8/16-cell stage

- late phases: globular stage, heart stage, torpedo stage, walking stick and upturned-U stages (Fig. 9). In these phases three major plant tissues developed: the protoderm that becomes the epidermis; the fundamental meristematic tissue

and the innermost procambium, that forms vascular tissue. Two major organ systems are also formed during this phase: the cotyledons and the axis comprising the shoot apical meristem (SAM), hypocotyls, root and root apical meristem (RAM) (Harada, 2001) (Fig. 9).

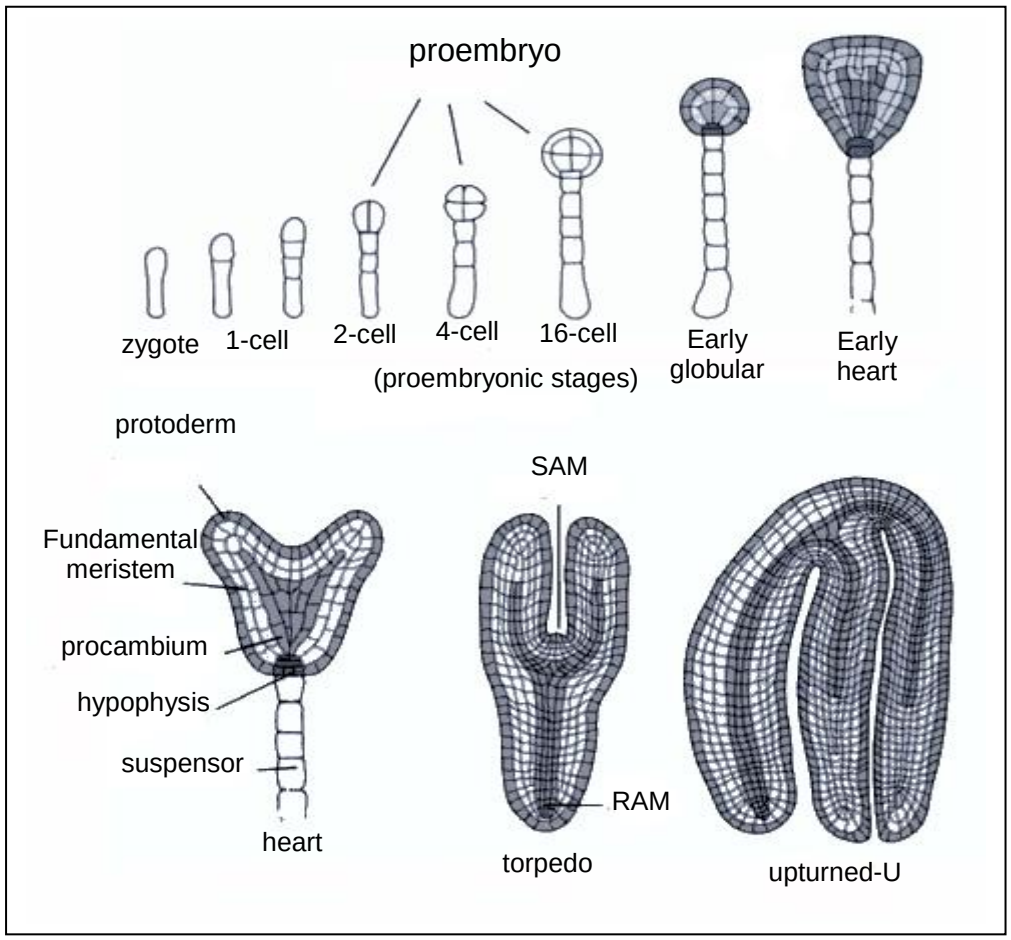

Fig. 9 – Stages of embryo development. Early stages are called according to the number of proembryo cells, late stages according to the embryo shapes. The tissutal differentiation is indicated (histogenesis) with different degrees of grey (figure modified from Howell, 2000).

Proembryo phases begin with the double fertilization event: two gametes of the pollen grain enter in the ovule carried by pollen tube and passing through the

micropyle, aperture formed by non complete overgrowth of ovule integuments (Fig. 10). Pollen tube releases the two sperm nuclei: one of sperms fuses with the egg cell to form zygote diploid and the second sperm fuses with the diploid central cell to produce the triploid endosperm mother cell (Bowman, 1994; Harada, 2001).

Endosperm development progresses through four phases: syncytial, cellularization, differentiation and death. The syncytial stage of endosperm development is initiated by successive divisions of the triploid nucleus without cytokinesis. This generates a multinucleate syncytium where the cytoplasm is organized into a nuclear cytoplasmatic domain (NCD) defined by a radial microtubule system. The cytoplasm is then compartimentalized into tube-like structures called alveoli. Periclinal divisions in the alveoli lead to the process of cytokinesis and cellularization of the endosperm. Cellularization begins in the region surrounding the developing embryo and coincides with the initiation of cotyledon development in the embryo. A wave of cellularization then spreads toward the chalazal region. Then, during cotyledons expansion and elongation, endosperm cells undergo apoptosis, a process triggered by ethylene (Chaudhury et al, 2001).

The roles for the endosperm include maintenance of high osmotic potential around the embryo, mechanical support during early embryo growth and storage of reserves, nutrients and hormones to facilitate and support seed germination. In Arabidopsis, where the endosperm does not persist and the cotyledons form the primary storage organ, the endosperm promotes early growth of the embryo until sufficient reserves are stored in the cotyledons (Chaudhury et al, 2001).

Following fertilization, the diploid zygote divides asymmetrically and transversely, producing a small apical or terminal (chalazal) cell and an elongate basal (micropylar) cell. The smaller apical cell will give rise to the embryo, except for the root. The larger basal cell will give rise to the radicle as well as

the extraembryonic suspensor through which nutrients are supplied to the developing embryo from the mother plant (Fig. 10).

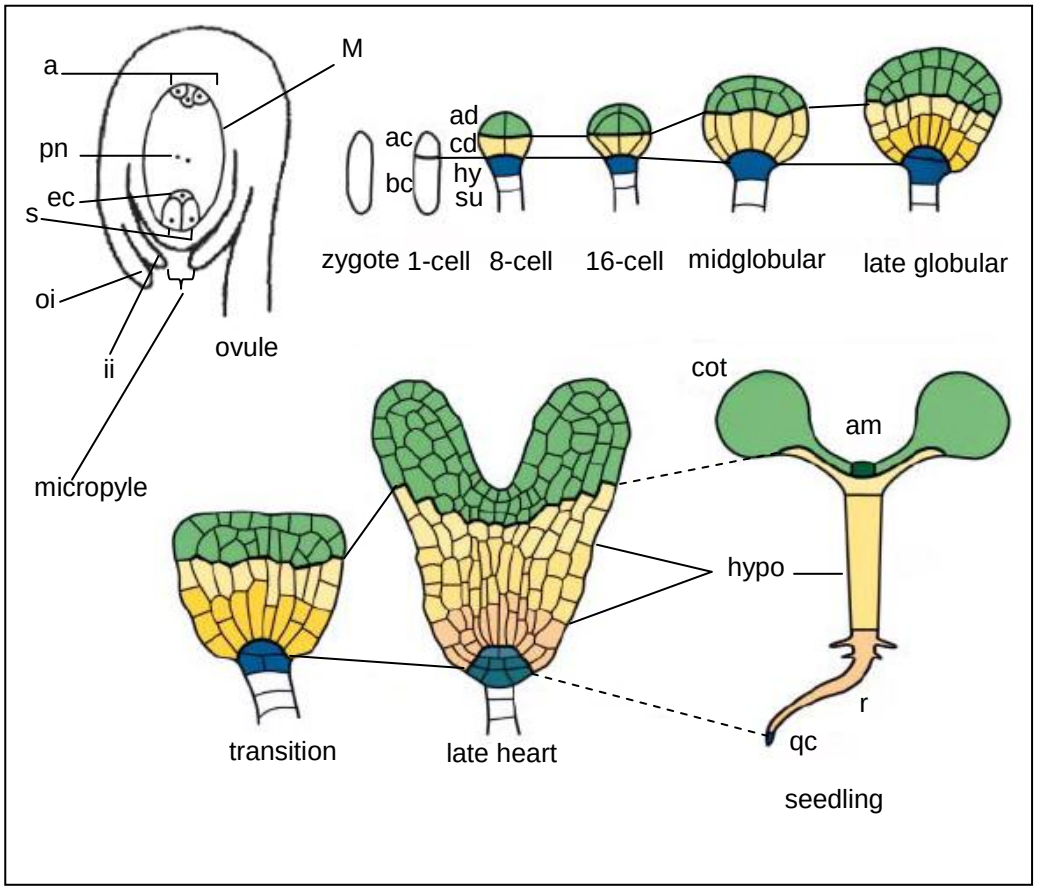

Fig. 10 – Ovule organization and apical-basal development of *A. thaliana* embryo.
Schemes of longitudinal median sections. The upper and lower thick lines represent clonal boundaries between the descendants of the apical and basal daughters cells of the zygote and between the apical and central embryo domains, respectively. **a**, antipodal cells; **ac**, apical daughter cell; **ad**, apical embryo domain; **am**, apical meristem; **bc**, basal daughter cell; **cd**, central embryo domain; **cot**, cotyledon; **ec**, egg cell; **hy**, hypophysis (embryo basal domain); **hypo**, hypocotyl; **M**, megagametophyte; **pn**, polar nuclei; **qc**, quiescient center; **r**, root; **s**, synergids; **su**, suspensor (figure modified from Laux et al., 2004).

The apical cell undergoes a longitudinal division to produce the two-terminal cell embryo, each cell being equal in size. Each of the cells of the two-terminal

cell embryo then undergo longitudinal divisions resulting in a quadrant embryo. Each of the four apical cells divides transversely, producing the octant embryo composed of two tiers of four cells each. The shoot meristem and the flanking cotyledon primordia origins from the upper tier cells (apical region); the lower tier (central region) gives rise the hypocotyl, the embryonic root and the upper tiers of root meristem cells. The basal cell of the zygote divides transversely to produce a file of 7-9 highly vacuolated cells; the uppermost daughter of the basal cell, called hypophysis, becomes part of the primary root system, whereas the remaining daughters form the extraembryonic suspensor (Jürgens, 2001) (Fig. 10).

Each of the eight cells derived from the apical cell of the octant embryo divides periclinally to produce a 16-cell embryo (dermatogen or early globular stage) (Fig. 10) with eight inner and eight outer, or protodermal cells. Development of the suspensor is complete by the early globular stage. The inner cells give rise to ground tissue and procambium while the epidermis derived from the outer cells. In this stage fundamental meristem interposes between protoderm and vascular primordium (Fig. 9).

Then, the cells continue to undergo division producing 32-cell (midglobular stage) and 64-cell stage embryo (late globular stage); at this time cell division in the protoderm increases and this marks the site of the future cotyledons. The cells division producing the cotyledon primordia are rapid and leads to the heart stage embryo (Fig. 9-10). During the heart stage, the cotyledon primordia enlarge, cell divisions occur and cells in the hypocotyl and radicle elongate (Bowman, 1994). The embryo assumes an elongated shape, typical of the torpedo stage (Fig. 9). In the later stages, the embryo increases in size and the cells differentiate (Mansfield and Briarty, 1991, 1992). Moreover, differentiation of tissues begins to accelerate with the plastid differentiation leading to the “greening” of embryo.

The root and shoot meristems, both of which have their origins in embryogenesis, are ultimately responsible for the postembryonic architecture of the plant. The two meristems differ markedly in the timing of their origin as well as their cellular organization. The root meristem is formed relatively early in embryogenesis (by the mid-heart stage) (Fig. 10); in contrast, the shoot apical meristem arises later in embryogenesis, during the torpedo stage (Fig. 9). The shoot meristem is easily identifiable in later stage embryos as storage granules do not accumulate in the cells of the shoot meristem (Bowman, 1994).

The embryo enters now in the maturation phase characterized by cell differentiation and an increase in embryo size: the torpedo stage embryo elongates into "walking stick" embryo, then the cotyledons grow and curve over forming the "upturned-U" embryo (Fig. 9). This passage is due to cell division and subsequent cell expansion; these stages are also characterized by deposition of protein, lipid, starch and sugar reserves in the embryo that takes place over several days (Bowman, 1994). In particular lipid deposition starting in the torpedo stage and protein deposition at the upturned-U stage (Fig. 8). During these stages, expansion of the embryo forces it into the fully cellularized endosperm. Continued cell expansion throughout the embryo causes it to fill most of the embryo sac, crushing the endosperm formed earlier during embryogenesis and presumably absorbing it (Mansfield and Briarty, 1992). By maturity, the cotyledons are tightly appressed to the radicle, leaving little open space in the embryo sac, with only a single persistent layer of endosperm remaining (Bowman, 1994).

Finally, with desiccation, the late embryogenesis begins. In this stage the rate of reserve deposition is curtailed significantly and there is a slight decrease in embryo size (Bowman, 1994). The embryo enter a metabolically quiescent state that may persist before favourable hormonal and environmental conditions, as light and humidity, induce germination.

In plants, regulation of embryogenesis is studied by isolation and characterization of mutants with embryo defects. Literally hundreds of mutations have been isolated that disrupt embryogenesis in Arabidopis. The spectrum of isolated mutants can be broken down into categories: (1) embryonic lethal mutants in which embryogenesis is arrested; (2) mutants defective primarily in the accumulation of storage products; (3) abnormal seedling mutants with altered pigmentation; (4) mutants with altered seed morphology but normal maturation programs; (5) mutants with fundamental defects in the regulation of late embryogenesis; for example *leafy cotyledon* mutant, in which cotyledons are partially transformed into leaves (Bowman, 1994; Meinke et al., 1994).

Early embryogenesis and the establishment of the an apical-basal axis of polarity

Early embryogenesis is the critical developmental phase during which the basic features of the plant body are established: the apical-basal axis of polarity, different tissue layers, and both the root pole and the shoot pole.

Several genes are involved in the early events of embryo development and their mutations determine specific defects in apical-basal embryo axis determination. For example, homeodomain genes of the *WUSCHEL* (*WUS*) family, called *WUSCHEL RELATED HOMEOBOX* (*WOX)* genes, are expressed in specific domains of the embryo. *WOX2* and *WOX8* are expressed in egg cell and in the zygote and, after its division, are expressed in the apical cell and in the basal cell, respectively (Haecker et al., 2004). *WOX9* is expressed in the basal daughter cell of the zygote and then it shifts into derivatives of the apical cell

apparently in response of signals from the embryo proper (Haecker et al., 2004; Wu et al., 2007). Defects in the *wox2* mutant can be observed in the apical cell of zygote, but developmental abnormalities arise at the later stages, for example during formation of protoderm, an embryo region that will originate the epidermis. *wox8 wox9* double mutant and strong *wox9* single mutant embryos show more pronunced defects than *wox2* embryos, in fact the embryo development is arrested from the zygote stage. *WOX8* and *WOX9* appear to have additional, WOX2-independent functions in early development (Breuninger et al., 2008; De Smet et al., 2010; Haecker et al., 2004; Wu et al., 2007). During plant embryogenesis region-specific transcription programs are initiated very early and the *WOX* genes play an important role in this process (Haecker et al., 2004). Is known that the transcription factor WRKY DNA–BINDING PROTEIN 2 (WRKY 2) is involved in transcriptional activation of *WOX8* in the zygote and it is assumed to have as target also *WOX9* (Ueda et al., 2011, Lau et al., 2012).

An other developmental pathway is linked to apical-basal axis establishment after zygote division and it is auxin dependent. Auxin is a plant hormone implicated in various aspects of plant development, inculding embryogenesis, root development and vascular differentiation. During embryogenesis, axis formation involves the signalling molecule auxin, which undergoes polar auxin transport within plant tissues mediated by the asymmetric localization of PINFORMED (PIN) family auxin-efflux regulators (De Smet and Jürgens, 2007). Immediately after the zygote division, auxin is localized in the apical cell (Fig. 11a) and then in the developing proembryo (Fig. 11b,c). At the 32-cell stage it undergoes to basal shift to the hypophysis (Fig. 11d,e) and finally, at later stages of embryogenesis, is also concentrated in the tips of cotyledons and in the provascular strands (Fig 1f). When the quiescent centre of the root

meristem was established, the auxin maximum shifted further basally into the adjacent columella precursors (Fig. 11f, inset) (Friml et al., 2003).

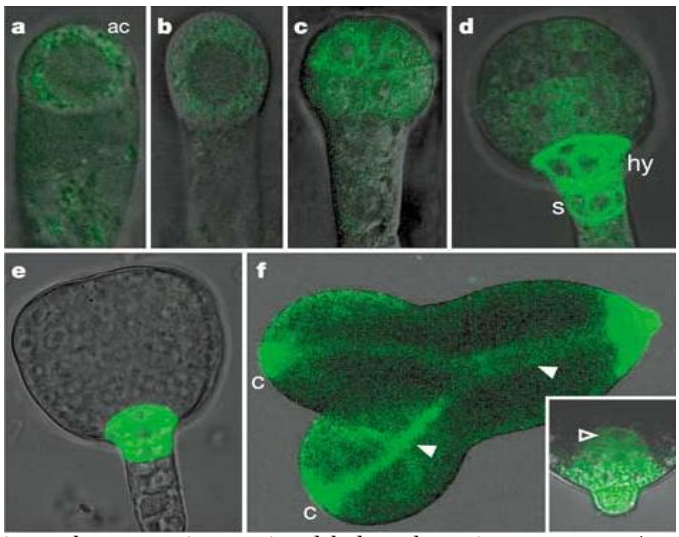

Fig. 11 – Expression pattern of *$DR5_{rev}$::GFP* in the wild-type *A. thaliana* embryo. *DR5* is a synthetic auxin-responsive promoter and is used to visualize the spatial pattern of auxin response and, indirectly, the auxin distribution. Signal after zygotic division (a), at the one-cell stage (b), at the eight-cell stage (c), at young globular stage (d), at late globular stage (e) and at the torpedo stage (f). Arrowheads indicate additional signal in cotyledon tips and provascular. inset shows maximum signal below the quiescent centre (open arrowhead). **ac**, apical cell; **s**, suspensor cells; **hy**, hypophysis, **c**, cotyledon. (Friml et al., 2003).

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; interfering with auxin transport compromises apical-basal polarity (De Smet et al., 2010). 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 PIN7, 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 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 (Fig. 12).

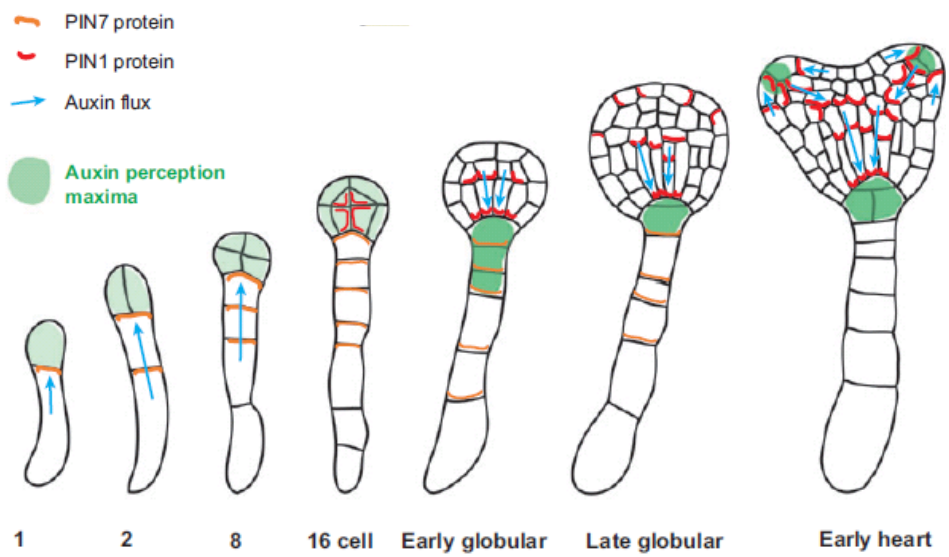

Fig. 12 – Auxin flow in the embryo and the PIN localization. Auxin transport is initially directed upward and mediated by PIN7, at the globular stage, apical-to-basal auxin transport becomes established and persists throughout the life cycle. This is marked by shift of the PIN1 localization and the reversal of PIN7 polarity (Jenik et al., 2007).

Mild defects at the basal embryo pole were observed in the *pin1* 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 vertical division (Fig. 13B). In most cases, the defects are confined to the lower region of the proembryo and in some case two proembryos developed on top of each other: the lower maintains the morphology of the suspensor and the limit between apical and basal structures is not defined (Fig 13B). At the globular stage *pin7* mutant shows a recovery of normal phenotype; then, the defects are confined to the basal part of the embryo. Others *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, in particular in the progeny of the hypophysis and in provascular initials 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 recover and showed serious defects in apical-basal embryo axis determination producing aberrant filamentous globular embryos (Fig 13C) (Friml et al., 2003).

One of the better-studied protein of Arabidopsis is GNOM, a membrane-associated guanine-nucleotide exchange factor acting on ADP ribosylation-factor G protein (ARF GEF) that is involved in the very early events of zygote development. GNOM regulates vesicle membrane trafficking required for the coordinated polar localization of auxin efflux carriers PIN-FORMED (PIN) along the apical-basal axis, in particular of PIN1 protein, that determines the direction of auxin flow. GNOM is specifically involved in endosomal recycling of the auxin-efflux carrier PIN1 to the basal plasma membrane in provascular cells, which in turn is required for the accumulation of auxin at the future root pole through polar auxin transport (Richter et al., 2010). The *gnom* mutant shows defects in coordinated polar localization of PIN1 and strongly pertubed directional auxin transport: these aspects result in compromises apical-basal embryo axis: individual cells within the *gnom* embryo distribution are not aligned with one other, nor with the axis of the embryo (De Smet et al., 2010; Steinmann et al., 1999).

Axialization of the embryo requires also *AUXIN RESPONSE FACTOR* (*ARF*) genes: *MONOPTEROS* (*MP*)*/AUXIN RESPONSE FACTOR 5* (*ARF5*) and its *AUXIN/INDOLE-3-ACETIC ACID* (*AUX/IAA*) inhibitor *BODENLOS* (*BDL*)*/IAA12* (Hamann et al., 2002; Lau et al., 2008). *MP* encodes a transcription factor that mediates auxin-responsive gene expression; auxin-dependent degradation of the interacting BDL protein releases MP from

inhibition and allows for the activation of target genes (Schelereth et al., 2010). Hypophysis specification and embryonic root formation critically depend on MP and BDL interacting proteins. *MP* and *BDL* transcripts accumulate in the proembryo rather then in the adjacent cell whose fate is to be specified. *mp* mutants fail to specify the hypophysis, although neither *MP* nor *BD* mRNA accumulates in the hypophysis prior to its division (Hamann et al., 2002). This suggests that *MP* and *BDL* genes might act non-cell-autonomously in hypophysis specification. *mp* and *bdl* embryos show defects in the embryonic axis; the cells at the base of the proembryo divide abnormally, starting as early at the 4-cell stage, and never elongate or become organized in files.

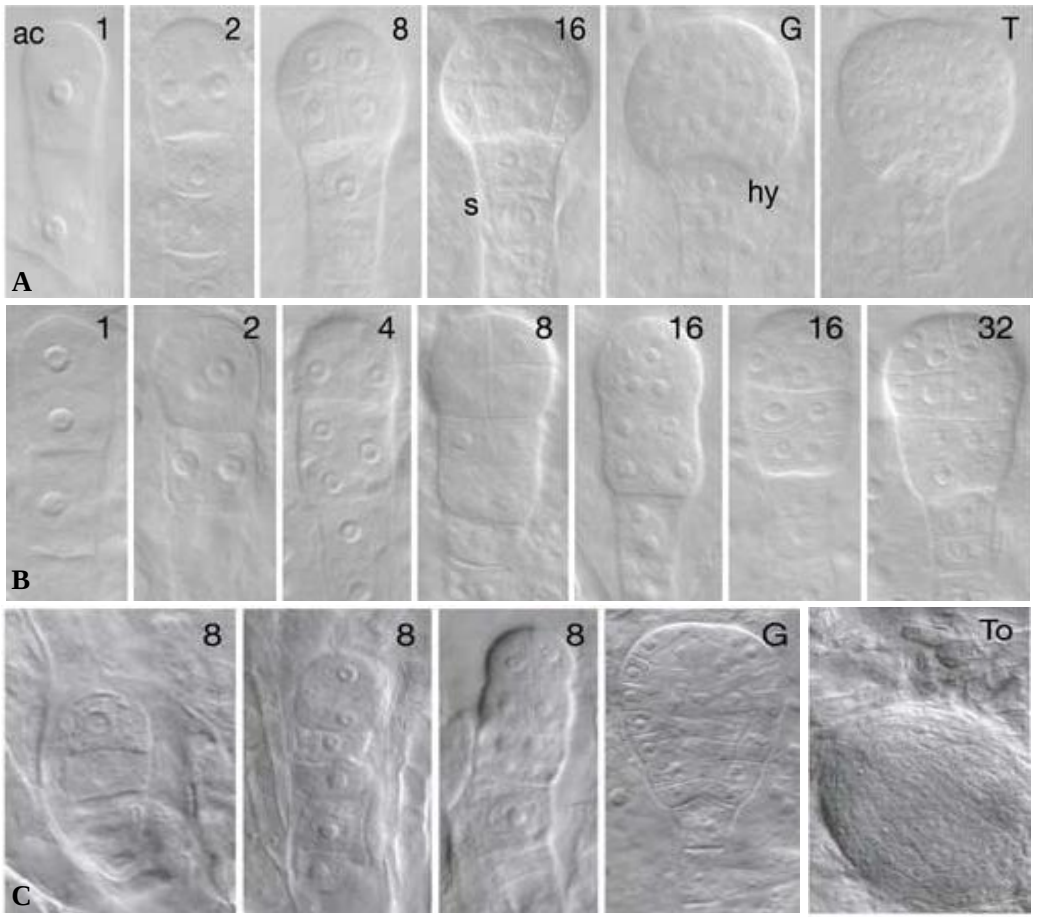

Fig. 13 – Abnormal embryogenesis in *pin7* and *pin1 pin3 pin4 pin7* mutants. (A) Wild-type, (B) *pin7* and (C) *pin1 pin3 pin4 pin7* embryo development. Numbers inidicate the developmental stage according to actual number of proembryo cells of the corresponding wild-type stage; **G**, globular; **T**, late globular; **To**, torpedo. **ac**, apical cell; **s**, suspensor; **hy**, hypophysis (Friml et al., 2003).

Mutant seedlings produce a short peg of undifferentiated cells in the place of a hypocotyls and root and also show fused cotyledons with a reduced vasculature. Dominant mutation that render the BDL protein insensitive to auxin dependent degradation result in very similar phenotypes, suggesting the BDL acts as inhibitors of MP (Hamann et al., 2002; Weijers et al., 2006). In the proembryo cells, auxin-induced BDL degradation allows MP to activate transport of auxin through PIN1 to the future hypophysis and hypothetical signal to the adjacent

extra-embryonic cell. Here, an auxin-activated ARF-AUX/IAA pair and hypothetical signal presumably specify hypophysis fate (Weijers et al., 2006; Schelereth et al., 2010). Recently, detailed expression analysis revealed ARF candidates expressed in the hypophysis (Rademacher et al., 2011; Lau et al., 2011).

Characterization of *NF-Y* genes in Arabidopsis thaliana

The relative amounts of transcript in different *Arabidopsis thaliana* organs or tissues were evaluated by quantitative RT-PCR assay by using gene specific probes (Fig.14). Taken together, these data suggest a correlation between the relative mRNA levels and the tissue distribution. Inside each family, certain members are expressed ubiquitously, during both vegetative and reproductive growth, while others are activated after the switch to reproductive growth and preferentially expressed in flowers and siliques (Gusmaroli et al., 2002).

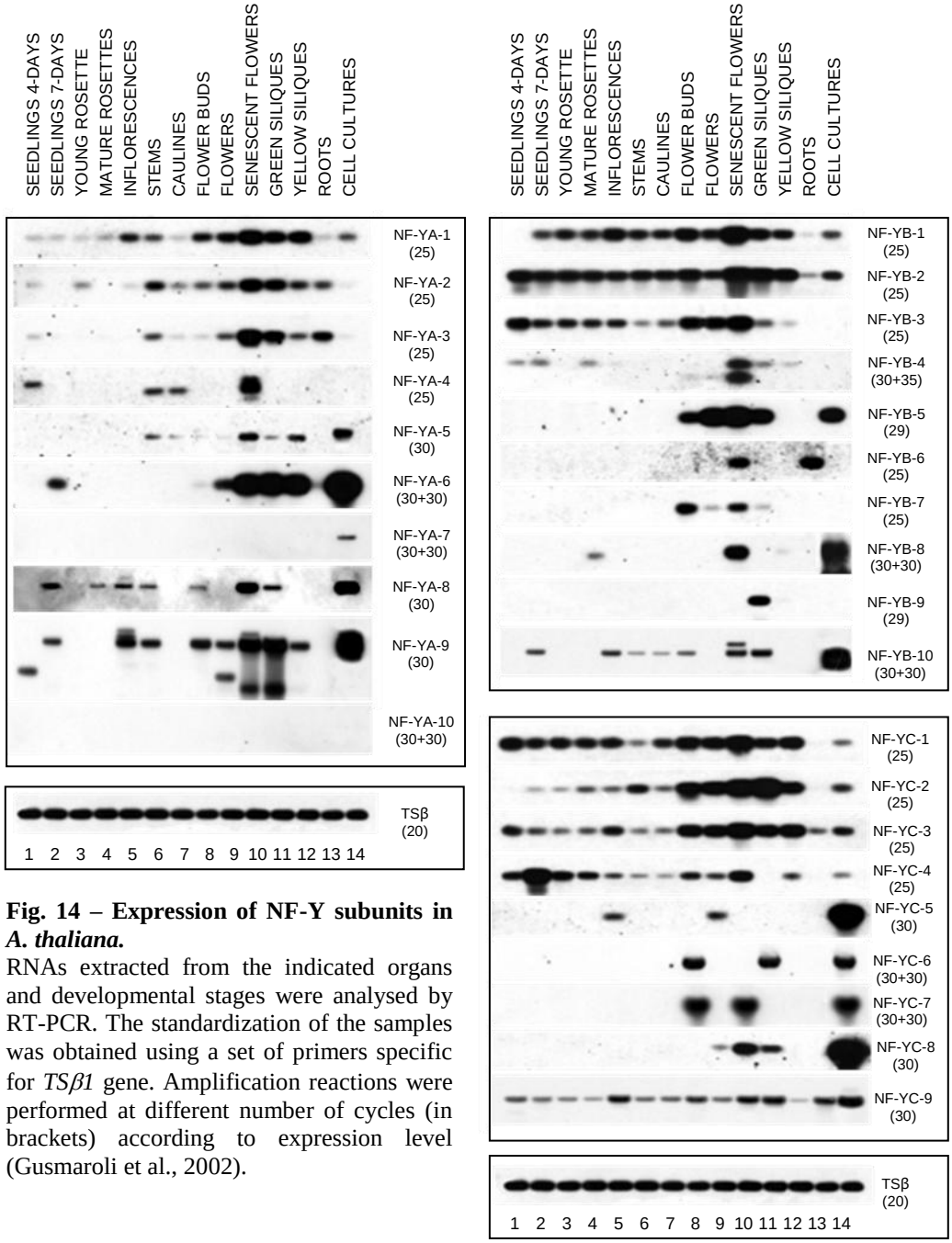

Fig. 14 – Expression of NF-Y subunits in *A. thaliana.*
RNAs extracted from the indicated organs and developmental stages were analysed by RT-PCR. The standardization of the samples was obtained using a set of primers specific for *TSβ1* gene. Amplification reactions were performed at different number of cycles (in brackets) according to expression level (Gusmaroli et al., 2002).

Individual subunits are known to be involved in a number of important processes.

***LEAFY COTYLEDON1* (*LEC1* or *NF-YB9*)** was the earliest cloned and described plant *NF-Y*. *LEC1* has strong expression in the developing embryo and it is necessary for controlling the transition from embryo to adult status. *lec1* mutation is pleiotropic, indicating several roles in late embryo development (Fig. 15).

lec1 embryos are intolerant of desiccation and normally die if dried on the plant; moreover, they show defects in the expression of some, but not all, maturation-specific genes (Meinke, 1992; West et al., 1994; Parcy et al., 1997). Desiccation-intolerant *lec1* embryos can be rescued from plants before desiccation and germinated to produce homozygous mutant plants that are fertile and that do not display any obvious vegetative or floral mutant phenotypes (Lotan et al., 1998) (Fig. 15).

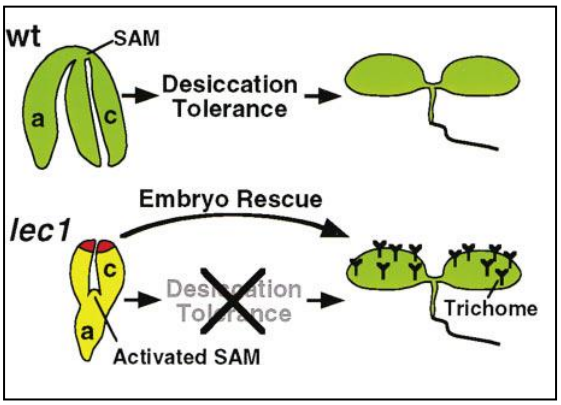

Fig. 15 – Pleiotropic effects of the *lec1* mutation on embryo development.
The axes of mutant embryos are short, and their cotyledons are round and do not curl; the shoot apical meristems of *lec1* embryos are activated in that they are domed and possess leaf primordial. *lec1* mutant embryos are intolerant of desiccation, but if they are isolated before desiccation can be germinated; *lec1* seedlings possess trichomes on cotyledons.
a, axis; c, cotyledon; SAM, shoot apical meristem (Lotan et al., 1998).

Mutant seeds are indistinguishable from wild-type until early embryo development stages, with the exception of suspensor cells. In particular, the wild-type suspensor is a structure consisting of a single file of six to eight cells that are identical genotypically to embryo proper cells. By contrast, *lec1* globular and transition stage embryos have abnormal suspensors: cell walls parallel to the suspensor axis are observed, suggesting that aberrant cell divisions occur in the mutant suspensor (Fig. 16 A,B) (Lotan et al., 1998).

Nevertheless, serious abnormalities are first detected in the embryo at the torpedo stage (Fig. 16 A); vacuolation of hypocotyl cells became pronounced at this stage and the shape of mutant embryos is distorted. Additional defects become apparent as development proceeded. The hypocotyl failed to elongate during the cotyledon stage and is reduced in length at maturity; several protoderm cells on the adaxial surface of mutant cotyledons enlarg and differentiate into trichome initials (Fig. 16 D-G). Internal cells of mutant cotyledons begin to enlarge, vacuolated, and resemble leaf mesophyll cells. Intercellular spaces are prominent in mutant cotyledons and the dense packing of cells observed in wild-type cotyledons is disrupted (Fig. 16 H). The shoot apical meristem (SAM) is greatly enlarged and often included leaf primordial. The root apex is normal in appearance but become active prematurely in viviparous seeds.

LEC1 plays a role in the specification of embryonic organ identity as well, in particular in cotyledon identity determination. In fact, embryonic leaves or cotyledons of *lec1* mutant possess stomata, that in wild-type cotyledons do not appear until after germination, and thricomes, epidermal hairs, which normally form only on leaves and stems in *Arabidopsis*. The anatomy of *lec1* mutant cotyledons is intermediate between those of wild-type cotyledon and a leaf (Lotan et al., 1998; West et al., 1994).

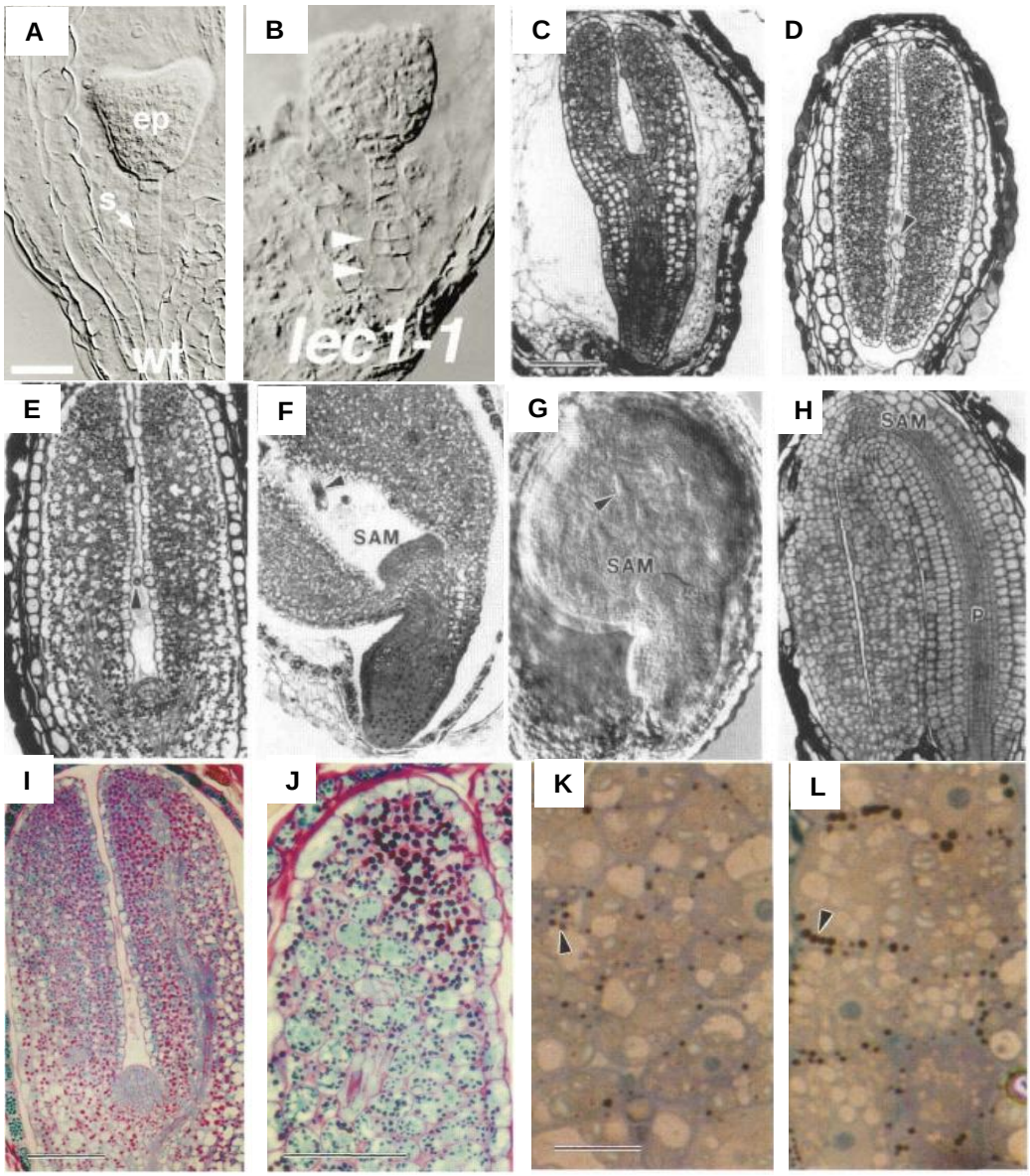

Fig. 16 – Development anatomy of *lec1* mutant embryo and storage product accumulation. (A,B) Cleared seeds from the indicated plants. Arrowheads indicate sites of abnormal suspensor cell divisions. Bar =25 µm. ep, embryo proper; s, suspensor. (C-G) *lec1* embryo anatomy. (C-F) Light micrographs of mutant seeds at the torpedo stage in (C) and cotyledon stage in (D) to (F); (G) nomarski image of a cleared mutant seed at the cotyledon stage; (H) longitudinal section of a wild-type embryo. SAM, shoot apical meristem; P, procambium; arrowhead, thricome initial. Bars= 100 µm. (I-J) Light micrographs of protein and starch accumulation in *lec1* developing embryo. Staining with toluidine blue and periodic acid-Schiff's reagent. Nucleoli and protein bodies are dark blue; starch grains are red. (I) *lec1* embryo at the cotyledon stage; (J) enlarged view of starch grains at the tip of *lec1* cotyledons. Bars= 100 µm. (K-L) Light micrographs of lipid bodies in *lec1* cotyledons (K) and hypocotyls (L) tissue stained with sudan black. Arrowhead, lipid bodies. Bars= 20 µm. (Meinke et. al, 1994; Lotan et al., 1998).

lec1 mutant exhibits defects also in storage product accumulation. Wild-type embryos are normally filled with protein and lipid bodies but contain relatively small amounts of starch. The hypocotyls and cotyledons are generally indistinguishable with respect to major storage products (Meinke et al., 1994). Starch is the primary storage product in *lec1* embryos. Plastid with large starch grains are abundant in the mesophyll region of mutant cotyledons (fig. 16 I) and the starch is most pronounced at the tips of mutant cotyledons (Fig. 16 J).

Only few lipid bodies are present in cotyledons of *lec1* embryos at maturity (Fig. 16 K) ad they are slightly more abundant in the *lec1* hypocotyl (Fig. 16 L).

Ectopic expression of the *LEC1* gene in *lec1* null mutants shows that *lec1-1 35S::LEC1* mutants is tolerant of desiccation, but viable seed production was drastically low. These *35S::LEC1* seedlings are smaller than wild-type and they possess cotyledons that remain fleshy and failed to expand; their roots often do not extend and sometimes green. Some *35S::LEC1* seedlings produce a single pair of cotyledon-like organs on the shoot apex at positions normally occupied by leaves. Unlike wild-type leaves, these organs do not expand and do not possess trichomes or mature stomatal structures; morphologically, they closely resemble embryonic cotyledons. Finally, were discovered embryo-like structures, multiple embryonic cotyledon-like organs, on the leaves of progeny plant from *35S::LEC1* lines (Lotan et al., 1998). These data confirm that LEC1 is a specific regulator of embryo development.

An other subunit characterized is **LEC1-LIKE (L1L or NF-YB6)**. By using amino acid sequence of LEC1 it was found that the gene encoding L1L was related most closely to LEC1. Sequence similarity among NF-YB proteins is limited primarily to the central B domain, consistent with comparisons of HAP3 subunits from other organisms (Li et al., 1992; Kwong et al., 2003). HAP3 subunits are recognised by their central B domain, an 90-amino acid region of the protein that is conserved across eukaryotic organisms. Closed examination

of B-domain sequence alignments shows that L1L and LEC1 define a distinct class of NF-YB subunits. In fact, the two proteins share 83% sequence identity with each other but only 52 to 71% identity with the other Arabidopsis NF-YB subunits (Kwong et al., 2003). On the basis of sequence identity within B domain, it defins two classes of NF-YB subunits: the LEC1-type and non-LEC1-type; in particular 16 specific amino residues within the B domain permit to do this distinction (Lee et al., 2003).

L1L is expressed in vegetative organs at low levels and, similar to *LEC1*, is expressed primarily during seed development. The RNA accumulation patterns suggests a role for *L1L* in embryogenesis. Suppression of *L1L* gene expression by RNA interference (RNAi) induces defects in embryo development that differs from those of *lec1* mutants, suggesting that *LEC1* and *L1L* play unique roles in embryogenesis. In particular, RNAi mutants are arrested at a number of different embryonic stages with a range of morphological phenotypes. Some embryos arrested at the globular stage (Fig. 17B and C) but had extra cells in the suspensor. Other mutants arrested at later embryonic stages and had reduced cotyledons (Fig. 17D). Seeds containing these defective embryos do not germinate, nor do immature seeds collected before desiccation germinate in culture (Kwong et al., 2003).

L1L expressed under the control of DNA sequences flanking the *LEC1* gene, is able to rescue the desiccation intolerance of suppressed genetically the *lec1* mutation, suggesting that the LEC1- type B domains of L1L and LEC1 are critical for their function in embryogenesis.

Moreover, ectopic expression of *L1L* shows seedlings remaining arrested in their development with only two cotyledons, and seedlings that developed multiple pairs of fleshy, cotyledon-like structures at positions normally occupied by leaves. These morphological defects reproduced those observed in transgenic

seedlings overexpressing *LEC1*, although are not detect somatic embryos on *35S:L1L* seedlings (Kwong et al., 2003).

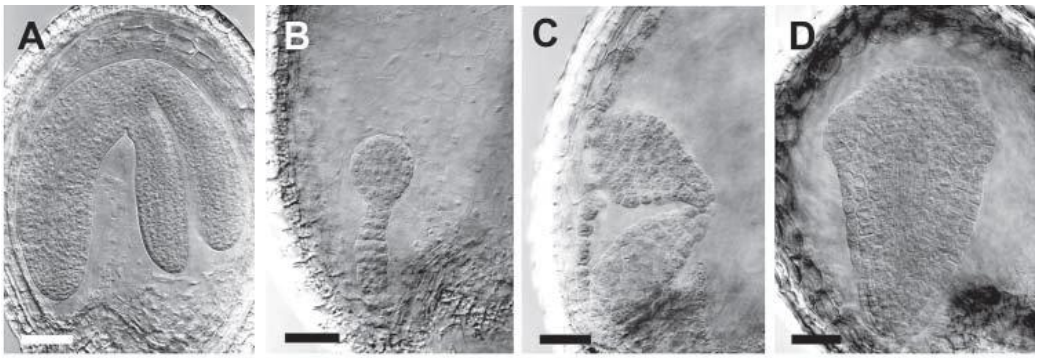

Fig. 17. RNAi suppression of *L1L* gene expression induces embryo defects.
(A) Seed with a wild-type embryo at a upturned-U stage.
(B) to (D) Cleared seeds containing defective embryos from lines containing the *L1L* RNAi constructs. Progeny segregating with wild-type phenotype in the same silique were at the upturned-U stage.
Nomarski images. Bars= 50 µm in (A) and (D) and 25 µm in (B) and (C). (Kwong et al., 2003).

Taken together, these data demonstrated that *L1L* is a regulator of embryo development; it is expressed predominately during embryo development and it is required for the completion of embryogenesis and that B domain is critical for *L1L* and *LEC1* functions. Although *L1L* can function redundantly with *LEC1* when expressed ectopically, the two subunits have distinct functions during embryogenesis: LEC1 is a embryonic regulator that mediates the switch between embryo and vegetative development (Lotan et al., 1998); L1L is a regulator protein that plays an essential role during embryogenesis (Kwong et al., 2003).

LEC1 and L1L, but not other NF-YBs, in combination of NF-YC2 can activate the promoter of *CRUCIFERIN C* (*CRC*), which encodes a seed storage protein and the promoter of *SUCROSE SYNTHASE 2* (*SUS2*) through the interaction with a seed-specific Abscisic Acid (ABA)-Response Element (ABRE) binding

bZIP factor, bZIP 167. This activation is specific to LEC1 and L1L and is inhibited by the expression of *NF-YA* subunits (Yamamoto et al., 2009).

Recent Affymetrix GeneChip, chimeric promoter-β-*glucoronidase* (*GUS*) transgene experiments and laser capture microdissection (LCM) show that 48 Transcription Factor (TF) genes, including *LEC1* and *L1L*, are seed-specific transcription factors genes and that they are active in specific seed compartments and tissues, suggesting that they may have an important role in the differentiation and/or function of unique seed parts (Le et al., 2010).

Other NF-Y subunits are known to have functions in various Arabidopsis processes.

Li et al. (2008) demonstrated that ***NF-YA5*** is strongly induced by drought stress in an (ABA)-dependent manner (in fact, ABA accumulation is required for some drought stress-induced upregulation of gene expression). *NF-YA5* is highly expressed in vascular tissues and guard cells and the transcript is induced in response to drought stress and ABA. *NF-YA5* contains a target site for miR169, which targets mRNAs for cleavage or translational repression. *miR169* is downregulated by drought stress through an ABA-dependent pathway and in these conditions the *NF-YA5* transcript increases. *nf-ya5* knockout and plant overexpressing *miR169a* (one member of miR169 family in Arabidopsis) shows enhanced leaf water loss and is more sensitive to drought stress than wild-type. Thus, *NF-YA5* is important for drought resistance and its induction by drought stress occurs at both transcriptional ad post-transcriptional levels.

It is also showed that a nuclear factor Y heterotrimer comprised of NF-YA5, NF-YB9 and possibly C9 is involved in signal transduction chain shared by blue light and abscisic acid in Arabidopsis seedlings. Moreover, ***NF-YC4*** has a role in the germination process: *nf-yc4* mutants have increased sensitivity to ABA in plate germination assay (Warpeha et al., 2007).

Also ***NF-YB1*** is identified as a putative regulator of drought-related stress tolerance; transgenic Arabidopsis plants that constitutively express *NF-YB1* show increased tolerance to water deprivation. *NF-YB1* is not regulated by drought stress tolerance pathways mediated by transcriptional factor, as CBF (C-repeat binding factor) or ABA and it is a factor that acts through undescribed transcriptional mechanism (Nelson et al., 2007).

NF-YB2 and ***NF-YB3*** are involved in promotion of flowering (Kumimoto et al., 2008); they share similar activities and are both necessary and sufficient for the promotion of flowering in response to inductive photoperiodic conditions (long-days). In particular *NF-YB2* and *NF-YB3* act through the activation of the key floral regulator *FLOWERING LOCUS T* (*FT*), gene responsible in vegetative to floral meristem conversion. This study supports an emerging model in which NF-Y complexes provide a component of the DNA target specificity for transcriptional regulators such as CONSTANS (CO), key regulator of photoperiod-induced flowering time. In this way, under long-day conditions, CO protein accumulates and induces the expression of important downstream components of photoperiodic floral promotion pathway such as *FT*.

Recently, three *NF-YC* were identified, ***NF-YC3***, ***NF-YC4*** and ***NF-YC9***, that are also required for flowering and physically interact *in vivo* with both NF-YB2 and NF-YB3. Furthermore, *NF-YC3*, *NF-YC4* and *NF-YC9* can physically interact with CO and are genetically required for CO-mediated promotion. In particular, CO utilizes NF-Y transcription factor complexes for the activation of FT during photoperiod-dependent floral initiation (Kumimoto et al., 2010).

In a yeast three-hybrid system and *in vivo* bimolecular fluorescence complementation analyses (BiFC), it was demonstrated that bZIP128 interacts with the trimer composed by **NF-YA4/NF-YB3/NF-YC2**. bZIP128 is one bZIP Arabidopsis transcriptional factor associated to endoplasmic reticulum (ER) membrane and involved in unfolded protein response (UPR). In plants, adverse

environmental conditions can produce ER stress and UPR; when unfolded or misfolded proteins accumulate in the ER, UPR is activated to produce protein folding factors and other factors to aid in protein folding. In response to stress, the N-terminal portion of bZIP128 is released into cytoplasm by proteolysis and relocates to the nucleus to form transcriptional complexes on target genes involved in ER protein folding and secretion. In this model, bZIP28 activates *NF-YC2*, and the dimerization with NF-YB3 allows the latter to be translocated to the nucleus. The NF-YB3/NF-YC2 dimer interacts with NF-YA4, which likely enters independently. The trimer NF-Y forms with bZIP28 dimers a transcriptional complex that upregulates the expression of ER stress-induced genes (Liu and Howell, 2010).

Interestingly, recent study showed a role of ***NF-YB2*** in regulation of root growth. *NF-YB2* is expressed in the tip region of the roots, where cell division and elongation occur. *NF-YB2*-overexpression enhances primary root elongation and the rate of root elongation is increased compared to wild-type. Detailed analysis also showed that *NF-YB2*-overexpression do not affect the length of the root elongation zone and the cell length along the elongation zone: a faster root elongation is due to a faster cell division and/or elongation. In particular, kinematic analysis indicated that root cell in *NF-YB2*-overexpressors elongate faster than the cells in wild-type roots (Ballif et al., 2011).

NF-Y genes in other plants

Sequencing of genomes and bioinformatics analyses allow to study *NF-Y* genes that have an important role in development and physiology of increased number of other plants.

In *Zea mays* has been characterized an orthologous of Arabidopsis *NF-YB1*, *ZmNF-YB2*, that is shown to have an equivalent activity. Under waterlimited conditions, transgenic maize plants with increased *ZmNF-YB2* expression show tolerance to drought (Nelson et al., 2007). Moreover, expression of *ZmLEC1*, a maize homologue of the Arabidopsis *LEC1*, in somatic and zygotic embryo, but not in callus, suggests its involvement during embryogenesis in maize (Zhang et al., 2002).

In carrot and in cocoa (*Theobroma cacao L.*) an orthologue of Arabidopsis *LEC1* and a homologue of *L1L*, respectively, have been isolated. In carrot, C-LEC1 can complement the Arabidopsis *lec1* mutant (Yazawa et al., 2004) and it is demonstrated that can interacts *in vitro* with C-HAP2B and C-HAP5A or C-HAP5B (homologues of HAP2 and HAP5). In this way, these proteins form heterotrimeric complex that binds specifically to DNA fragments containing a CCAAT and that regulates gene expression during carrot embryo development (Yazawa et al., 2007). Moreover, in cocoa the expression of *TcL1L* has been analysed: the transcripts mainly accumulates in embryonic cells of young embryos and in the protoderm and epidermis of young and immature embryos, either zygotic or somatic. Ectopic expression of the *TcL1L* gene could partially rescue the Arabidopsis *lec1* mutant phenotype, suggesting a similarity of function in zygotic embryogenesis and the similar roles of *TcL1L* ad *L1L* during embryogenesis (Alemanno et al. 2008).

Also in *Helianthus annuus HaL1L*, a homologue of Arabidopsis *L1L* has been identified and it is expressed primarly at an early stage of sunflower embryogenesis. Experiments performed by Fambrini et al. (2006) provided evidence that *HaL1L* is expressed both in zygotic and in somatic embryogenesis, suggesting its involvement in these developmental processes. Nonetheless, its specific function in embryo development remains to be determined. Studies performed on ephyphyllous clone (EMB-2) derived by *in vitro* tissue culture of

the interspecific hybrid *Heliantuus annuus* x *H. tuberous* allowed to investigate if the ectopic expression of *HaL1L* gene and auxin activity are correlated with establishment of embryogenic competence. This clone produces epiphyllous (EP) leaves, both *in vitro* and *in vivo*, that exhibit prominent ectopic embryo- and shoot-like structures. *HaL1L* is highly expressed in EP embryos of the EMB-2 clone and, in particular, is highly expressed in early developmental stages of somatic embryo-like structures. Moreover, high amount of auxin endogenous was detected in single cells or in small groups of cells along the epidermis of EP leaves and accompanied the early stages of embryo development. Exogenous auxin treatment on non-epiphyllous leaves slightly increases *HaL1L*. Although *HaL1L* seems to be not directly mediated by auxin level *per se*, it is clear that localized *HaL1L* expression and auxin accumulation in EP leaf epidermis domain represent early events of somatic embryogenesis displayed by the epiphyllous EMB-2 clone (Chiappetta et al., 2009). Recently, it was demonstrated that during plant development *HaL1L* transcription could be under epigenetic regulation through methylation of cytosine residues and by potential interactions with hormones and Transcription Factors (TFs). Interestingly, the WUSATA target site (target sequence for the WUSCHEL TF), located in the intron of *HaL1L*, is able to bind the TF WUS, and the auxin and abscissic acid responsive motifs present in the *HaL1L* promoter region, suggested that this gene maybe additionally under hormonal control (Salvini et al., 2012).

10 *HAP2* (*NF-YA*), 11 *HAP3* (*NF-YB*) and 7 *HAP5* (*NF-YC*) were identified in the rice genome. All the three HAP family genes encode a protein with a conserved domain in each family and various non-conserved regions in both length and amino acid sequence (Miyoshi et al., 2003; Thirumurugan et al., 2008). Three genes *HAP3/NF-YB*, named *OsHAP3A*, *OsHAP3B* and *OsHAP3C*, with high homology in the conserved region, have been isolated in rice and they

are expressed in various organs including leaves. In the transgenic plants with RNAi construct of *OsHAP3A*, reduced expression of not only *OsHAP3A* but also *OsHAP3B* and *OsHAP3C* is observed. These plants show reduced amount of chlorophyll and chloroplast degeneration accompanied by reduced expression of nuclear-encoded photosynthesis genes, while expression of chloroplast-encoded genes is not affected or rather increased. These data suggest that one or more *OsHAP3* genes regulate the expression of nuclear-encoded chloroplast-targeted genes and normal development of chloroplasts (Miyoshi et al., 2003). It was demonstrated that *OsHAP2E* is a target of two miRNAs: miRNA-169g and miRNA-169n-o, that are induced by high salinity. After high salinity signal, *OsHAP2E* transcripts are cleaved and therefore down-regulated demonstrating involvement of *OsHAP2E* gene in salt stress (Zhao et al. 2009).

In *Triticum aestivum* 10 *NF-YA*, 11 *NF-YB* e 14 *NF-YC* were identified. Quantitative expression analysis revealed that some of the wheat *NF-Y* genes are expressed ubiquitously, while the others are expressed in an organ-specific manner, in particular each TaNF-Y subunit family has members that are expressed predominantly in the endosperm. Moreover, nine *NF-Y* appear to be responsive to drought stress and three of these genes are up-regulated under drought conditions, indicating that these members of the NF-Y family are potentially involved in plant drought adaptation (Stephenson et al., 2007). Additional studies showed that six wheat NF-YC members *TaNF-YC (TaNF-YC3, 5, 8, 9, 11 e 12)* are regulated by light in both leaf and seedling. The potential target genes for *TaNF-YC11* include subunit members from all four thylakoid membrane-bound complexes required for the conversion of solar energy into chemical energy and rate-limiting enzymes in the Calvin cycle. *TaNF-YC11* seems to be potentially involved in regulation of photosynthesis-related genes (Stephenson et al., 2010). Stephenson at al. (2011) identified two NF-YB members (*TaNF-YB3* and *7*) which are markedly upregulated by light in

the leaves and seedling shoots. Transgenic wheat lines constitutively overexpressing *TaNF-YB3* had a significant increase in the leaf chlorophyll content, photosynthesis rate and early growth rate. In addition, they showed that *TaNF*-YB3 is involved in the positive regulation of a number of photosynthesis genes in wheat. Recently, it was demonstrated interactions between wheat VRN2 (one of vernalization gene) and eight different NF-Y proteins; *VRN2* locus includes two tandemly duplicated genes *ZCCT1* and *ZCCT2*, both involved in flowering repression and characterized by a CCT domain - first identified in Arabidopsis CONSTANS (CO) - in C-terminus. This CCT domain is also present in CO2 protein, closely related CO protein and involved in photoperiod pathway accelerating flowering under long days. CCT domains present in VRN2 and CO2 proteins interact with the same subset of eight NF-Y proteins: NF-YA7, A9; NF-YB1, B3, B9; NF-YC7, C10, C11. It was demonstrated that ZCCT1 and CO2 compete for interaction with NF-Y protein in yeast and that these CCT proteins compete also with NF-YA for interactions with NF-YB proteins. Moreover, the interaction between VRN2 and NF-YB1 and NF-YB9 was also confirmed *in planta*. In addition, mutations in CCT domain of VRN2 eliminate its ability to repress flowering and also reduce the strength of the interactions between VRN2 and NF-Y proteins and the ability of VRN2 to compete with CO2. These results suggest that the CCT-NF-Y protein interactions play an important role in the integration of vernalization and photoperiod seasonal signals in the temperate cereals (Li et al., 2011).

In the model legume *Medicago truncatula* was identified *MtHAP2-1*, a HAP2-type transcription factor, expressed in the nodule meristematic zone and essential for the differentiation of nodule cells (Combier et al., 2006). Spatial and temporal expression of this gene is controlled by miR169 (Jones-Rhoades and Bartel, 2004) and by a small peptide codified by *MtHAP2-1* 5' leader

sequence that may contribute to spatial restriction of its expression within the nodule (Combier et al., 2008).

It is known that overexpression of *AtLEC1* and its orthologs in canola (*Brassica napus*), *BnLEC1* and *BnL1L*, causes an increased fatty acid level in transgenic Arabidopsis plants, which, however, also show sever developmental abnormalities. Conditional expression of *BnLEC1* and *BnL1L* (that is driven by a specific promoter that retains the seed-specific expression pattern but with a reduced expression level) in transgenic canola increases the seed oil content and has no detrimental effects on major agronomic traits. Consistent with these results, expression of a subset of genes involved in fatty acid biosynthesis, glycolisys and sugar metabolism is increased in developing seeds and in the silique wall of the transgenic rapeseed suggesting that *BnLEC1* and *BnL1L* are reliable targets for genetic improvement in seed oil production of rapeseed (Tan et al., 2011).

In *Brachypodium distachyon* were identified, annotated and further characterized 36 NF-Y proteins: 7 NF-YA, 17 NF-YB and 12 NF-YC. By examining phylogenetic relationships, orthology predictions and tissues-specific expression pattern, numerous example of likely functional conservation between dicots and monocots have been proposed. It was demonstrated that overexpression of *BdNF-YB6* (predicted to share a relatively recent ancestor with two positive regulators of flowering, *NF-YB2* and *NF-YB3*) resulted in significantly earlier flowering in the wt Arabidopsis plants, and in rescue of late flowering phenotype in *nf-yb2 nf-yb3* mutant plants. The current data serves as an entry point for translating many NF-Y function from dicots to the genetically tractable monocot model system Brachypodium and, in turn, to the agriculturally important grasses (Cao et al., 2011).

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The Arabidopsis *NF-YA3* and *NF-YA8* genes are functionally redundant and control 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 embryogenesis, 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* play an important role in Arabidopsis 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 being 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 are arrested at the globular stage and that both proembryo and suspensor are characterized by a disordered cell cluster with an irregular

shape, suggesting defects in the determination of the apical-basal embryo axis. 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 globular-arrested *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 transport in early developing embryos. Our findings indicate that *NF-YA3* and *NF-YA8* are functionally redundant regulators of the globular-heart transition that marks the acquisition of axiality during embryo development in *Arabidopsis thaliana*.

Keywords

NF-YA3; *NF-YA8*; embryogenesis; apical-basal axis; auxin.

Highlights

NF-YA3 and NF-YA8 are phylogenetically closely-related genes.

NF-YA3 and *NF-YA8* are both highly expressed in the embryo from the globular to the torpedo stages.

Double mutant embryos are arrested at the globular stage and show defects in axiality.

Defects in *nf-ya3 nf-ya8* embryos are correlated to a defective auxin transport.

NF-YA3 and *NF-YA8* are redundant genes that regulate the globular-heart embryo transition.

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 (Remenyi et al., 2004; Siefers et al., 2009; Singh, 1998; Wolberger, 1998).

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 (Bucher, 1990; Mantovani, 1998). 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 Histon Fold Motif (HFM), a conserved protein-protein and DNA-binding interaction module (Luger et al., 1997; Schade et al., 1997). This dimer translocates to the nucleus, where it offers a complex surface for the association of the NF-YA subunit (Frontini et al., 2004; Goda et al., 2005; Steidl et al., 2004; Tuncher et al., 2005). The resulting mature NF-Y heterotrimer is then competent to bind with high specificity and affinity the CCAAT motif in promoters (Mantovani, 1999). NF-YA and NF-YC subunits contain large domains rich in glutamines and hydrophobic residues with transcriptional activation function (Mantovani, 1999). 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 (Gusmaroli et al., 2001a; Mantovani, 1999). In animals and yeast, each subunit of NF-Y is encoded by a single gene, whereas in the Arabidopsis genome 10 NF-YAs, 13 NF-YBs and 13 NF-YCs are annotated (Gusmaroli et al., 2001a, 2002a; Siefers et al., 2009). 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 embryogenesis, drought resistance, ABA signaling, flowering time and primary root elongation (Ballif et al., 2011; Kumimoto et al., 2008; Kumimoto et al., 2010; Kwong et al., 2003; Lotan et al., 1998; Nelson et al., 2007; Warpeha et al., 2007; Yamamoto et al., 2009). *NF-YA* genes are involved in drought resistance (Li et al., 2008), in Endoplasmic Reticulum (ER) stress response and in blue light and ABA responses with *NF-YB* and *NF-YC* (Liu and Howell, 2010; Warpeha et al., 2007).

Embryogenesis in higher plants is a developmental process divided into three distinct phases: morphogenesis, during which embryo pattern formation occurs; maturation phase, during which macromolecules, particularly storage proteins, lipids and carbohydrates are synthesized and finally, late embryogenesis, a preparation phase for seed desiccation, inhibition of precocious germination, and establishment of dormancy (Goldberg et al., 1994; West and Harada, 1993).

During the morphogenesis phase, the basic body plan of the plant is established with the specification of the shoot-root axis of polarity and the formation of the embryonic organ and tissue systems. The early embryonic development of Arabidopsis proceeds through series of cell divisions and this reveals a tight control of the process (Mansfield and Briarty, 1991; Mansfield et al., 1991). Following fertilization, the zygotic division generates a smaller apical and a larger basal cell. The apical cell divides vertically and generates an eight-celled

proembryo composed of two tiers of four cells each. The shoot meristem and the flanking cotyledon primordia originates from the upper tier cells (apical region); the lower tier (central region) gives rise to the hypocotyl, the embryonic root and the upper tiers of root meristem cells. The basal cell of the zygote divides transversely to produce a file of 7-9 cells; the uppermost daughter of the basal cell, called the hypophysis, becomes part of the primary root system, whereas the remaining daughters form the extraembryonic suspensor (Jürgens, 2001). Each of the eight cells derived from the apical cell divides periclinally to produce a 16-cell embryo (early globular stage), with eight inner and eight outer cells. The inner cells give rise to ground tissue and procambium, while the epidermis derives from the outer cells. Then, the cells continue to undergo division producing 32-cell (midglobular stage) and 64-cell stage embryo (late globular stage); at this time cell division in the protoderm increases and this marks the site of the future cotyledons. The cell divisions producing the cotyledon primordia are rapid and lead to the heart stage embryo; during the heart stage, the cotyledon primordia enlarge, cell divisions occur and cells in the hypocotyl and radicle elongate (Bowman, 1994). The embryo assumes an elongated shape, typical of the torpedo stage. In the later stages, the embryo increases in size and the cells differentiate (Mansfield and Briarty, 1991, 1992). 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. This transition is due to cell divisions and subsequent cell expansions; these stages are also characterized by the deposition of protein, lipid, starch and sugar reserves in the embryo (Bowman, 1994). 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 (Breuninger et al., 2008; De Smet et al., 2010; Haecker et al., 2004; Wu et al., 2007). Another developmental pathway is auxin dependent. Auxin is a signalling hormone implicated in the establishment of the embryonic body axis (Jenik et al., 2007) which undergoes polar transport mediated by asymmetric localization of specific cellular efflux carrier proteins, the PINFORMED (PIN) protein family (Rubery and Sheldrake, 1974; Friml and Palme, 2002). 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 (Friml et al., 2003). It is known that interfering with auxin transport causes defects in the determination of the apical-basal poles during embryo development (Friml et al., 2003;Weijers et al., 2005; Xiang et al., 2011). Axialization of the embryo requires also *AUXIN RESPONSE FACTOR* (*ARF*) genes: *MONOPTEROS* (*MP*)*/AUXIN RESPONSE FACTOR 5* (*ARF5*) and its *AUXIN/INDOLE-3-ACETIC ACID* (*AUX/IAA*) inhibitor *BODENLOS* (*BDL*)/*IAA12*. It is known that *mp* and *bdl* embryos show defects in the embryonic axis (Hamann et al., 2002; Lau et al., 2008).

In this paper, we present the functional characterization of *NF-YA3* and *NF-YA8*, two members of the Arabidopsis *NF-YA* family, very closely related in the phylogenetic tree (Gusmaroli et al., 2002a; Siefers et al., 2009). 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* embryos are arrested at the globular stage and that this arrest is due to defects in the apical-basal embryo axis. Our data indicate that *NF-YA3* and *NF-YA8* are redundant regulators of the globular-heart transition and that they are involved in the determination of apical-basal axis during embryogenesis.

Materials and methods

Plant materials and growth conditions

Wild-type *Arabidopsis thaliana* seeds (Col-O) and *DR5rev::GFP* 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 (14h light/10 h dark) at a fluence rate of 100 µE $m^{-2}s^{-1}$.

In situ hybridization

Flowers and siliques of wild-type Arabidopsis plants were collected at various developmental stages and were fixed and embedded in Paraplast Plus embedding media as described by Cañas et al. (1994). Digoxygenin-labeled

antisense 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 synthetized by PCR with the following primers: a forward primer A3NoT7a (5'-GTTAGCCAGAGAGCCTTATTC-3') and a reverse primer containing a T7 promoter adapter A3+T7a) (5'-TAATACGACTCACTATAGGGGTAATCGATGAAGCGAAGAAG-3') for *NF-YA3*; a forward primer A8NoT7-A2 (5'-GCTAATTGTTGCCTCTGAG-3') and a reverse primer containing a T7 promoter adapter A8+T7-a (5'-TAATACGACTCACTATAGGGGAATCCTGGTCCTGGAAAC-3') for *NF-YA8*. Hybridization and immunological detection were performed as described previously (Cañas et al., 1994). The sections were mounted with a coverslip and subsequently observed using a Zeiss Axiophot D1 microscope equipped with differential interface 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 amplified by PCR with a forward primer containing a *Sal*I restriction site at the 5' end (5'-GTCGACACGGCTTTGTAGAG-3') and a reverse primer containing a *Xba*I site at the 3' end (5'-TCTAGAACGTCTTCCTTAACA-3'). The *pNF-YA8::GUS* fusion was constructed from a 1000-bp genomic region upstream of the *NF-YA8* start codon amplified by PCR with a forward primer containing a *Sal*I restriction site at the 5' end (5'-GTCGACGACTTAAACTGTGTGC-3') and a reverse primer containing a *Xba*I site at the 3'end (5'-TCTAGACAACAATTAGCTCTCT-3'). The PCR fragments were cloned in the

pCR4-TOPO (Invitrogen), excised with *Sal*I - *Xba*I and subcloned between the *Sal*I - *Xba*I sites preceding the *GUS* gene in the binary vector pGPTV-Kan (Becker et al., 1992).

The constructs for *35S::NF-YA3* and *35S::NF-YA8* lines were obtained using the Gateway cloning system. *NF-YA3* and *NF-YA8* cDNAs were amplified by PCR using the following primers: A3TH5 (5'-AATTCCAGCTGACCACCATGATGCATCAGATGTTGA-3') and A3TH3 (5'-GATCCCCGGGAATTGCCATGTCAGATATGGACAGAG-3') for *NF-YA3* and A8TH5 (5'-AATTCCAGCTGACCACCATGGATAAGAAAGTTTCAT-3') and A8TH3 (5'-GATCCCCGGGAATTGCCATGTCAGATATGGACAGAG-3') for *NF-YA8*. The PCR products were cloned in the binary vector in the pGD625 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* gene was amplified by PCR using the forward primer 8-3 RNAi F (5'-GCGTTAATCTCTTTGGACAC-3') in combination with the reverse primer 8-3 RNAi R (5'-AGTAGCAGCCAAGGATGACT -3'). The fragment was placed in both orientations into the pFRH 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 (Labereke et al., 1974).

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 (Clough and Bent, 1988). Homozygous T_3 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 T_1 independent *RNAi::A3/A8* lines were selected on MS agar plates supplemented with 1% (w/v) sucrose containing 20 µg ml^{-1} hygromycin.

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-glucuronic acid, 0.1% Triton X-100, and 0.5mM ferrocyanidine 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 (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 Axiocam 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 (Masiero et al., 2004). Wild-type alleles were amplified using the following primers: atACF3 (5'-CTCTGCAATCGTGTTTTGGTT-3') and atACR1 (5'-GTTAGGCCAACAGGTAAACAG-3') for the *NF-YA3* allele and NF-YA8F2 (5'-CTTTGACAGACACATGTATCATCAA-3') and NF-YA8R2 (5'-TGGCTGCTACTTCGCTTATTAGC-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'-TAGCATCTGAATTTCATAACCAATCTCGATACAC-3') together with the *NF-YA3*-specific primers atACR1 for the *nf-ya3* allele or with the *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'-TCTAGACAAGACCCTTCCTC -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 crossed with a *DR5rev::GFP* marker line, using Leica TCS SP5 AOBS and DMI 6000 CS microscope.

Semi-quantitative RT-PCR analysis

Total RNA was isolated (van Tunen et al., 1988) from developing siliques of *nf-ya3* and *nf-ya8* plants and of *35S::NF-YA3* and *35S::NF-YA8* overexpression lines as previously described (Gusmaroli et al., 2001a). Approximately 5 µg were reverse-transcribed using RT Superscript II (Invitrogen) and an oligo dT, as previously described (Procissi et al., 1997). After first strand cDNA synthesis, samples were diluted 50 times and used as templates for semi-quantitative RT-PCR, with *NF-YA3*-specific primers atACF2 (5'-ATCTTATACTGAAGTTGCTAGTAG-3') and atACR2 (5'-

TTCTGCAGCATATGAGCTTCGA-3') and *NF-YA8*-specific primers NF-YA8F2 and NF-YA8R2. *ACTIN2* specific primers, ACT2-F (5'-TGCTTCTCCATTTGTTTGTTTC-3') and ACT2-R (5'-GGCATCAATTCGATCACTCA-3') were used as a control of cDNA concentration. The amplifications were carried out within linear ranges (25 cycles). The PCR products were transferred onto Hybond N+ nylon membranes (Amersham) and hybridized with gene-specific probes labelled using the DIG-High Prime kit (Roche).

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 20µl containing 5µl of 50-fold diluted cDNA, 0.25–0.4 µM of each primer and 10µl 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 NFYA3_RT-R and *NF-YA8*-specific primers NFYA8_RT-F (5'-CACACACCATTTCTCTGTCCA-3') and NFYA8_RT-R (5'-TTGAAGCTTGAAGGTTTTTGG -3'). *ACTIN2* and *RCE1* were used as the reference genes. Specific primers for *ACTIN2* gene are ACT2 F and ACT2 R (see above), for *RCE1*we used RT 147 (5'-CTGTTCACGGAACCCAATTC-3') and RT 148 (5'-GGAAAAAGGTCTGACCGACA-3') (Pribil et al., 2010).

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 30s. 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 PRISM7700 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 siliques). Standard errors of ΔΔCt 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 (Gusmaroli et al., 2001b, 2002b). 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 segmental duplicated regions of the Arabidopsis genome (Arabidopsis Genome, 2000).

In order to characterize the *NF-YA3* and *NF-YA8* genes in Arabidopsis, we first analysed in detail 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 and 6 for the *pNF-YA8::GUS* construct were analysed. For both constructs, five transgenics showed similar GUS expression patterns in vegetative and reproductive tissues (Fig. 1). In particular, a similar GUS staining was detected in rosette leaves, which showed high signal in midrib, hydathodes 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 axils 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 for both constructs in the embryo at the 2-4 cell stage (Fig. 1 L,l), then the signal increased and was high from the globular to the torpedo stages (Fig. 1 M-P and m-p), after which GUS staining decreased (Fig. 1 Q,q).

To further investigate the temporal and spatial expression profile of *NF-YA3* and *NF-YA8* during flower and seed development, we performed *in situ* hybridization analyses on developing flowers and siliques (Fig. 2). Similar weak hybridization signals were observed during ovule and pollen development. *NF-*

YA3 and *NF-YA8* were both expressed in the placenta (Fig.2 A,F), in the ovule primordia and in the septum (Fig. 2 B,G) and in subsequent stages of ovule development (Fig. 2 C-E and H-J). In addition, *NF-YA8* was also expressed in carpels (Fig. 2 F,H). Both genes were expressed in anthers at floral stage 8 (Bowman,1994) (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* transcripts were also detected during embryo development. *In situ* analysis revealed a low signal for both genes in the embryo at the early globular stage (Fig. 2 K,Q). The expression level increased in the globular stage (Fig. 2 L,R) and was high until the torpedo stage (Fig. 2 M-O and S-U); in particular, in these stages, *NF-YA3* and *NF-YA8* were expressed 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 the sense probe gave no signal (not shown).

Overall, the *in situ* analyses 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 insertional 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 *nf-ya3* 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 *nf-ya3* developing siliques compared to wild-type (Fig. 3 B). However, there were no obvious morphological alterations in *nf-ya3* mutants and genotypic analysis of 247 progeny plants from *NF-YA3*/*nf-ya3* 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 downstream of the *NF-YA8* start codon in the linker region of the conserved domain between the two sub-domains (Subunit Association Domain and DNA binding domain).

Furthermore, sequencing of *NF-YA8* T-DNA flanking regions also showed a duplicated genomic region of 54 bp close to the RB border (corresponding to the sequence comprised between position +1050 bp and +1104 bp from the start codon) (Fig. 3 A). RT-PCR analysis showed that the *NF-YA8* transcript was absent in *nf-ya8* 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*/*nf-ya8*

heterozygotes showed a normal genotypic 1:2:1 ratio (51:69:38, χ^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 345 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 (χ^2 = 1.986, 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.

Siliques of 17 plants *nf-ya3/nf-ya3 NF-YA8/nf-ya8* and siliques 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 (χ^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 apical-basal pattern during embryo 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 arrested embryos (Fig. 5). Phenotypic deviations from the wild-type were first identified in embryos at the 4-cell stage: the early mutant embryo presented defects in the lower region of the proembryo that was more enlarged than in wild-type, suggesting incorrect cell divisions (Fig. 5 A,B). In addition, embryos at 8-cell stage 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 embryos

appeared to be substantially arrested at the globular stage (Fig. 5 G-R), and showed 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, the apical-basal pattern formation during embryogenesis.

RNAi suppression of *NF-YA3* and *NF-YA8* causes embryonic arrests

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 T_1 primary transformants were recovered on hygromycin selection. Four out of 51 T_1 *nf-ya3 RNAi::A3/A8* transgenic lines and four out of 49 T1 *nf-ya8 RNAi::A3/A8* transgenic lines produced defective T_2 seeds. More specifically, T_1 plants from *nf-ya3 RNAi::A3/A8* lines segregated 5.36%, 18.18%, 17% and 24.78% arrested T2 seeds and T*1* 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: they were arrested at globular stage and 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-ya3 RNAi::A3/A8* lines displaying embryos with the mutant phenotype possessed undetectable levels of *NFY-A3* and *NF-YA8* and siliques of *nf-ya8 RNAi::A3/A8* line possessed a low level of *NF-YA3* and an undetectable level of *NF-YA8* (Fig. 6 B). Together, these results suggest that concurrent suppression and reduction of *NF-YA3* and *NF-YA8* gene expression induced defects in the apical-basal pattern formation.

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::CDS-NF-YA3* 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* T_1 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 (KAN^R:KAN^S, 15:1) and showed 7.5%, 13.71%, 17.53% of arrested seeds, respectively. One line, with one copy of the construct (KAN^R:KAN^S, 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* T_2 plants homozygous for the complementation construct. Siliques of *nf-ya3/nf-ya3 NF-YA8/nf-ya8* T_3 plants showed a percentage of arrested seeds lower than 25%, confirming a partial complementation. Green seedlings of these four lines (T_4 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% 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* T_2 plants homozygous for the complementation construct. The analysis of siliques from *nf-ya3/nf-ya3 NF-YA8/nf-ya8* T_3 plants indicated that lines 16, 21 and 52 were partially complemented. Also in this case, PCR analysis confirmed that some of T_4 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. 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.

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 carried out by Nomarski microscopy did not reveal morphological defects in plant organs and in ovule, pollen and embryo development of these overexpression lines (data not shown).

Auxin distribution is altered in the *nf-ya3 nf-ya8* embryos

Defective apical-basal pattern in *nf-ya3 nf-ya8* embryos raised the question whether auxin transport 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 (Friml et al., 2003). Our analysis showed that in wild-type embryos GFP signal was restricted to the hypophyseal region from the 32-cell globular stage onwards (Fig. 8 A), whereas no GFP signal was detected in *nf-ya3 nf-ya8* defective embryos (Fig. 8 C). 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. 8 B), whereas again no GFP signal was observed in *nf-ya3 nf-ya8* embryos at later stages showing aberrant cell divisions and development (Fig. 8 D). We can conclude that defects in the apical-basal pattern of *nf-ya3 nf-ya8* embryos are correlated to a defective auxin transport.

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. Since the *in situ* analyses confirmed these GUS expression results, we concluded that the genomic region upstream of the start codon fused to the *GUS* reporter gene

contained all the regulatory sequences essential for the gene expression of *NF-YA3* and *NF-YA8* and that the genes may have an hypothetical function during embryo development.

Identification of the factors that control embryogenesis is of significant interest, since they are essential to produce a viable plant with a normal phenotype under diverse conditions. Therefore, several large-scale screenings have been performed to identify these factors (Tzafrir et al., 2004). Embryo mutants are typically identified using two criteria: reduced seed set and segregation distortion (Errampalli et al., 1991). The *nf-ya3* and *nf-ya8* single mutants did not show any phenotypic defect and the segregation analyses revealed that the observed genotypic ratios were not statistically different from the expected. However, in the siliques produced by *nf-ya3/nf-ya3 NF-YA8/nf-ya8* plants we observed a number of arrested seeds higher than in wild-type plants and the segregation of *nf-ya3/nf-ya3 NF-YA8/ NF-YA8* versus *nf-ya3/nf-ya3 NF-YA8/nf-ya8* genotypic classes was 1:2, whereas the double mutants were totally absent. Detailed analysis of siliques produced by *nf-ya3/nf-ya3 NF-YA8/nf-ya8* plants showed that 25% of the seeds were arrested and this percentage correlated with the double mutant genotypic class. The fact that neither single mutant displayed a phenotype, while the *nf-ya3 nf-ya8* double mutant was embryo lethal, implies that *NF-YA3* and *NF-YA8* are functionally redundant, which is not surprising considering the high homology they share and that these genes lie within segmental chromosomal duplications. Taken together, these results indicate that *NF-YA3* and *NF-YA8* have an essential function during embryogenesis.

_NF-YA3_ and _NF-YA8_ control the apical-basal pattern formation during 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 embryo development. In particular all the arrested seeds analysed contained embryos blocked at the globular stage, in which the division plan of cells and number of cells in proembryo and suspensor were incorrect. Therefore, the arrested embryo showed an abnormal division of the uppermost region of the suspensor close to the proembryo (i.e. the hypophysis cell). The transition from early globular embryo to heart-stage embryo marks the acquisition of axiality. These morphological defects suggests that *NF-YA3* and *NF-YA8* are involved in the determination of the apical-basal embryo axis, which is essential to obtain a correct embryo development.

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 (De Smet and Jürgens, 2007). 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 (De Smet et al., 2010). 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 PIN7, 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 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. Mild defects at the basal embryo pole were observed in the *pin1* 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 (Friml et al., 2003).

Promoter-reporter gene studies using *DR5rev::GFP* revealed that the GFP signal was completely absent in *nf-ya3 nf-ya8* embryos arrested at globular stage and defective in apical-basal axis determination. These findings suggest that mutations in *NF-YA3* and *NF-YA8* may interfere with auxin response and auxin transport 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 could affect the auxin transport by PIN carriers, leading to embryo patterning defects. It is interesting to note that the abnormal phenotype of *nf-ya3 nf-ya8* mutant embryos is similar to that of *pin* mutant defective embryos. A second possibility is that the *NF-Y* and *PIN* genes 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, 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 involved in the globular-heart transition

The complementation experiment performed on *nf-ya3/nf-ya3 NF-YA8/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 (Albert et al., 1999; Chen et al., 2001; Kim and Huang, 2004). 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 (Odell et al., 1994; Scofield et al., 1993). Interestingly, in this work we showed that overexpressing *NF-YA3/NF-YA8* under the *35S* promoter was sufficient to partially rescue *nf-ya3 nf-ya8* embryos arrested at the globular stage, thus suggesting that *NF-YA3* and *NF-YA8* have a critical role in the

globular-heart transition that marks the acquisition of axiality, but probably the proteins 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. However, although not all embryos of *RNAi::A3/A8* transgenic plants were arrested, we showed that the suppression of the two *NF-YA* 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 or high reduction of *NF-YA3* and *NF-YA8* endogenous transcripts, respectively, are necessary and sufficient to obtain globular arrested embryos.

Conclusions

Our results show that *NF-YA3* and *NF-YA8* are functionally redundant regulators of Arabidopsis embryo development. In particular, we have shown that the *nf-ya3 nf-ya8* embryos have an irregular shape and are arrested at the globular stage showing defects in cell divisions and in embryo axis poles specification.

We also found that mutations in both *NF-YA3* and *NF-YA8* affect the auxin transport in early developing embryo, known to be involved in apical-basal embryo axis establishment by its polar transport, thus suggesting their specific function in the acquisition of axiality. Finally, complementation and RNA interference experiments demonstrated that *NF-YA3* and *NF-YA8* are new functionally redundant genes specifically required for globular-heart transition and axis formation during embryogenesis.

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Database linking and Accession numbers

Sequence data and protein data from this article can be found in the TAIR database and the UniProt database respectively, under the following accession numbers: *NF-YA3* (TAIR ID: AT1G72830), (UniProt ID: Q93ZH2); *NF-YA8* (TAIR ID: AT1G17590), (UniProt ID: Q9LNP6); *ACT2* (TAIR ID: AT3G18780); *RCE1* (TAIR ID:AT4G36800).

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Figure legends

Fig. 1. GUS staining in *pNF-YA3::GUS* and *pNF-YA8::GUS* plants.

In capital letters GUS staining in *pNF-YA3::GUS* plants; in small letters GUS staining in *pNF-YA8::GUS* plants. GUS staining in vegetative and reproductive tissues: in rosette leaves (A and a), in cauline leaves (B and b), in axils of stem branches (C and c), in roots (D and d), in flowers (E and e) and in siliques (F and f). (G and g) GUS staining in anthers at 8 floral stage. (H-K and h-k) GUS staining in the different stages of ovule development. (H and h) ovule primordia of flower at stage 9-10, (I and i) developing ovules of flower at stage 11, (J and j) ovule of flower at stage 12 and (K and k) mature ovule of flower at stage 13. (L-Q and l-q) GUS staining during seed development. (L and l) Embryo at 2-4 cell stage, (M and m) globular, (N and n) mid-heart, (O and o) late-heart, (P and p) torpedo and (Q and q) mature embryo. Stages of flower development are according to Bowman, 1994. h, hydathodes; t, trichomes. Bars in (A) to (F) and (a) to (f) = 250µm; bars in (G) to (K) and (g) to (k) = 20µm; bars in (L) to (Q) and (l) to (q) =10µm.

Fig. 2. *In situ* analyses of *NF-YA3* and *NF-YA8* expression in developing flower and seed.

Longitudinal sections of developing flowers, showing *NF-YA3* (A-E) and *NF-YA8* (F-J) expression in flower at stage 8 (A,F), in flower at stage 9 (B,G), stage 11 (C,H), 12 (D,I) and stage 13 (E,J). Stages of flower development are according to Bowman, 1994. Longitudinal sections of developing seeds, showing *NF-YA3* (K-P) and *NF-YA8* (Q-V) expression in different stages of embryo development: (K,Q) embryo at early globular stage, (L,R) globular, (M,S) mid-heart, (N,T) late-heart, (O,U) torpedo and (P,V) cotyledon stage. Arrows indicate endosperm cells. Abbreviations***:*** p, placenta; c, carpel; op, ovule primordia; o, ovule; a, anther; tm, tetrad of microspores; pg, pollen grains; t, tapetum. Bars = 50µ.

Fig. 3. Structure of the T-DNA insertional mutants and semi-quantitative RT-PCR analyses.

A) Schematic representation of the *NF-YA3* and *NF-YA8* genes. Position of the T-DNA insertion sites for *nf-ya3* and *nf-ya8* mutant alleles are indicated. Exons are boxed and lines between boxes represent introns. Red boxes correspond to coding sequence exons, black boxes correspond to 5' UTR and 3'UTR. LB, left border; RB, right border. As determined by sequencing of the T-DNA/gene junctions, the duplicated genomic region of 54 nucleotides in the *nf-ya8* mutant allele is shown. The highly conserved domain, composed by Subunit Association Domain (SAD) and DNA binding domain (BD) and the linker region (lined box), is indicated. B) RT-PCR of *NF-YA3* and *NF-YA8* transcript in *nf-ya3* and *nf-ya8* mutants. Total RNA was extracted from developing siliques of wild-type and mutant plants and analyzed by RT-PCR. All cDNA samples were standardized using a set of primers specific for the *ACTIN 2* (*ACT2*) gene.

Fig. 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.

Fig. 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. Arrows indicate cells that have undergone abnormal division. Bars = 10μm.

Fig. 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 = 50μm. (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.

Fig. 7. RT-PCR of *NF-YA3* and *NF-YA8* transcripts in *35S::NF-YA3* and *35S::NF-YA8*. RT-PCR analysis showing transcript levels of *NF-YA3* and *NF-YA8* in developing siliques of transgenic and wild-type plants. PCR products were blotted and hybridized with gene-specific random primed probes. The *ACTIN 2* (*ACT2*) gene was used as a control.

Fig. 8. Expression pattern of *DR5rev::GFP* in wild-type and *nf-ya3 nf-ya8* embryo.

(A,B) *DR5rev::GFP* expression in wild-type embryos. Embryo at globular stage (A) and at cotyledon stage (B). (C,D) Absence of *DR5* signal in *nf-ya3 nf-ya8* defective embryos. Confocal laser scanning images; bars = 25μm.

Tables

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	36.81/25
nf-ya3/nf-ya3 NF-YA8/nf-ya8	217/172.5	62.90/50
nf-ya3/nf-ya3 nf-ya8/nf-ya8	0/86.25	0/25
Total	344/344	100/100
P value[a]	0.2> P >0.1	

[a] Chi-square test for a 2:1 segregation hypothesis.

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
P value[a]	0.5>P>0.2	

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

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

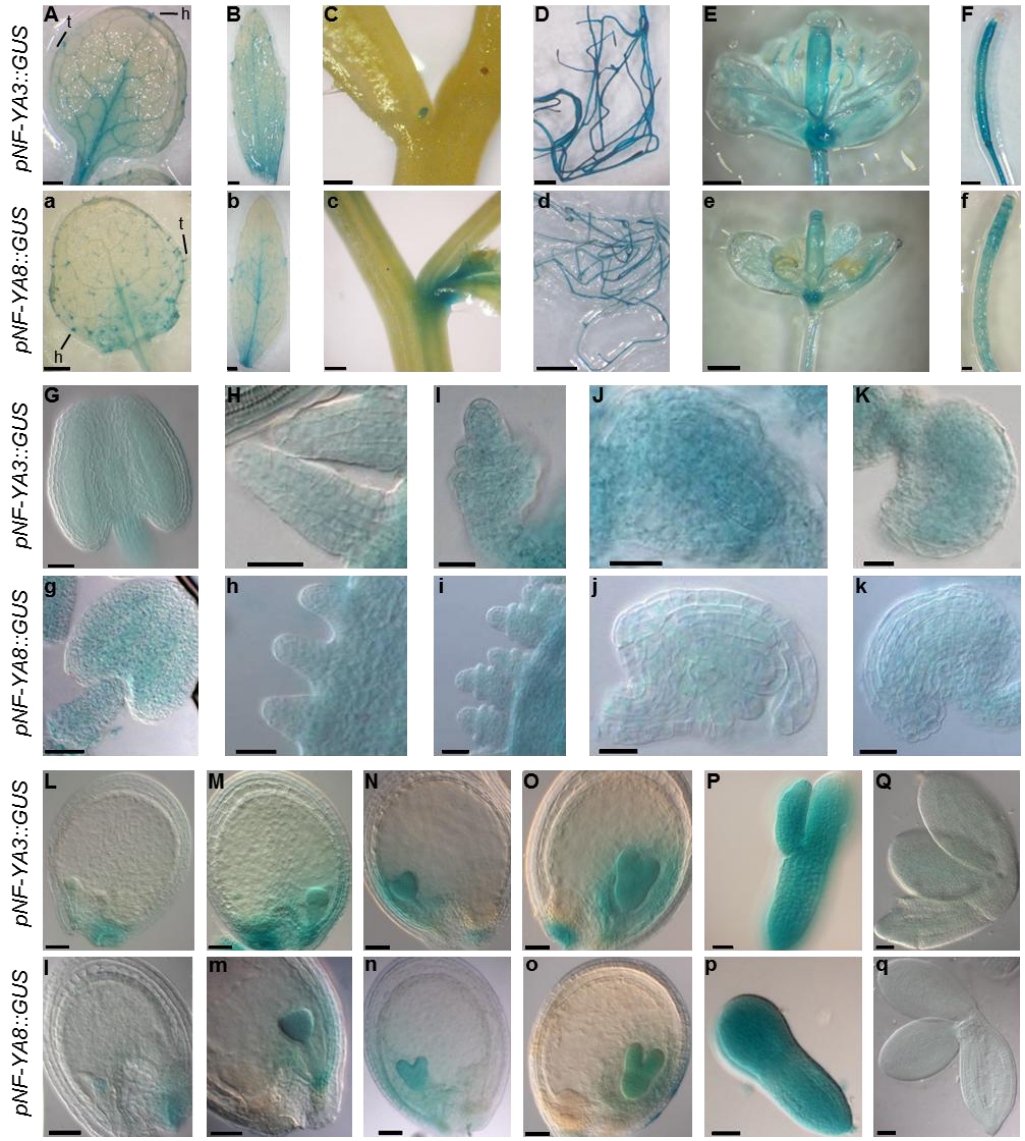
		Progeny phenotype (observed/expected[b])				
T_1 line[a]	KAN^R/KAN^S	Normal seeds	Arrested seeds	Total	P value[c]	Observed arrested seeds (%)
11	15:1	74/78.75	6/1.25	80/80	P<0.0001	7.5
13	15:1	258/294.33	41/4.67	299/299	P<0.0001	13.71
16	15:1	160/190.97	34/3.03	194/194	P<0.0001	17.53
48	3:1	126/137.81	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

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

		Progeny phenotype (observed/expected[b])				
T_1 line[a]	KAN^R/KAN^S	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* T1plants 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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pNF-YA8::GUS
pNF-YA3::GUS
pNF-YA8::GUS
pNF-YA3::GUS
pNF-YA8::GUS
A
t
h
B
C
D
E
F
a
t
h
b
c
d
e
f
G
H
I
J
K
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Q
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m
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o
p
q

Fig. 1

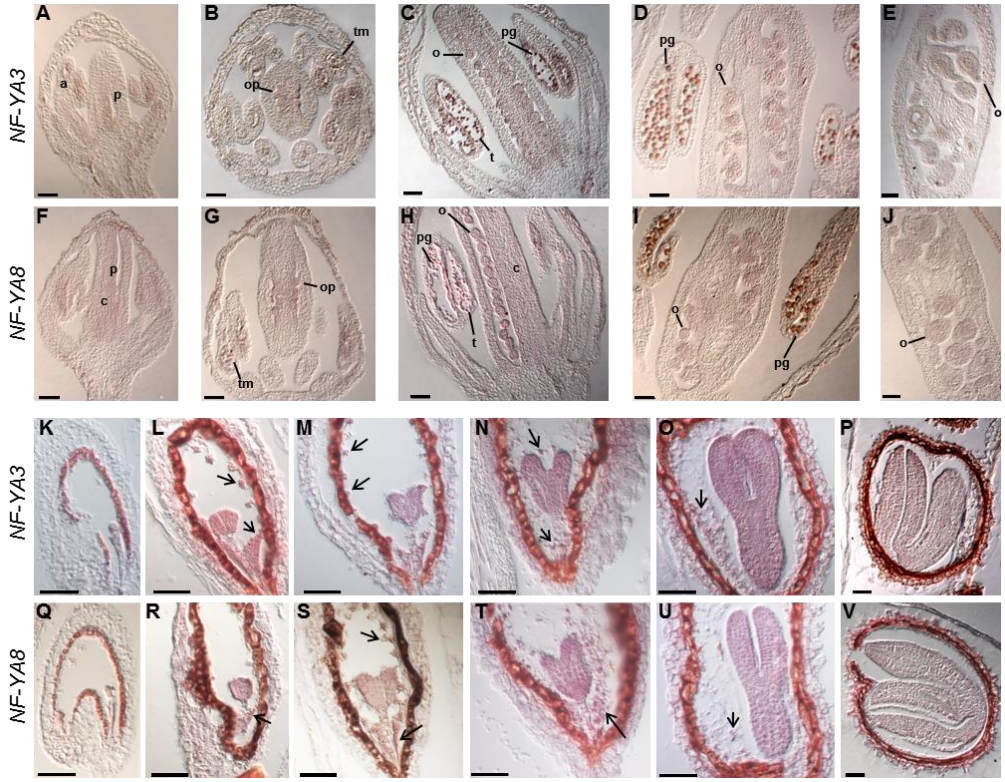
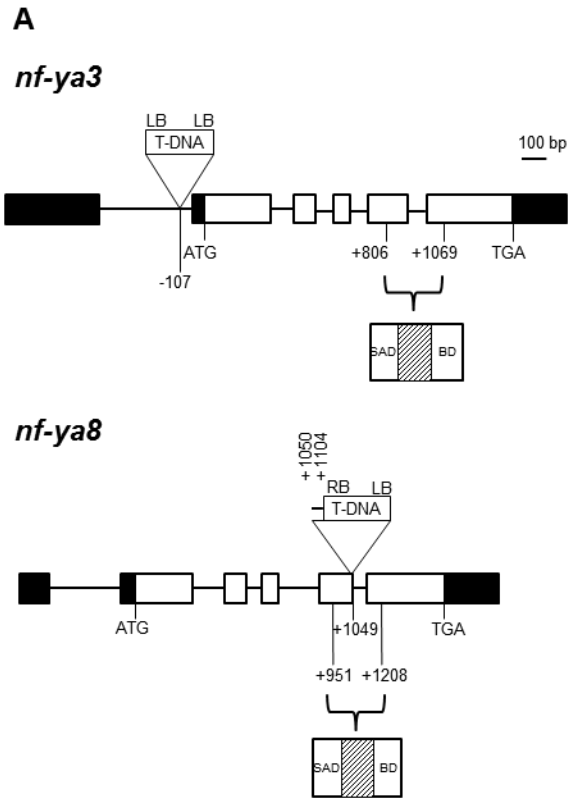

NF-YA3
NF-YA8
NF-YA3
NF-YA8
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
a
p
tm
op
o
pg
t
c

Fig. 2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nf-ya3
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LB
T-DNA
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ATG
-107
+806
+1069
TGA
SAD
BD
nf-ya8
+1050
+1104
RB
LB
T-DNA
ATG
+1049
TGA
+951
+1208
SAD
BD
B
WT
nf-ya3
WT
nf-ya8
NF-YA3
NF-YA8
ACT2
ACT2

Fig. 3

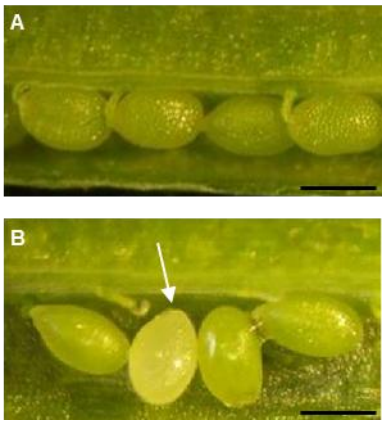

Fig. 4

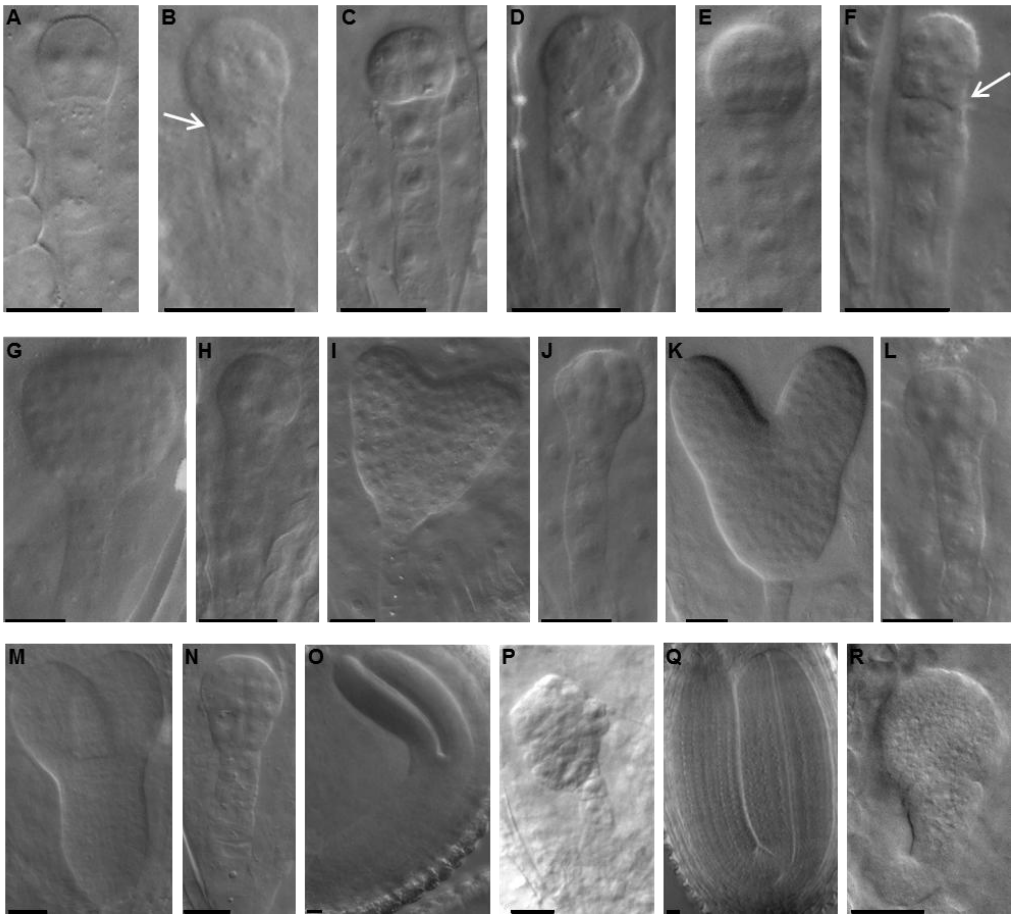

Fig. 5

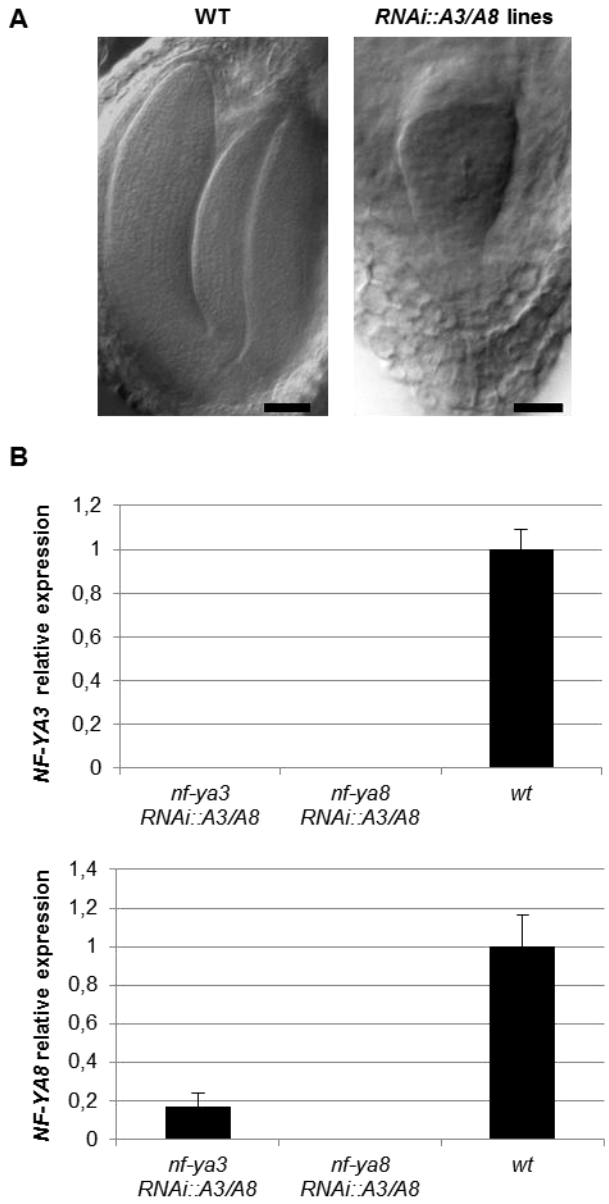
A
WT
RNAi::A3/A8 lines
B
NF-YA3 relative expression
1,2
1
0,8
0,6
0,4
0,2
0
nf-ya3 RNAi::A3/A8
nf-ya8 RNAi::A3/A8
wt
NF-YA8 relative expression
1,4
1,2
1
0,8
0,6
0,4
0,2
0
nf-ya3 RNAi::A3/A8
nf-ya8 RNAi::A3/A8
wt

Fig. 6

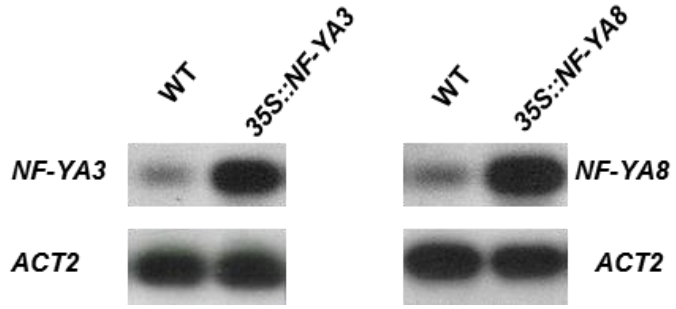

Fig. 7

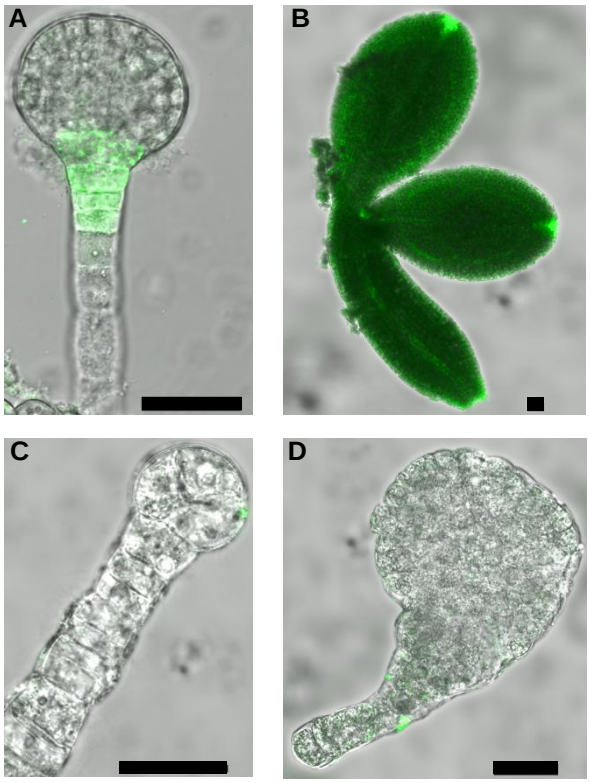

Fig. 8