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Divergent Expression Patterns of *miR164* and *CUP-SHAPED COTYLEDON* Genes in Palms and Other Monocots: Implication for the Evolution of Meristem Function in Angiosperms

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Abstract

In order to understand how the morphology of plant species has diversified over time, it is necessary to decipher how the underlying developmental programs have evolved. The regulatory network controlling shoot meristem activity is likely to have played an important role in morphological diversification and useful insights can be gained by comparing monocots and eudicots. These two distinct monophyletic groups of angiosperms diverged 130 Ma and are characterized by important differences in their morphology. Several studies of eudicot species have revealed a conserved role for *NAM* and *CUC3* genes in meristem functioning and pattern formation through the definition of morphogenetic boundaries during development. In this study, we show that *NAM*- and *CUC3*-related genes are conserved in palms and grasses, their diversification having predated the radiation of monocots and eudicots. Moreover, the *NAM-miR164* posttranscriptional regulatory module is also conserved in palm species. However, in contrast to the *CUC3*-related genes, which share a similar expression pattern between the two angiosperm groups, the expression domain of the *NAM-miR164* module differs between monocot and eudicot species. In our studies of spatial expression patterns, we compared existing eudicot data with novel results from our work using two palm species (date palm and oil palm) and two members of the Poaceae (rice and millet). In addition to contrasting results obtained at the gene expression level, major differences were also observed between eudicot and monocot *NAM*-related genes in the occurrence of putative *cis*-regulatory elements in their promoter sequences. Overall, our results suggest that although *NAM*- and *CUC3*-related proteins are functionally equivalent between monocots and eudicots, evolutionary radiation has resulted in heterotopy through alterations in the expression domain of the *NAM-miR164* regulatory module.

Key words: evo-devo, meristem, microRNA, palms, CUC, miR164.

Introduction

Angiosperm species display an enormous diversity in their architecture and in the structure of their individual organs. One of the most important evolutionary questions is to understand how this morphological diversity arises during evolution. Plant form diversity has resulted in part from variations in the organogenic activity of meristems (apical and/or axillary) during the vegetative and reproductive phases of development. In order to understand how angiosperm morphology has radiated during evolution, it is therefore interesting to investigate how meristem function has diverged between different groups. One important morphological dichotomy observed during angiosperm evolution is the split between the monocots and eudicots lineages, which diverged 130 Ma.

In angiosperms, the shoot meristem is typically a dome-shaped structure with a characteristic inner organization of

tissues, containing a group of undifferentiated and dividing cells (Kwiatkowska 2008). Its activity is tightly controlled in order to carry out two processes: first, its self-maintenance; and second, the formation of lateral organs. The formation of an organ primordium without disrupting the integrity of the meristem requires the creation of boundaries that separate the primordium from surrounding tissues, in order to delineate territories, which will undergo different cell fates.

Several genes involved in the definition of the morphogenetic boundaries have been identified in a number of species (Aida and Tasaka 2006). One such class identified includes the *Petunia hybrida* gene *NO APICAL MERISTEM* (*PhNAM*; Souer et al. 1996), the *Arabidopsis thaliana* genes *CUP-SHAPED COTYLEDON 1, 2, and 3* (*AtCUC1*, *AtCUC2*, *AtCUC3*; Aida et al. 1997; Takada et al. 2001; Vroemen et al. 2003) and the *Antirrhinum majus* gene *CUPULIFORMIS* (*AmCUP*) (Weir et al. 2004). All these genes encode

related members of the NAC family (for NAM, ATAF1/2, CUC), which constitute one of the largest plant-specific families of transcription factors (105 genes in *A. thaliana* and 75 in rice; Ooka et al. 2003).

In eudicot species, NAM/CUC3 genes are known to be involved in shoot apical meristem (SAM) establishment during embryogenesis, in axillary meristem establishment at later stages, and also in the establishment and maintenance of meristem-organ (M-O) and organ-organ (O-O) boundaries during plant development as a whole (Souer et al. 1996; Aida et al. 1997, 1999; Takada et al. 2001; Vroemen et al. 2003; Weir et al. 2004; Hibara et al. 2006; Keller et al. 2006; Blein et al. 2008; Raman et al. 2008; Berger et al. 2009). They are also involved in lateral organ enlargement (Larue et al. 2009), in leaf shape patterning in both simple and compound-leaved species (Nikovics et al. 2006; Blein et al. 2008; Berger et al. 2009), and in the postmeristematic maintenance of phyllotaxy through the inhibition of cell division and elongation in their expression domain, thereby leading to structural separation (Peaucelle et al. 2007). The two classes of CUC gene are expressed in narrow domains and in the M-O and O-O boundaries, and their contribution may differ between species and/or tissues, as illustrated by *AtCUC1* and *AtCUC2* versus *AtCUC3* involvement in *A. thaliana*, indicating both partial redundancy and subfunctionalization of the NAM and CUC3 genes (Hibara et al. 2006; Nikovics et al. 2006).

Several studies have shown that some NAM-related genes are posttranscriptionally regulated by a microRNA, *miR164*, which limits boundary expansion by cleavage of their mRNAs (Laufs et al. 2004; Mallory et al. 2004; Baker et al. 2005; Nikovics et al. 2006; Peaucelle et al. 2007; Sieber et al. 2007). In *A. thaliana*, *miR164* acts by regulating transcript abundance in a pre-existing transcriptional pattern, leading to a dampening of expression and therefore the reduction or stabilization of target gene expression levels (Baker et al. 2005; Nikovics et al. 2006; Sieber et al. 2007; Raman et al. 2008). This suggests that the spatial regulation of NAM genes depends to a large extent on initial transcriptional activity; however, nothing is known as yet of the factors directly involved in this process. A different regulatory process occurs in tomato, in which the *GOBLET* gene, the tomato NAM ortholog, and *miR164* are expressed in complementary domains in the SAM and also in the developing leaf primordium during leaflet formation (Berger et al. 2009). This suggests that the regulatory mechanism of the NAM-*miR164* module might vary between species, from the dampening system in *A. thaliana*, to an exclusive system as in tomato (Voinnet 2009). However, at this point, it cannot be concluded which regulatory system is more typical in eudicots.

In *Zea mays* and *Oryza sativa* (Poales, Poaceae), the CUC3-related genes present an expression pattern similar to that observed in eudicots (i.e., M-O and O-O boundaries) (Hibara and Nagato 2005; Zimmerman and Werr 2005; Hibara and Nagato 2006; Oikawa and Kyo-zuka 2009). In contrast, the expression patterns of the NAM-related genes from these two species show some divergence compared

with eudicots (Hibara and Nagato 2005; Zimmerman and Werr 2005; Hibara and Nagato 2006; Oikawa and Kyo-zuka 2009). During maize reproductive development, the NAM-related genes are expressed within the spikelet and floral meristems and not in the M-O and floral O-O boundaries (Zimmerman and Werr 2005). These results suggest that the NAM/CUC3 genes from maize and rice may be partially divergent in their function compared with the situation in eudicots. It is not clear, however, whether this is a general characteristic of monocots or if it is limited to these grass species. Furthermore, no data are yet available on *miR164*-dependent regulation of NAM-related genes in maize and rice; thus, we do not yet know whether the NAM expression domains seen in these two species are associated with a similarly divergent *miR164* expression pattern.

In order to broaden current knowledge of NAM/CUC3 genes and *miR164* function in monocotyledons, we chose to characterize these genes in an order distinct from the Poales: the Arecales (Arecaceae or palm family). The palms are a group of plants of particular interest because they have certain distinctive features compared with other monocot groups (Tomlinson 2006; Jouannic et al. 2007). In this study, we focused mostly on the reproductive development of oil palm (*Elaeis guineensis*), a monoecious species producing male and female inflorescences at different times in an alternating cycle, and date palm (*Phoenix dactylifera*), a dioecious species. Both produce highly branched inflorescences consisting of a central rachis bearing rachillae, which carry the unisexual flowers. Male and female inflorescences are strongly dimorphic, the former being characterized by a higher density of rachillae and flowers due to the larger number of axillary and floral meristems initiated (Adam et al. 2005; Daher et al. 2010).

We describe here the isolation of NAM- and CUC3-related genes from oil palm and date palm, on the basis of a phylogenetic sequence analysis. We characterized their expression patterns during development and investigated the biological activities of their encoded proteins. We established that the NAM-*miR164* posttranscriptional regulatory module is present in palms. To broaden our studies to other monocot phyla, we included two additional monocot species from the Poales in our expression studies, namely pearl millet (*Pennisetum glaucum*) and rice (*O. sativa*). In contrast to the situation for CUC3-related genes, the NAM-*miR164* module displays a divergent spatial expression pattern in the monocot meristems when compared with eudicots. We provide evidence suggesting that this may be due in part to the divergence of *cis*-regulatory sequences in the monocot and eudicot gene promoters since these two plant groups separated during evolution.

Materials and Methods

Plant Material

Oil palm (*E. guineensis*) vegetative shoot apex-containing samples and leaf samples were harvested from 6-month-old plants

(C1001 genotype) grown in the glasshouse in Montpellier (France). Oil palm tissues enriched in rachis, rachilla, and floral meristems were excised from inflorescences collected from the axils of leaves ranging from the leaf number F−20 to F−10, F−6 to F+2, and F+6 to F+7, respectively, on 20-year-old plants at the INRAB plantation (Pobé, Benin). The youngest expanding leaf was numbered as leaf F0. The leaf produced immediately before leaf F0 is thus leaf F+1 and the leaf produced subsequently is leaf F−1 (this leaf is not yet expanded). For a histological description of the corresponding stages, see Adam et al. (2005). In vitro somatic embryos from oil palm were produced from embryonic cell suspensions as described in Aberlenc-Bertossi et al. (1999).

Date palm (*p. dactylifera*) inflorescences were collected from both male and female plants in Sanremo (Italy). Pearl millet (*p. glaucum*; ligui ecotype) and rice (*O. sativa*; Nipponbare ecotype) inflorescences were harvested from 2-month-old plants grown in the glasshouse in Montpellier (France).

Arabidopsis thaliana plants of the ecotype Columbia (Col) were supplied by the Nottingham Arabidopsis Stock Centre (see <http://nasc.nott.ac.uk> and the *cuc2-1* mutant line (*cuc2-1* *Ler* allele back-crossed in Col background) was kindly provided by M. Aida.

Complementary DNA and Gene Cloning

Oil palm and date palm NAM/CUC3-related cDNA fragments were amplified by polymerase chain reaction (PCR) from phage DNA of male inflorescence cDNA (complementary DNA) libraries constructed in the λZAPII vector (Stratagene) using the degenerate primers CUCS1 and CUCAS1 (see [supplementary table S1, Supplementary Material](#) online) designed against the NAC domain. After cloning and sequencing, one of the PCR products of interest was used as a probe to screen the oil palm male inflorescence cDNA library, using standard conditions (Sambrook et al. 1989). The phagemids were then isolated according to the manufacturer's instructions (Stratagene). Date palm full-length NAM/CUC3-related cDNAs were obtained by 5' and 3' RACE (rapid amplification of cDNA ends) PCRs using male inflorescence mRNA-derived reverse transcription (RT) obtained with the SMARTer RACE cDNA Amplification kit (Clontech).

The corresponding genes were isolated according to a nested PCR-based cloning approach using total DNA from a male date palm and an oil palm genomic DNA library (FN4 genotype) in conjunction with the Genome walker kit (Clontech) according to the manufacturer's instructions. The primers used for the promoter, the terminator, and the coding region of the corresponding genes are listed in [supplementary table S1 \(Supplementary Material](#) online).

All the PCRs were performed using the Advantage 2 PCR Kit (Clontech). The PCR products were cloned using a pGEM-T easy ligation kit (Promega, France) with *Escherichia coli* JM109 competent cells (Promega) and sequenced by Beckman Coulter Genomics (UK).

Computational and Database Analysis

Similarity searches were performed using the BlastP and BlastX programs with default parameters in conjunction with protein sequence databases and TBlastN with nucleotide sequence collections, expressed sequence tags and high throughput genomic sequence databases provided by the NCBI server (<http://www.ncbi.nlm.nih.gov/blast/>). Multiple sequence alignments were performed using ClustalW with default parameters (<http://www.ebi.ac.uk/clustalw/>). Phylogeny and molecular evolutionary analyses were based on neighbor joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) methods using Sea-view program version 4 (<http://pbil.univ-lyon1.fr/software/seaview.html>; Gouy et al. 2010). ML phylogenies were generated using LG (after authors) evolutionary model, a model of amino acid replacement model proved to be better than previously proposed model (Le and Gascuel, 2008). Statistical support of all phylogenetic analyses was provided by performing 100 bootstrap replicates. Accession numbers of all the sequences used for the NAM/CUC3 relationship analysis are listed in [supplementary table S2 \(Supplementary Material](#) online).

Promoter sequence comparisons of the NAM- and CUC3-related genes from the different species were performed using mVISTA web server (<http://genome.lbl.gov/vista/index.shtml>; Frazer et al. 2004).

RLM-RACE PCRs

To determine the transcription initiation sites of *EgNAM1* and *EgCUC3*, nested RACE PCRs were conducted using total RNA obtained from male inflorescences in conjunction with a GeneRacer kit (Invitrogen) according to the manufacturer's instructions. *EgNAM1AS3* and *EgNAM1AS4* primers, and *EgCUC3AS2* and *EgCUC3AS3* primers were used for *EgNAM1* and *EgCUC3*, respectively (see [supplementary table S1, Supplementary Material](#) online). To detect putative truncated mRNAs from *EgNAM1* at the *miR164* site, nested RACE PCRs were conducted using RNA ligase-mediated (RLM)-based RTs without CIP and TAP treatment (GeneRacer kit; Invitrogen) in conjunction with the *EgNAM1AS7* and *EgNAM1AS8* primers (see [supplementary table S1, Supplementary Material](#) online). All the PCRs were performed using the Advantage 2 PCR Kit (Clontech). PCR products were cloned using pGEM-T easy ligation kit (Promega) with *E. coli* JM109 competent cells (Promega) and sequenced by Beckman Coulter Genomics (UK).

RNA Extraction and Expression Analysis

Total RNAs (mRNAs and smallRNAs) were extracted using the RNeasy Plant Mini Kit with the RLT and RWT buffers (Qiagen, France). DNase treatments were performed using the RNase-free DNase set (Qiagen). The mRNA-derived RTs were obtained using the ImProm-II Reverse Transcription System (Promega) from 1 μg of total RNA according to the manufacturer's instructions. The miRNA-derived RTs were obtained using a miR-X miRNA First-Strand Synthesis Kit (Clontech) from 1 μg of total RNA according to the

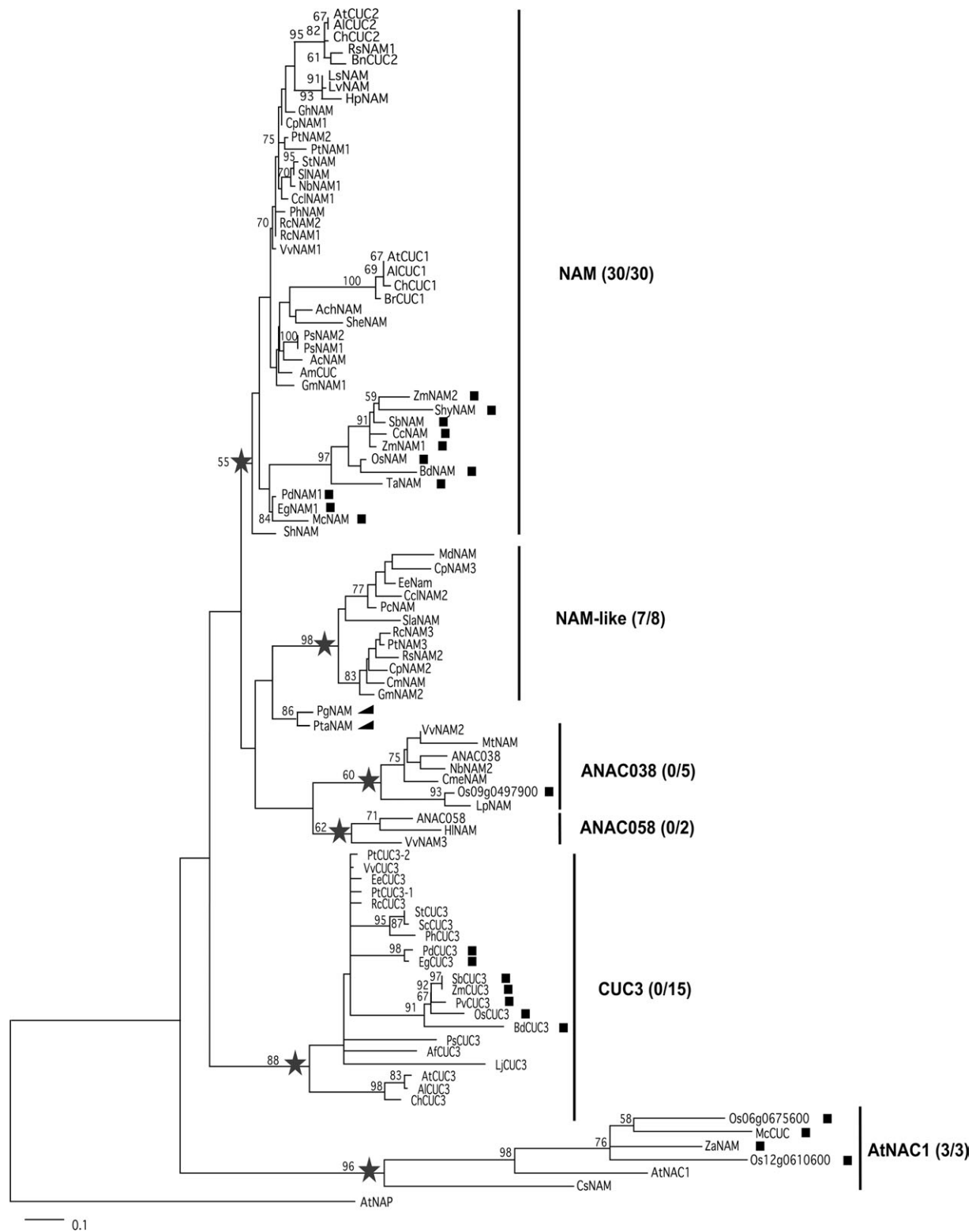


Fig. 1. Phylogenetic analysis of the NAM/CUC3 proteins. The tree is a bootstrap consensus from maximum likelihood analysis performed on the basis of NAC domain amino acid alignment, using LG evolutionary model. Bootstrap confidence values were obtained by 100 replicates and only bootstrap values greater than 50% are indicated. Black squares indicate sequences from monocot species and black triangles indicate sequences from gymnosperms. Asterisks indicate the nodes of the clades defined here. Values in brackets to the right of clade names indicate the number of full-length sequences possessing the *miR164*-binding site and the number of full-length sequences, respectively, for the corresponding clade. A list of the protein accession numbers used is detailed in [supplementary table S2 \(Supplementary Material online\)](#). Species name abbreviations are as follows: *Actinidia chinensis*, Ach; *Antirrhinum majus*, Am; *Arabidopsis lyrata*, Al; *Arabidopsis thaliana*, At; *Aquilegia coerulea*, Ac; *Aquilegia formosa*, Af; *Brachypodium distachyon*, Bd; *Brassica napus*, Bn; *Brassica rapa*, Br; *Cardamine hirsuta*, Ch; *Carica papaya*, Cp; *Castanea mollissima*, Cm; *Cenchrus ciliaris*, Cc; *Citrus clementina*, Ccl; *Crocus sativus*, Cs; *Cucumis melo*, Cme; *Elaeis guineensis*, Eg; *Euphorbia esula*, Ee; *Glycine max*, Gm; *Gossypium*

manufacturer's instructions. RT synthesis was performed twice for each RNA sample and tested independently for PCR amplifications to avoid artifacts related to the quality of RT synthesis.

Semiquantitative RT-PCRs using mRNA-derived RTs as templates were performed using GoTaq polymerase (Promega) and the following conditions: 3 min 94 °C (30 s 94 °C; 30 s 55 °C; 30 s 72 °C) for 25–35 cycles, 10 min 72 °C. Nonquantitative RT-PCRs using miRNA-derived RTs were performed using the LightCycler 480 SYBR Green I Master kit (Roche, France) according to the manufacturer's instructions and the following conditions: 10 s 95 °C, (10 s 95 °C, 15 s 60 °C) 40 cycles. The primer pairs EgNAM1S1–EgNAM1AS5, EgCUC3S5–EgCUC3AS4, EgKNOX1S4–EgKNOX1AS3, and EgEF1aS2–EgEF1aAS2 were used for specific amplification of *EgNAM1*, *EgCUC3*, *EgKNOX1* (accession number DQ890420) and *EgEF1- α 1* (accession number AY550990) transcripts, respectively. The primer pair EgNAM1S10–EgNAM1AS5 was used for the specific amplification of the 5'-untranslated region (UTR) and coding region of *EgNAM1*. MIR164A and MIR164C primers were used for specific *miR164* amplification in conjunction with the mRQ 3' primer from the miR-X miRNA First-Strand Synthesis Kit (Clontech). PCR amplifications of *EgEF1- α 1* and *U6* reverse transcripts were used as RT controls. Amplified cDNAs were cloned and sequenced to check the specificity of the amplification products. The sequences of all the primers used for RT-PCRs are listed in [supplementary table S1 \(Supplementary Material online\)](#).

RNA In Situ Hybridization

To obtain DNA templates for the RNA probe synthesis, PCR amplifications were performed with gene-specific antisense primers tailed with a T7 RNA polymerase binding site. PCRs were performed with the EgNAM1S9–EgNAM1AST7, EgCUC3S3–EgCUC3AST7, PdNAM1S2–pDNAM1AS2T7, and EgH4S1–EgH4AST7 primer pairs for *EgNAM1*-, *EgCUC3*-, *PdNAM1*-, and *EgH4*-specific probes, respectively (see [supplementary table S1, Supplementary Material online](#) for primer sequences). The resulting DNA fragments were used directly as templates for synthesizing antisense riboprobes incorporating UTP–digoxigenin (Roche) as the label in conjunction with a T7 Maxi Script kit (Ambion). The amplification products were tested by Southern hybridization to evaluate the specificity of the probe. For *miR164* detection, 0.02 μ M of a 5' digoxigenin–labeled miRCURY LNA microRNA detection probe complementary to *ath-miR164a* (Exiqon, Denmark) was used. In situ hybridization experiments were carried out as described by Adam et al. (2007). An LNA complementary to mouse *miR124* (5'-TTAAGGCACGCGTGAATGCCA-3') with no predicted target sequences in plants was used as a negative control.

No signal was observed in the different plant tissues tested for the in situ hybridization experiments.

Detection was performed using the Vector Blue Alkaline Phosphatase Substrate Kit III (Vector Laboratories). Photos were taken using an Evolution MP 5.0 cooled camera (MediaCybernetics) in conjunction with a Leica DMRB microscope and images were processed with Photoshop software (Adobe, France).

Plant Transformation

The complete open reading frames (ORF) of *EgNAM1* and *EgCUC3* were amplified by PCR using the Advantage 2 PCR Kit (Clontech) with the EgNAM1S5–EgNAM1AS1 and EgCUC3S4–EgCUC3AS1 primer pairs for *EgNAM1* and *EgCUC3*, respectively (see [supplementary table S1, Supplementary Material online](#) for the primer sequences) and cloned using the pENTR-D-TOPO cloning kit (Invitrogen). The corresponding ORFs were transferred to the pMDC32 binary vector (Curtis and Grossniklaus 2003) using the Gateway LR Clonase enzyme mix (Invitrogen) according to the manufacturer's instructions. The resulting constructs were named 35S:*EgNAM1* and 35S:*EgCUC3*, respectively. In order to obtain the *CUC2* promoter fusion constructs, the *EgNAM1* ORF was cloned in the pCR2.1-TOPO vector (Invitrogen). The resulting plasmid was digested by *EcoRI*, and the *EcoRI*–*EcoRI* insert fragment was ligated to the pGREEN0129 binary vector (Hellens et al. 2000) containing a 3.7-Kb-long fragment, the *CUC2* promoter region (*pCUC2*; Nikovics et al. 2006). The resulting construct was named *pCUC2:EgNAM1*. The *pCUC2:CUC2* construct was done as previously described (Nikovics et al. 2006).

Wild-type *A. thaliana* plants of the ecotype Columbia and *cuc2-1 A. thaliana* mutant plants were transformed using the floral dip method (Clough and Bent 1998), with the 35S and the *pCUC2* constructs, respectively. Transformants that survived in the medium containing hygromycin were checked for transgene insertions by PCR. Selected plants were transferred to the glasshouse and grown under standard conditions for flowering. Plant phenotypes were observed in the T0 and T1 generations for the overexpression lines. The floral phenotype of transformed *cuc2-1* plants was calculated as the average of the sepal fusion score (Laufs et al. 2004) of ten flowers of the main stem of T1 plants (the untransformed *cuc2-1* line has a score of 6.2 ± 0.7 [standard deviation]). For the scoring of the leaf phenotype of transformed *cuc2-1* lines, 20 T2 plants of each line were grown alongside with four standard lines showing increasing dissection levels. At bolting, a serration score ranging from 1 to 5 was given to each line by comparing it with a smooth line: *cuc2-1*, serration score = 1; normal: wild type, serration score = 2; moderate increase of dissection: *mir164a-4*, serration score = 3; intermediate increase of

←
hirsutum, Gh; *Humulus lupulus*, Hl; *Helianthus paradoxus*, Hp; *Lactuca sativa*, Ls; *Lactuca virosa*, Lv; *Lolium perenne*, Lp; *Lotus japonicus*, Lj; *Malus domestica*, Md; *Medicago trunculata*, Mt; *Musa cachaco*, Mc; *Nicotiana benthamania*, Nb; *Oryza sativa*, Os; *Paullinia cupana*, Pc; *Panicum virgatum*, Pv; *Petunia hybrida*, Ph; *Phoenix dactylifera*, Pd; *Picea glauca*, Pg; *Pinus taeda*, Pta; *Pisum sativum*, Ps; *Populus trichocarpa*, Pt; *Raphanus sativus*, Rs; *Ricinus communis*, Rc; *Saccharum hybrid*, Shy; *Saruma henryi*, Sh; *Silene latifolia*, Sla; *Solanum chacoense*, Sc; *Solanum lycopersicum*, Sl; *Solanum tuberosum*, St; *Sorghum bicolor*, Sb; *Striga hermonthica*, She; *Triticum aestivum*, Ta; *Vitis vinifera*, Vv; *Zantedeschia aethiopica*, Za; *Zea mays*, Zm.

dissection: *CUC2g-m4*, serration score = 4; stronger increase of dissection, serration score = 5.

Results

Palm *NAM/CUC3* Homologs and Related Genes from Other Plant Species

The use of degenerate PCR primers designed on the basis of highly conserved amino acid motifs within the NAC domain (FRFHPTDE and MKKTLVYF) allowed the isolation of *NAM/CUC3*-related cDNAs, namely *EgNAM1* and *EgCUC3* from oil palm (GenBank accession numbers HM622270 and HM622271, respectively) and *PdNAM1* and *PdCUC3* from date palm (GenBank accession numbers HM622272 and HM622273, respectively). Blast analyses revealed that the deduced *EgNAM1* and *PdNAM1* polypeptides were related to the proteins *AtCUC1* and *AtCUC2* from *A. thaliana*, whereas *EgCUC3* and *PdCUC3* were more related to the *A. thaliana* *AtCUC3* protein. The NAC domain, which is subdivided into five subdomains (A to E; Ooka et al. 2003), is highly conserved (similarities ranging from 92% to 99%) between the different *NAM*- and *CUC3*-related proteins (supplementary fig. S1, Supplementary Material online).

Detailed phylogenetic analyses were performed in order to examine the relationship between the palm *NAM/CUC3* sequences and those of related proteins (fig. 1). Our analyses were based on the protein sequences of the NAC domain available in public databases from the various established genome sequences (ESTs and whole genomes available in June 2010). Several algorithms (NJ, MP, and ML) were used and all three methods produced a similar tree topology. The ML tree topology is shown in figure 1. Several noticeable clades are indicated with a star, notably in the cases of the *NAM* and the *CUC3* clades. As expected, *EgNAM1* and *PdNAM1* belong to the *NAM* clade, whereas *EgCUC3* and *PdCUC3* belong to the *CUC3* clade. Within the *CUC3* and *NAM* clades, the sequences identified in monocotyledonous plants were grouped into two subbranches, distinct from the eudicot sequences, with a cleavage between Poales and non-Poales sequences. Within the *NAM* and *CUC3* clades, the sequences from eudicot species were resolved in several subclades.

Within the *CUC3* clade, each species studied to date possesses only a single gene of this type with the exception of *Populus trichocarpa*. In contrast, several duplication events appear to have occurred in the *NAM* clade, as suggested by the presence of paralogs in species from the orders Brassicales (Malvids-Rosids I) and Rosids II (Fabids). In the monocot *NAM* subbranches, a single *NAM*-related gene is present per species with the exception of *Z. mays*, which possesses two closely related genes. In date palm, only one *NAM*-related gene has been found in the available genome sequence data (see <http://qatar-weill.cornell.edu/research/datepalmGenome/>).

The *NAM*-like clade contains sequences specifically from eudicot species but not from *A. thaliana*. *NAM*-related sequences from *Picea* and *Pinus* species from gymnosperms are branched at the base of the *NAM*-like clade. As regards

the three other identified clades, the *ANAC038* and *AtNAC1* groups contained both eudicot and monocot species, in contrast to the *ANAC058* clade that comprised only eudicot species.

In parallel to the *NAC* domain based relationship analysis, members of the *NAM* and *CUC3* clades were found to share distinct features in their C-terminal region outside the *NAC* domain. The C-terminal domain of the *CUC3*-related proteins is poorly conserved, but the *NAM*-related proteins share distinct conserved domains as already reported (Zimmermann and Werr, 2005; see supplementary fig. S1, Supplementary Material online). These motifs are conserved in both eudicots and monocots (comprising Poales and the two palm species), with the exception of motif I that is highly conserved in the eudicot and palm proteins but not in the Poales proteins (supplementary fig. S1, Supplementary Material online).

No *miR164*-binding site was found in the full-length gene sequences corresponding to the *CUC3*, *ANAC058*, and *ANAC038* clades (fig. 1). In contrast, a *miR164*-binding site was found in available full-length gene sequences corresponding to the *NAM*, *AtNAC1*, and *NAM*-like clades (fig. 1). The *miR164*-binding site alignment revealed an overall well-conserved sequence with a few variations, allowing the definition of different groups (fig. 2A). Eudicot sequences from the *NAM* clade belong mainly to groups 1 and 2 and monocot species to groups 3 (Poales) and 4 (palms). The *NAM*-related sequence from the gymnosperm species *Picea glauca* possesses a *miR164*-binding site identical to that observed in the two palm species (group 4). In accessions from the *NAM*-like clade, sequences displaying divergences compared with the *miR164*-binding site from *NAM* sequences were identified (fig. 2B). In *AtNAC1*, the *miR164*-binding site showed only one additional nucleotide change compared with the *miR164*-binding site identified in *LsNAM*. Interestingly, all the monocot sequences of the *NAM* clade, along with the entire *NAM*-like clade, *AtNAC1*, and the *NAM*-related sequence from *P. glauca*, share a C nucleotide at position 14, in contrast to the other eudicot species. Moreover, all the aforementioned sequences, plus the sequences from group 2, share a C nucleotide at position 9. These two substitutions lead to a better pairing between the mature *miR164* and its targets. In parallel, various paralogs of the mature *miR164*, characterized by single nucleotide substitutions, have been observed in individual species (fig. 2B). The set of paralogous mature *miR164* may differ between species. However, these sequence variations do not correlate with sequence modifications in the binding site of the target mRNA sequences (fig. 2). Finally, it was observed that the sequence of mature *Ath-miR164a/b* was conserved in all the species for which data were available from both eudicots and monocots. This mature *miR164* sequence may correspond to the ancestral form (fig. 2B).

Genomic Structure of *NAM*- and *CUC3*-related Genes

Genomic sequencing revealed that *EgNAM1* and *EgCUC3* were both organized into three exons and two introns

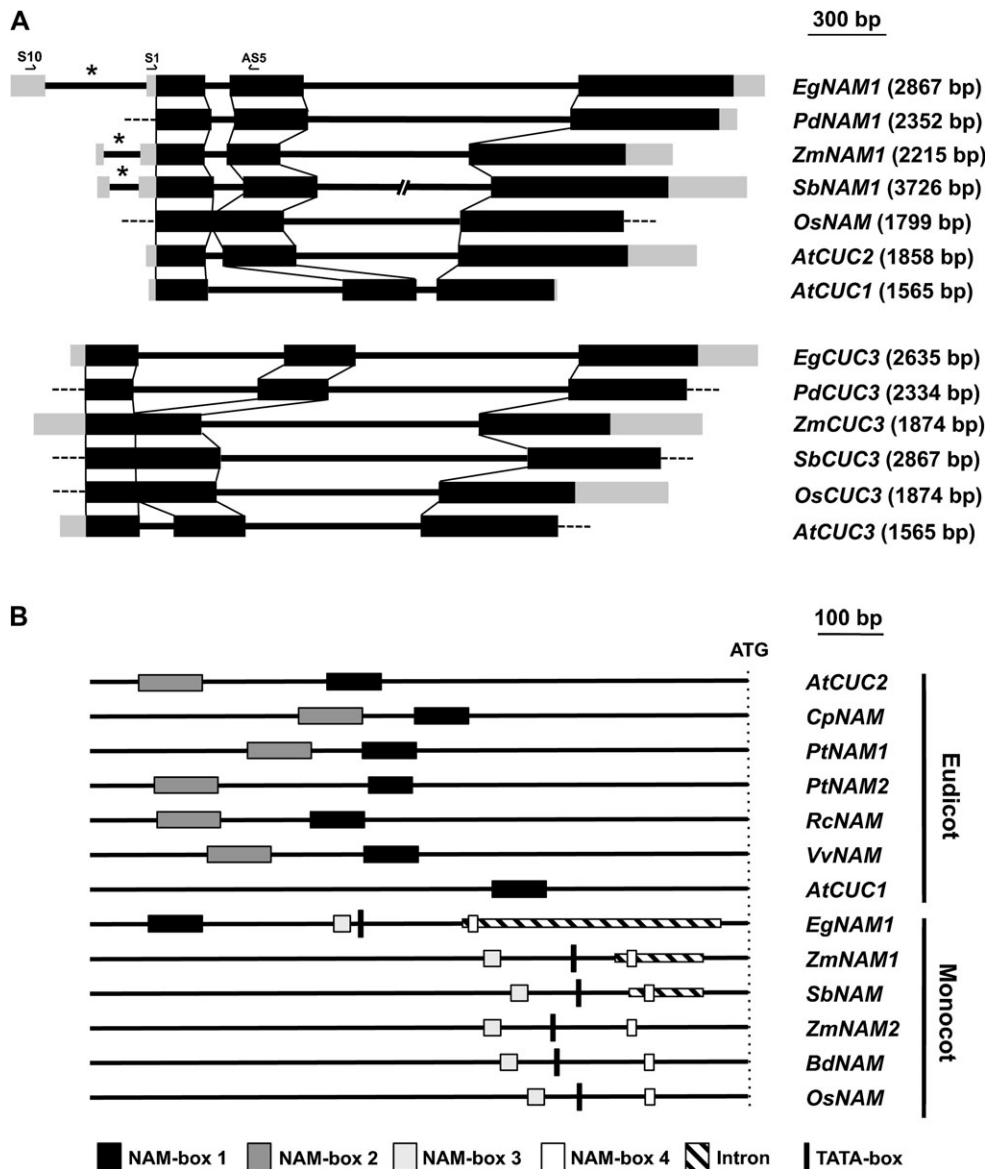


Fig. 3. Genomic structure of *NAM/CUC3*-related genes and CNSs in the 5' flanking regions of *NAM*-related genes. (A) Genomic structure of *NAM*- and *CUC3*-related genes in *Elaeis guineensis* and *Phoenix dactylifera* in comparison with *Zea mays*, *Oryza sativa* and *Arabidopsis thaliana*. Black lines correspond to intron sequences, light and dark gray boxes correspond to untranslated and translated exon sequences, respectively. Asterisks indicate the presence of a 5'-UTR intron. The positions of the primers used for the *EgNAM1* RT-PCR-based expression analysis are indicated by arrows (S10 = *EgNAM1*S10 primer; S1 = *EgNAM1*S1 primer; AS5 = *EgNAM1*AS5 primer). (B) The position of the four CNSs identified in the 5' upstream regions from the translation start codon of *NAM*-related genes are illustrated. The alignments of the corresponding sequences are shown in [supplementary fig. S1](#) ([Supplementary Material](#) online).

sequences (CNSs), which were named *NAM*-box 1, *NAM*-box 2, *NAM*-box 3, and *NAM*-box 4. Their relative positions with respect to the translation initiation codon are shown in [figure 3B](#) (see [supplementary fig. S1](#), [Supplementary Material](#) online for the sequence alignments). *NAM*-box 1 (ca. 130 bp long) was conserved in all the eudicot genes investigated and in the *EgNAM1* gene from oil palm. *NAM*-box 2 (ca. 110 bp long) is conserved specifically in the eudicot species, with the exception of *AtCUC1*. *NAM*-box 3 (ca. 39 bp long) and *NAM*-box 4 (ca. 30 bp long) were conserved specifically in the monocot genes investigated, upstream of the putative TATA-box and in the 5' half of the 5' UTR intron region, respectively. A BlastN search against

the complete genomic sequences from maize, rice, *Sorghum* and *Brachypodium* did not reveal any *NAM*-box 1 or 2 sequences in the more distant 5' flanking regions of their *NAM* genes, nor anywhere else in these genomes. In the same way, no *NAM*-box 3 or 4 related sequences were identified in the *Arabidopsis*, *Populus*, *Carica*, or *Vitis* genomic sequences. None of these sequences share similarities with any known regulatory domains. Taken together, these data indicate a wide divergence between eudicots and monocots with respect to the organization of their *NAM*-related gene 5' flanking regions.

As regards the *CUC3*-related genes, our comparisons showed that their overall exon-intron organization is similar

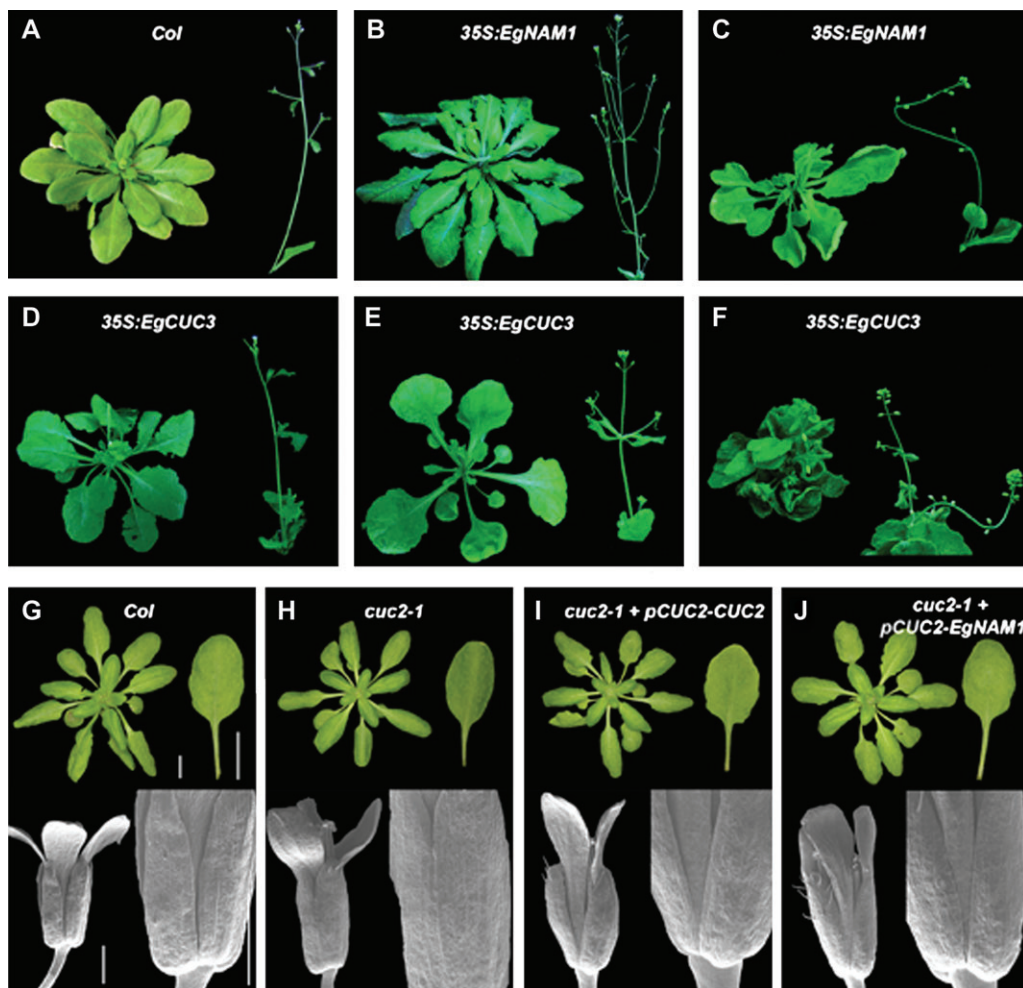


Fig. 4. Functional analysis of *EgNAM1* and *EgCUC3* genes in *Arabidopsis thaliana*. (A–F) Leaf and inflorescence phenotypes of independent *A. thaliana* Col0 plants transformed with the 35S-*EgNAM1* (two independent lines) and 35S-*EgCUC3* constructs (three independent lines). (G–J) Leaf and flower phenotypes of independent *A. thaliana* *cuc2-1* mutant plants transformed with the *pCUC2*–*CUC2* and *pCUC2*–*EgNAM1* constructs. Scale bars: 1 cm (rosette and leaf); 1 mm (flower and sepals).

to that of the *NAM*-related genes, with three exons, one intron at a common position in the NAC domain coding region, and a second intron in the C-terminal coding region. The exceptions were the *ZmCUC3*, *OscUC3*, and *SbCUC3* genes from maize, rice, and *Sorghum*, respectively, for which the intron in the NAC domain coding region was absent, suggesting a loss of this intron in the Poales. No 5′-UTR introns were observed in either the monocot or the eudicot *CUC3*-related genes, nor were any CNSs detected in their 5′ flanking regions. Interestingly, an intron is present at a conserved position in the NAC domain coding region of both *CUC3*- and *NAM*-related genes (fig. 3A) and also in some other NAC-related clades (data not shown), suggesting an ancient origin for this feature within the NAC gene family.

Are *EgNAM1* and *EgCUC3* the Functional Equivalents of the *AtCUC2* and *AtCUC3* Proteins?

To investigate the biological functions of the proteins encoded by the oil palm *EgNAM1* and *EgCUC3* genes, *A. thaliana* transgenic plants ectopically expressing each

of them under the control of the CaMV 35S promoter were generated (fig. 4A–F). In both cases, alterations to leaf shape and inflorescence architecture were observed in comparison with wild-type plants. In less severely affected lines, plants were affected only in their leaf shape with a highly pronounced serration of the leaf edges (fig. 4B and D). In more severely affected lines, the overall leaf shape was altered to produce larger leaves and petioles and the leaves were often crenated. Plants of this type also displayed a modified inflorescence structure with alterations to phyllotaxy and branching (fig. 4C, E, and F). These phenotypic alterations have also been observed in plants overexpressing the *AtCUC2* gene (Laufs et al. 2004), in plants expressing a *miR164*-resistant form of *AtCUC2*, *CUC2g-m4* (Nikovics et al. 2006; Peaucelle et al. 2007), and in plants expressing *AtCUC3* under the control of the *AtCUC2* promoter (B. Adroher and P. Laufs, unpublished data).

A complementation assay of the *A. thaliana* *cuc2-1* mutant with *EgNAM1* under the control of *AtCUC2* promoter confirmed that *EgNAM1* is functionally equivalent to *AtCUC2*. This was witnessed by the complementation of the

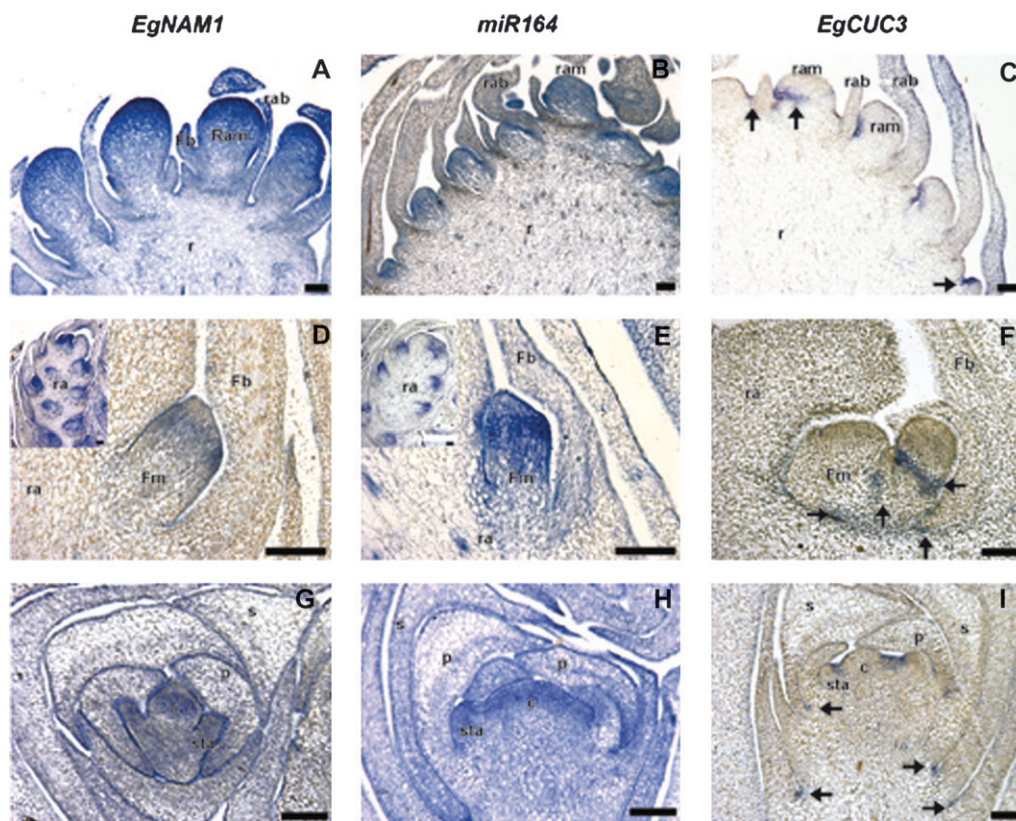


Fig. 6. In situ hybridization analysis of the expression of *EgNAM1* (A, D, G), *Egu-miR164* (B, E, H) and *EgCUC3* (C, F, I) in the oil palm female inflorescence. (A, B, C) Female inflorescence rachilla meristems initiated from the rachis at the axil of rachilla bracts. (D, E, F) Female inflorescence floral triad meristem initiated along the rachilla at the axil of floral bracts. (G, H, I) Pistillate flowers with developed sepals and petals and reproductive organ primordia. Arrows indicate *EgCUC3*-associated signals. c, carpel; Fb, floral bract; Fm, floral meristem; p, petal; r, rachis; ra, rachilla; rab, rachilla bract; rap, rachilla primordium; ram, rachilla meristem; sta, staminode; s, sepal; bars = 100 μm .

and the conservation of the *NAM-miR164* regulatory module in a monocot species.

Expression Patterns of *EgNAM1*, *Egu-miR164*, and *EgCUC3* in Reproductive Organs

Oil palm inflorescences are compound structures consisting of a central rachis that gives rise to branches known as rachillae. The oil palm produces functionally unisexual male and female inflorescences in alternation. During reproductive development, the rachis produces a series of rachilla bracts that subtend in their axils the meristems from which rachillae will be produced. The density of floral meristems produced on the rachilla is related to the sex of the inflorescence; thus, male rachillae can be seen to display a larger number of floral bracts than the future female rachillae (Adam et al. 2005). While the male inflorescence produces individual functional male flowers, the rachillae of the female inflorescence bear triads of flowers, each consisting of a functional pistillate flower flanked by two abortive staminate flowers.

EgNAM1, *Egu-miR164*, and *EgCUC3* transcript localization was studied in longitudinal sections of female inflorescences at different stages of development: at rachilla meristem initiation (fig. 6A–C), in the floral triad meristem (fig. 6D–F), and in the pistillate flower (fig. 6G–I). In the rachilla meristem, a signal corresponding to *Eg-*

NAM1 transcripts was detected in the apical cells of rachillae and at a lower level in the cells of rachilla bracts (fig. 6A). To follow in situ *miR164* expression, we used a probe complementary to *Osa-miR164a/b/f*, which recognizes at least a subset of the palm *miR164* pool (fig. 5B). Mature transcripts of *Egu-miR164* were also detected in the apical cells of rachilla meristems but not in the rachilla bracts (fig. 6B). At the same developmental stage, *EgCUC3* transcripts were detected specifically in the boundary cells between the rachilla bracts and the rachilla meristem (fig. 6C). At the floral triad meristem stage, *EgNAM1* and *Egu-miR164* transcripts were detected in the same cells of floral meristems. Transcripts were located in the cells surrounding the floral meristem (i.e., in a ringlike zone) and at a lower level in the apical tip of the meristem (fig. 6D–E). In contrast, *EgCUC3* transcripts were detected in the cells separating adjacent organs: between the rachillae and the floral triad meristems and in the zone dividing the three floral triad meristems (fig. 6F). At the flower development stage in the female inflorescence, both *EgNAM1* and *Egu-miR164* transcripts were accumulated in the cells of the reproductive organ primordial, but *EgNAM1* transcripts were not detected in the L1 cell layer of these organs (fig. 6G–H). At the same developmental stage, a signal corresponding to *EgCUC3* transcripts was

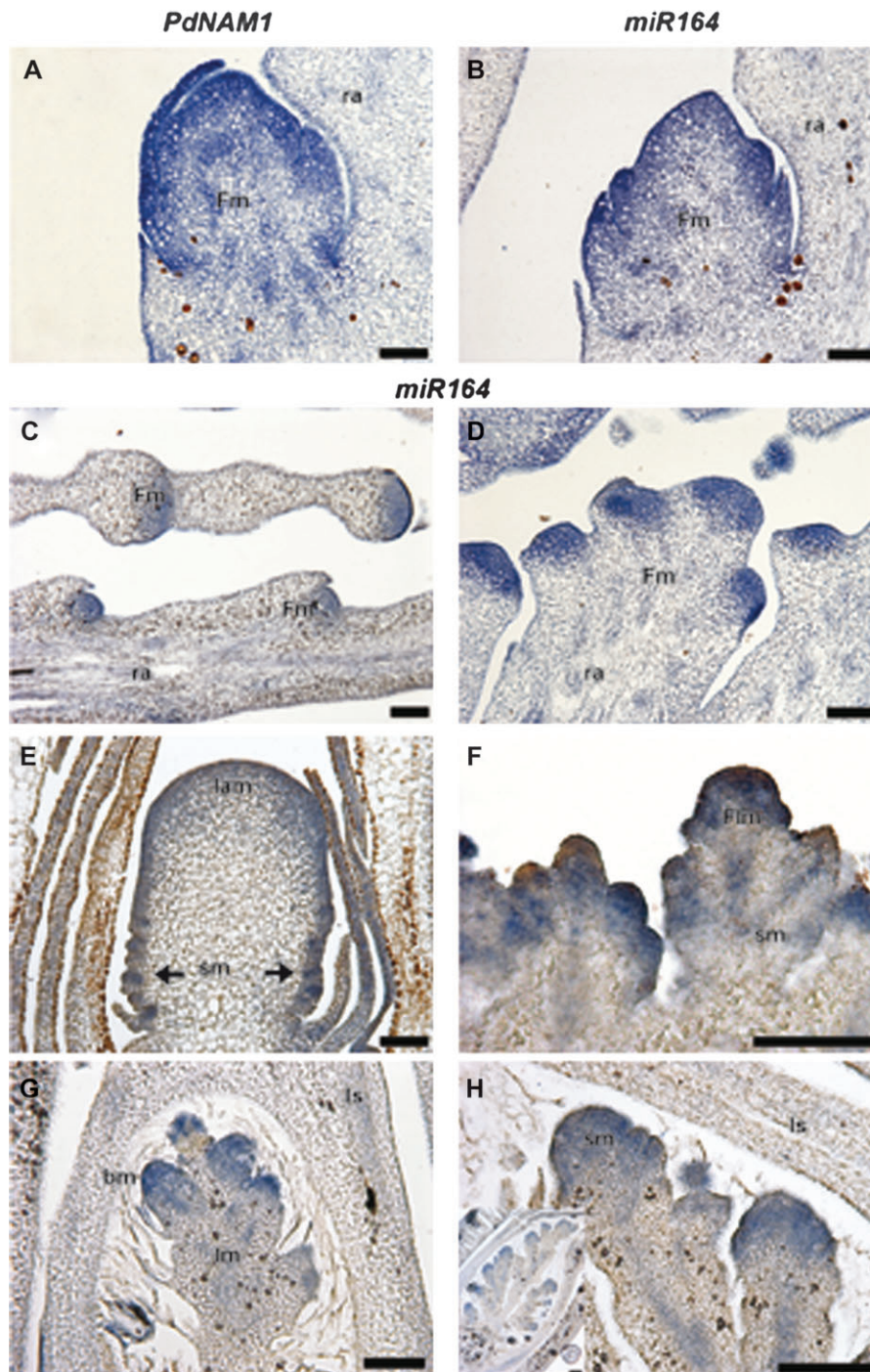


Fig. 7. In situ hybridization analysis of the expression of *PdNAM1* (A) and *miR164* (B to H) in the inflorescence meristems of other monocot species. (A, B) Date palm pistillate flower showing initiation of floral organs. (C) Date palm female rachilla bearing floral meristem. (D) Date palm male rachilla bearing floral meristem. (E, F) Millet reproductive meristems; E, millet inflorescence meristem, spm are initiated at the base of inflorescence, and initiate later the spikelet meristem, F, millet spikelet meristem with floral meristems. (G, H) Rice inflorescence meristem, Im initiated branch meristem which later bears the spikelet meristem. The SM initiates sterile glumes, followed by lemmas, containing florets in their axils. lam, Inflorescence apical meristem; Im, inflorescence meristem; Flm, Floret meristem; Fm, floral meristem; bm, branch meristem; ls, leaf sheath; sm, spikelet meristem; spm, spikelet pair meristem; ra, rachilla; bars = 100 μ m.

detected in the cells separating adjacent organs (i.e., the whorl-whorl boundary) and in the residual floral meristem of carpel primordia (fig. 6I).

Studies of the localization of *EgNAM1* and *EgCUC3* transcripts and mature *Egu-miR164* were also performed, using equivalent developmental stages, on the

male inflorescence. In this case, the patterns of expression observed were similar to those seen for the female inflorescence (supplementary fig. S3, Supplementary Material online).

The above data collectively indicate that in both female and male inflorescences and flowers, *EgNAM1* and *Egu-*

miR164 transcripts are colocalized in meristematic zones. A notable difference is seen between the indeterminate rachilla meristem, in which *EgNAM1* and *Egu-miR164* were detected in the apical tip of the meristem, and the determinate floral meristems in which transcripts were detected mainly in a ringlike zone below the apex. In contrast to *EgNAM1* and *Egu-miR164* transcripts, mRNAs of *EgCUC3* showed a distinct spatial pattern of accumulation in meristem–organ boundaries (in the case of rachillae and floral meristems) and whorl boundaries (in the case of the developing flower).

Expression Patterns of *miR164* and NAM-related Genes in Reproductive Organs of Other Monocot Species

To assess whether the expression pattern of the NAM-*miR164* module observed in oil palm was conserved in another palm species, the expression of *PdNAM1* and of *miR164* orthologs was studied in reproductive tissues of date palm (fig. 7A and B). The signals visualized on longitudinal sections of the pistillate floral meristem showed that *PdNAM1* and *miR164* transcripts were present in the same cells, in the floral meristem, and in the developing sepal and petal primordia (fig. 7A and B). *MiR164* transcripts were also detected at earlier stages of floral meristem development in both the male and female inflorescence (fig. 7C and D). These accumulation patterns were similar to those observed in inflorescences of oil palm. Although date palm is strictly dioecious, as opposed to the temporally dioecious oil palm, no distinction between male and female inflorescences was observed in our studies, these data again mirroring those obtained with oil palm.

To determine whether the expression pattern of *miR164* was also conserved in reproductive organs of other monocot species, we selected two species from the family Poales: pearl millet (*P. glaucum*) and rice (*O. sativa*) (fig. 7E–H), which both produce hermaphrodite flowers. In pearl millet, a signal corresponding to *miR164* was detected in the cells of spikelet meristems and in the apical region of the inflorescence meristem (fig. 7E). Later during development, *miR164* transcripts were also detected in the cells of the floral meristem but were apparently excluded from the L1 cell layers (fig. 7F). In rice inflorescences, *miR164* transcripts were observed to accumulate in a similar way: in the cells of the lateral meristems, in the spikelet meristems, and in the floral meristem.

These data indicate that the similar *miR164* expression patterns observed in two different palm species belonging to distinct subfamilies (Arecoideae and Coryphoideae) are also conserved in two species of the Poales, again belonging to distinct subfamilies (Panicoideae and Ehrhartoideae).

Discussion

NAM/CUC3 Gene Relationship and Evolution of the NAM-*miR164* Regulatory Module

Our phylogenetic reconstruction based on NAC domain sequences identified three main groups of NAM- and

CUC3-related genes: the NAM, the NAM-like, and the CUC3 clades. Genes belonging to the NAM and CUC3 clades are present in both eudicots (core and basal eudicots) and monocots (Arecales, Poales, Zingiberales), indicating that these clades predate eudicot–monocot separation (130 Ma; Moore et al. 2007). Within the NAM clade, several duplication events have occurred, notably in the Brassicales (*CUC1* vs. *CUC2* genes) and also in *Populus*, pea, and maize. In contrast, the NAM-like clade appears to have originated specifically in eudicots from an ancestral eudicot NAM/NAM-like gene, which may have been lost in the Brassicales. The weak support along the backbone of the phylogenetic tree, especially the branching of the gymnosperm sequences, unables us to conclude about the evolutionary diversification of the different clades. In order to reconstruct more precisely how NAM/CUC3 evolution occurred within the plant kingdom, it will be necessary to assemble more data from other monocot families, basal angiosperms, gymnosperms, and lower branching tracheophyte groups.

The *miR164*-binding site is globally well conserved in all the full-length sequences belonging to the NAM and AtNAC1 clades along with the NAM-related sequence from the gymnosperm species *P. glauca*. The fact that the *miR164* recognition site is conserved in terms of both transcribed mRNA and encoded protein sequences in NAM-related genes and other NAC domain encoding genes targeted by *miR164* adds further weight to the hypothesis of a common origin for the *miR164*-targeted genes rather than an evolutionary convergence. A few modifications of the *miR164* recognition site sequences were observable in relation to the phylogenetic relationships of the plant species (i.e., eudicot vs. monocots; Poales vs. Arecales). However, these modifications were not associated with changes to the mature *miR164* sequence in these species. This may be because *miR164* tolerates mismatches at the recognition site for its pairing to the target mRNA, leading to possible polymorphisms in the recognition site. A degenerate *miR164* recognition site was observed in full-length sequences belonging to the NAM-like clade. Unfortunately, it cannot be concluded whether these sites are still recognized by *miR164*. Interestingly, no polymorphism was observed in any of the seven positions adjoining the 3' end of the binding site (with the exception of a few NAM-like sequences), suggesting that the nucleotide sequence of this region is crucial to the activity of *miR164* or its cofactors. This is corroborated by studies of the effects of mutations in this region on the regulation of the target mRNA, as illustrated by the goblet mutant from tomato (Berger et al. 2009), the *A. thaliana cuc2-1D* mutant (Larue et al. 2009), or the *miR164*-resistant form of *AtCUC2* (Laufs et al. 2004).

MiR164-related sequences have been identified in a wide range of vascular plants, including core eudicots, monocots, basal angiosperm species such as *Amborella trichopoda* and *Cabomba aquatica* (Jasinski et al. 2010), and the gymnosperm species *P. glauca* and *Pinus resinosa* (Axtell and Bartel 2005; Sunkar and Jagadeeswaran 2008). In contrast, no *miR164*-related sequences were detected in the sequenced

genome of the bryophyte *Physcomitrella patens*. Based on the pattern of conservation observed within the vascular plant lineages for *miR164* and its recognition site in target mRNAs, it seems probable that the *miR164* regulatory module pre-existed gymnosperm/angiosperm separation but was acquired after bryophyte/gymnosperm separation. This *miR164* ancestral gene may have arisen from inverted duplications of an ancestral NAC gene (Axtell and Bowman 2008).

Functional Evolution of the NAM-miR164 Module in Monocots

No data have been published to date on the conservation of the *NAM-miR164* regulatory module and *CUC3* genes in monocots from a functional point of view. Indirect information on their function can be deduced from heterologous functional complementation (our study) and expression patterns (Hibara and Nagato 2005; Zimmermann and Werr 2005; Hibara and Nagato 2006; Oikawa and Kyojuka 2009; our study). The ability of the oil palm EgNAM1 and EgCUC3 proteins to interact with the other effectors of the AtCUC2 or AtCUC3 regulatory networks in *A. thaliana* (e.g., DNA binding sites and protein partners) imply, at least in part, a conservation of the *NAM* and *CUC3* regulatory networks between plant families/orders, which diverged 130 Ma (Moore et al. 2007). Furthermore, our expression study suggests that the *NAM*- and *CUC3*-related genes do not act redundantly in the functioning of meristems in monocots, in contrast to the situation observed in eudicots.

The conservation of *CUC3*-related gene expression patterns observed in eudicots, palms, and grasses suggests a similar function throughout angiosperms in the control of M-O and O-O boundaries and also in the regulation of SAM and axillary meristem establishment. However, functional analysis will be required in order to confirm this hypothesis.

The detection of EgNAM1 transcripts truncated at the *miR164*-directed cleavage site indicated that the *NAM-miR164* module is functionally conserved in the Arecales. Moreover, a recent study revealed the presence of OsNAM transcripts cleaved at the *miR164* recognition site in the rice mRNA degradome (Li et al. 2010). It seems reasonable to suppose that this is also the case with other monocot groups. Further evidence for functional conservation of the *NAM-miR164* module comes from the fact that the oil palm EgNAM1 gene is able to complement the *A. thaliana cuc2* mutant, which implies that there is binding of the *A. thaliana miR164* to the EgNAM1 mRNA. The task will now be to determine its function in monocot meristems, as it does not appear to be involved in the fine definition of postembryonic M-O and O-O boundaries (Zimmerman and Werr, 2005; our study). Indeed, *NAM*-related transcripts from palms and grasses are accumulated within the reproductive meristems proper rather than being confined to their boundaries. The colocalization of *NAM*-related transcripts with *miR164* inside the reproductive meristems in palms and the similar transcriptional ac-

tivity of *MIR164* inside the reproductive meristems of rice and pearl millet suggest that the “dampening” system of regulation of target mRNAs by *miR164* occurs in these monocot species as observed in *A. thaliana*. It has to be kept in mind that the probe used for in situ hybridizations was designed against *Ath-MIR164a*, which is also conserved in grasses (fig. 2B). Consequently, the signal detected may be specific to the *miR164a* form only, and it cannot be excluded that other putative forms of *miR164* are expressed in other domains. Alternatively, it is possible that different forms of *miR164* were being detected at the same time in these experiments. However, despite the divergence of transcript localization between monocots and eudicots, it will be important to determine whether the *NAM*-related proteins colocalize with their transcripts within meristems and in which way these proteins may still be involved (or not) in boundary definition in monocots.

Importantly, the divergence of expression patterns of *NAM*-related genes in eudicots and monocots may be due to divergence of their *cis*-regulatory sequences, leading to a divergence in their transcriptional regulatory networks. Indeed, major differences between eudicot and monocot *NAM*-related genes are observable within their 5′ flanking regions, notably with respect to the presence or absence of several different CNSs and an intron in the 5′ UTRs. Evolutionarily conserved motifs have been identified in the promoter regions of a range of genes affecting plant development, examples including the *LATE ELONGATED HYPOCOTYL* and *SHOOTMERISTEMLESS* genes from *A. thaliana* (Uchida et al. 2007; Spensley et al. 2009). However, in contrast to the latter two classes, *NAM*-related genes display a clear dichotomy between currently identified monocot and eudicot genes. Moreover, 5′-UTR introns are known to be involved in the transcriptional regulation of certain genes through the action of specific DNA sequences on the level and/or specificity of their expression (see Kooiker et al. 2005, Chung et al. 2006, and Karthikeyan et al. 2009 for examples). In future studies, it will be of great interest to elucidate the regulatory role of these CNSs in both the monocot and eudicot contexts. Since *NAM*-related transcripts and *miR164* both show a divergent expression pattern in monocots compared with eudicots, it can be inferred that there has been a coevolution of their transcriptional regulatory network. One explanation is the modification of the activity or the expression pattern of common regulatory factor(s) between the two genes during angiosperm evolution.

In the context of evolutionary developmental biology, the role of *NAM/CUC3* genes is of great interest due to their involvement in the determination of plant architecture, especially through their participation in meristem functioning and boundary definition. The description of *NAM-miR164* module and *CUC3* genes in a wider panel of angiosperm species has pointed to major differences between monocots and eudicots in the way these regulatory mechanisms have been recruited to regulate development.

Supplementary Material

Supplementary tables S1 and S2 and supplementary figs. S1–S3 are available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

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