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Identification of the arabidopsis RAM/MOR signalling network: adding new regulatory players in plant stem cell maintenance and cell polarization

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• **Background and Aims** The RAM/MOR signalling network of eukaryotes is a conserved regulatory module involved in co-ordination of stem cell maintenance, cell differentiation and polarity establishment. To date, no such signalling network has been identified in plants.
• **Methods** Genes encoding the bona fide core components of the RAM/MOR pathway were identified in *Arabidopsis thaliana* (arabidopsis) by sequence similarity searches conducted with the known components from other species. The transcriptional network(s) of the arabidopsis RAM/MOR signalling pathway were identified by running in-depth *in silico* analyses for genes co-regulated with the core components. *In situ* hybridization was used to confirm tissue-specific expression of selected RAM/MOR genes.
• **Key Results** Co-expression data suggested that the arabidopsis RAM/MOR pathway may include genes involved in floral transition, by co-operating with chromatin remodelling and mRNA processing/post-transcriptional gene silencing factors, and genes involved in the regulation of pollen tube polar growth. The RAM/MOR pathway may act upstream of the *ROP1* machinery, affecting pollen tube polar growth, based on the co-expression of its components with *ROP-GEFs*. *In silico* tissue-specific co-expression data and *in situ* hybridization experiments suggest that different components of the arabidopsis RAM/MOR are expressed in the shoot apical meristem and inflorescence meristem and may be involved in the fine-tuning of stem cell maintenance and cell differentiation.
• **Conclusions** The arabidopsis RAM/MOR pathway may be part of the signalling cascade that converges in pollen tube polarized growth and in fine-tuning stem cell maintenance, differentiation and organ polarity.

Key words: *Arabidopsis thaliana*, RAM/MOR signalling network, transcriptional networks, cell polarity, stem cell maintenance, floral transition, *in situ* hybridization.

INTRODUCTION

The RAM/MOR signalling network

Establishment and maintenance of cell polarity is essential for proper development of eukaryotic organisms. In the budding yeast *Saccharomyces cerevisiae*, the RAM network (*r*egulation of *ACE2*p activity and cellular *m*orphogenesis; see Appendix for list of abbreviations) has emerged as a central signalling module coordinating cell separation with establishment and maintenance of cell polarity and integrity (Racki *et al*., 2000; Bidlingmaier *et al*., 2001; Weiss *et al*., 2002; Nelson *et al*., 2003; Bourens *et al*., 2009). The RAM network has been shown to be essential for the correct asymmetric segregation of cell polarity determinants between mother and daughter cell, thus providing intrinsic cues for cell fate asymmetry (Jansen *et al*., 2006). Mutations in the yeast RAM components are lethal, causing cell lysis, except in the *ssd1* strain background where they are not lethal but lead to failure in cell separation, altered colony morphology and defects in polarized cell growth (Jorgensen *et al*., 2002).

The core of the yeast RAM signalling network consists of two kinases (CBK1 and KIC1) and four associated proteins (MOB, HYM1, TAO3 and SOG2) (Kurischko *et al*., 2005). The pivotal element of RAM is the kinase CBK1, belonging to the NDR (Nuclear Dbf2 Related) family of AGC kinases (Hergovich *et al*., 2006), and its activating protein MOB2. MOB2 has no intrinsic activity but its binding to CBK1 is necessary for the regulation of kinase activity. The CBK1–MOB2 complex has a dual role: the regulation of ACE2, a yeast-specific transcription factor driving the expression of genes

involved in mother/daughter cell separation, and the control of polarized morphogenesis by co-ordinating the organization of the actin cytoskeleton. These two functions are independent (Nelson *et al*., 2003), but rely on common upstream regulatory components that have been shown to be generally conserved in eukaryotes (Maerz and Seiler, 2010): the four proteins HYM1, TAO3, SOG2 and KIC1 (Kurischko *et al*., 2005). KIC1 is a member of the PAK1/Ste20 kinase family and phosphorylates CBK1. HYM1, TAO3 and SOG2 have unknown molecular functions (Kurischko *et al*., 2005) but seem to act upstream of CBK1 activation (Nelson *et al*., 2003). As ACE2 is exclusively present in budding yeast, while the remaining RAM components are conserved among eukaryotes and fungi, an MOR (Morphogenesis Orb6 Network) network has been proposed as the conserved pathway regulating cell separation and polarity in fungi and higher eukaryotic organisms (Maerz and Seiler, 2010). For clarity and completeness, we refer throughout this paper to the RAM/MOR signalling network.

Regulation of asymmetric cell division and of cell polarization by RAM/MOR components

In yeast, the asymmetric distribution of cell fate determinants during late mitosis relies largely on CBK1 activity and dynamic localization during the cell cycle. Asymmetrically localized CBK1 controls transcription and translation of daughter cell-specific mRNAs by activating ACE2 and by blocking SSD1, an RNA-binding protein with similarity to RNase II. SSD1 binds, primarily, mRNAs which encode proteins involved in cell-wall organization (e.g. chitinases and glucanases), thus possibly acting as a CBK1-regulated determinant of asymmetric mRNA localization to the bud tip during polarized growth and as an mRNA translational repressor (Kurischko *et al*., 2011*a*,*b*).

Genes activated by ACE2 code for daughter cell-specific proteins (Voth *et al*., 2007), degrading the septum from the side of the daughter cell (Fujita *et al*., 2004). RAM/MOR contributes to polarized growth also by CBK1–MOB2-dependent regulation of actin-based secretion of exocytotic vesicles (Nelson *et al*., 2003), Golgi trafficking and glycosylation (Kurischko *et al*., 2008). CBK1 can recruit and phosphorylate SEC2 (an SEC4 RAB-GEF), promoting SEC4-dependent exocytosis (Kurischko *et al*., 2008). In *Schizosaccharomyces pombe*, the CBK1 homologue ORB6 has been shown to control the spatial confinement of CDC42 GTPase activation at the cell tips, by regulating GEFs (GEF1 and SCD1) and GAPs (RGA4) (Das *et al*., 2009). In *Drosophila*, human and mouse, the homologues of RAM components have also been shown to be involved in asymmetric stem cell division (Yamamoto *et al*., 2008), cell and organ polarity, and cell shape establishment and maintenance (Yamamoto *et al*., 2008; Fang and Adler, 2010; Horne-Badovinac *et al*., 2012; recently reviewed by Hiemer and Varelas, 2013).

Different pathways have been shown to regulate the fine balance between stem cell maintenance, asymmetric cell division and cell polarity in eukaryotes. RHO family small GTPases and their accessory proteins (GEFs, GAPs), a regulatory module conserved from yeast to mammals, drive cytoskeleton dynamics/reorganization and vesicular trafficking required for polarity establishment and maintenance in members of all three domains of life (Brembu *et al*., 2006; Yang, 2008; Craddock *et al*., 2012). The balance between stem cell maintenance and differentiation in plants is specifically regulated in the shoot apical meristem (SAM) by the WUSCHEL (WUS)-CLAVATA (CLV) and the SHOOTMERISTEMLESS (STM) pathways (Sijacic and Liu, 2010; Yadav and Reddy, 2012) in which the negative feedback regulation exerted by CLV1/CLV2 receptors on the stem maintaining gene WUS is required to restrict stem cell specification. In an independent pathway, STM suppresses stem cell differentiation (Miwa *et al*., 2009; Sijacic and Liu, 2010). RHO GTPases were suggested to function in the WUS–CLV signalling pathway and therefore in balancing differentiation and stem cell maintenance in the shoot meristem (Trotochaud *et al*., 1999).

An additional pathway, termed MEN (and SIN network in budding and fission yeast), also regulates the balance between mitotic exit and cytokinesis. This signalling core cassette involves members of the PAK1/Ste20 kinase family, NDR kinase family and MOB family, different from those of the RAM/MOR pathway, and is conserved in *Drosophila melanogaster*, mammals and arabidopsis (Bedhomme *et al*., 2008; Hergovich and Hemmings, 2012; Avruch *et al*., 2012).

In a similar way, given that the components of the RAM/MOR pathway are widely conserved across eukaryotes (Fig. 1), it is conceivable that its key regulatory players may be conserved also in plants. Until now, a RAM/MOR-like signalling cascade has not been studied in plants nor has its action been related to the pathways involving RHO small GTPases and WUS–CLV. In this work we aimed to identify the putatively conserved RAM/MOR components of *Arabidopsis thaliana*, by sequence similarity searches, and by in-depth analysis of microarray expression data available for this species, to pinpoint transcriptional networks of genes co-regulated with the core RAM/MOR components.

MATERIALS AND METHODS

Identification of bona fide RAM/MOR pathway genes

Previously identified gene sequences belonging to the RAM/MOR pathway in *Saccharomyces cerevisiae*, *Drosophila melanogaster* and *Homo sapiens* were used for BLASTp and PSI-BLAST searches (Altschul *et al*., 1997). Identified genes in *A. thaliana* were used as query in a whole-genome transcription correlation map (Morandini *et al*., in preparation) to cluster genes having globally similar transcription regulation (Menges *et al*., 2008). These operations were conducted in correlation matrices derived from absolute and log-scaled gene expression values.

Data clustering

All data were clustered using the software R (www.r-project.org). A hierarchical clustering was performed to check whether a core containing at least one gene member for each family exists. To find the best correlators to this sub-cluster, a second analysis was performed to sub-clusters against correlators

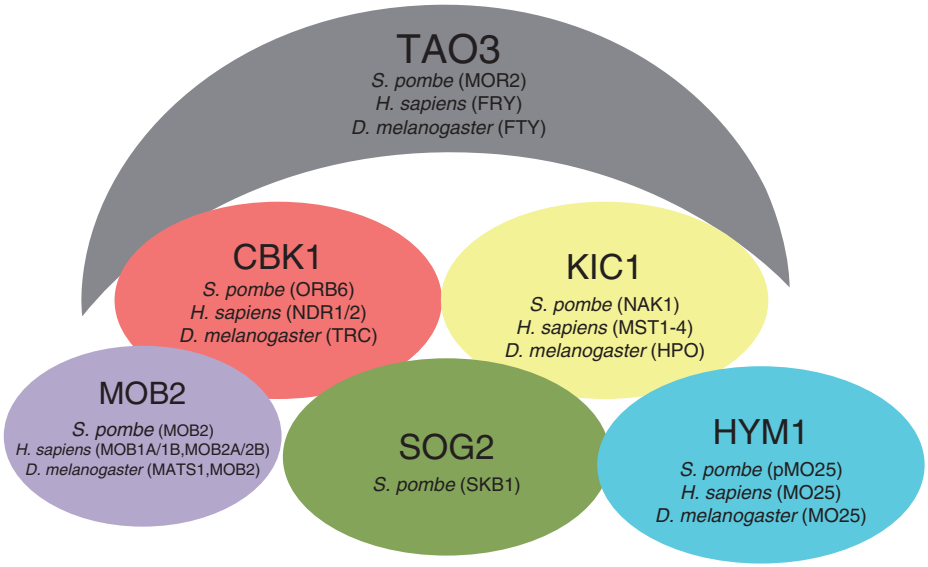

FIG. 1. The conserved elements of the RAM/MOR pathway of eukaryotes. The core of the network involves the two kinases CBK1 and KIC1, and the four associated proteins TAO3, MOB2, SOG2 and HYM1. TAO3 acts as a scaffold facilitating the interaction between the kinases CBK1 and KIC1, and MOB2 is a CBK1 co-activator. HYM1 may function as a scaffold. Most of the RAM/MOR components have been identified among different species: the names of members from *Saccharomyces cerevisiae* are in upper case letters to label each RAM/MOR component, while names of the characterized orthologues in *Schizosaccharomyces pombe*, *Homo sapiens* and *Drosophila melanogaster* are given in lower case letters in parentheses. Only one orthologue of the *S. cerevisiae* SOG2 has been identified exclusively in *S. pombe*. Interactions between proteins are symbolized by contact points.

exceeding a threshold of 0·9 or 0·55 for linear and logarithmic analyses, respectively. Heatmaps has been obtained with the package gplots (Warnes, 2012). Development (Schmid *et al*., 2005), hormones, abiotic stress, light (Kilian *et al*., 2007) and phatogen expression data was retrieved from the AtGenExpress Visualization Tool (AVT) (http://jsp.weigelworld.org/expviz/expviz.jsp). Clustering was done in both linear and log-transformed data.

Plant material and in situ *hybridization (ISH)*

Sections 7 μm thick of arabidopsis flowers at different developmental stages were hybridized with sense and antisense *AtSIK1*, *AtFRY*, *AtNDR3*, *AtNDR4* and *AtMO25-3* riboprobes labelled with digoxigenin-11-UTP using T3 polymerase following the protocol of the manufacturer (Roche, Basel, Switzerland). Probes were selected by PCR on leaf cDNA and contained a portion of the 3′ untranslated region. All ISH steps, with the exception of staining, were carried out using the Gene Paint suite accessories (Freedom EVO100, Tecan, Männedorf, Switzerland) as described by Begheldo *et al*. (2013). The signal was developed with detection buffer containing NBT-BCIP (Roche) following the manufacturer's instructions. NBT/BCIP staining was continued until a clearly detectable signal was visible under a light microscope. Sections mounted in 50 % (v/v) glycerol were observed with an Olympus BX50 microscope (Olympus, Tokyo, Japan) equipped with differential interference contrast optics. Images were captured with an MRc5 Axiocam colour camera (Carl Zeiss, Oberkochen, Germany), and processed with Adobe Photoshop CS4 (Adobe, San Josè, CA, USA).

Accession numbers

Sequence data of genes used in this article can be found in Tables 1–5, and Supplementary Data – Tables S1–S6.

RESULTS

Identification of core components of the putative arabidopsis RAM signalling network

To identify the putatively conserved RAM components in the arabidopsis genome, BLASTp/PSI-BLAST sequence similarity searches were conducted by using the full protein sequences of RAM components from other species (*S. cerevisiae*, *D. melanogaster* and *H. sapiens*) and their conserved domains as queries. We thus identified a first putative core of 18 conserved elements for the arabidopsis RAM network (Table 1).

Some of these proteins had already been classified by previous bioinformatics analyses, but they remain uncharacterized as there is no experimental evidence to relate them to a specific biological function or assign them to a signalling network. Similarity searches (adopting a cutoff expectation value of $\leq 10^{-40}$) pointed to the existence of a single gene (At5g15680) encoding a putative TAO3p-like homologue in the arabidopsis genome, which we have named *AtFRY* according to its human and *Drosophila* homologues, while four *HYM1* homologues were found to encode proteins that had already been independently classified (http://www.arabidopsis.org) as MO25-like proteins in arabidopsis (named *AtMO25-1* to *-4*) (Table 1). As far as STE20-like kinases are concerned, Karpov *et al*. (2009) found, based on sequence similarity, two genes encoding putative STE20-like proteins in *A. thaliana*, namely *SIK1* and Q9LQA1 (F4N2.17). However, we have found that the UniProt

TABLE 1. *RAM/MOR components identified in arabidopsis by sequence similarity searches based on identified elements from* S. cerevisiae, S. pombe, H. sapiens *and* D. melanogaster

	RAM components (*S. cerevisiae*)	*S. pombe* homologues	*H. sapiens* homologues	*D. melanogaster* homologues	*A. thaliana* homologues	Ref. for arabidopsis homologues
Ste20 family kinases	KIC1p	NAK1	MST1, MST2, MST3, MST4	HPO	At1g69220 (AtSIK1)	Karpov *et al*. (2009)
Scaffolds	TAO3p, HYM1p	MOR2, PMO25	FRY, MO25	FRY	At5g15680 (AtFRY) At2g03410 (AtMO25-1) At4g17270 (AtMO25-2) At5g18940 (AtMO25-3) At5g47540 (AtMO25-4)	This work
Co-activators	MOB2p	MOB2	Several MOB1 and MOB2	MATS, DMOB2	At5g45550 (AtMOB1A) At4g19045 (AtMOB1B) At5g20440 (AtMOB2A) At5g20430 (AtMOB2B)	Vitulo *et al*. (2007), Pinosa *et al*., (2013)
NDR kinases	CBK1p	ORB6	NDR1, NDR2	TRC	At4g14350 (AtNDR1) At1g03920 (AtNDR2) At3g23310 (AtNDR3) At2g19400 (AtNDR4) At2g20470 (AtNDR5) At4g33080 (AtNDR6) At1g30640 (AtNDR7) At5g09890 (AtNDR8)	Bögre *et al*. (2003)

RAM components were divided into four functional groups: Ste20 family kinases, scaffolds, co-activators and NDR kinases. The corresponding names of all components are reported in a separate column for each species. References to previously identified arabidopsis proteins with similarity to the reported functional categories are reported in the last column.

TABLE 2. *Genes involved in the specification or maintenance of stem cell identity in the shoot apical meristem (SAM) and positively co-regulated with a minimum cutoff value ≥0·55 (in the logarithmic analysis) with* AtFRY *and/or* AtSIK1

Gene name	AGI	Correlation coefficient		Known function	Ref.
		AtSIK1	*AtFRY*		
POL (Poltergeist)	At2g46920	*0·55*	*0·55*	Specification of asymmetric cell divisions in stem cells	Gagne and Clark (2010)
LIS (Lachesis)	At2g41500	**0·62**	**0·55**	Gametic cell fate determination	Gross-Hardt *et al*. (2007)
TPR2 (Topless-Related 2)	At3g16830	**0·56**	**0·66**	Establishment of embryonic polarity	Long *et al*. (2006)
LUG (Leunig)	At4g32551	0·39	**0·68**	Leaf adaxial cell identity, maintenance of SAM	Stahle *et al*. (2009)
SEU (Seuss)	At1g43850	0·18	**0·59**	LUG Interactor. Regulation of organ development from SAM	Bao *et al*. (2010)
SWP (Struwwelpeter)	At3g04740	*0·53*	**0·65**	LUG interactor, regulation of meristem pattern formation	Autran *et al*. (2002)
HR (Hedgehog Receptor)	At4g38350	0·43	**0·64**	Similar to Hedgehog receptor. Unknown function	Oh *et al*. (2005)
REV (Revoluta)	At5g60690	*0·46*	**0·56**	Establishment of organ polarity	Chandler (2012)
PAS1 (Pasticcino 1)	At3g54010	**0·66**	**0·59**	Control of cell proliferation and differentiation	Smyczynski *et al*. (2006)
KAPP (Kinase Associated Protein Phosphatase)	At5g19280	**0·55**	0·23	Negative regulator of CLV1 signalling	Carles and Fletcher (2003)
SYD (Splayed)	At2g28290	**0·62**	**0·67**	SNF2-class ATPase. Regulating WUS transcription	Kwon *et al*. (2005)

To highlight genes showing highly significant co-expression levels with *AtFRY* and *AtSIK1*, either alone or in combination, correlation coefficients >0·55 are reported in bold characters, while coefficients between 0·45 and 0·55 are in italics.

code Q9LQA1 (F4N2.17) corresponded to a BAC clone containing only the *SIK1* sequence. Therefore, *SIK1* (At1g69220) appears as the only gene encoding a bona fide STE20-like kinase in the *A. thaliana* genome, which we renamed *AtSIK1*. Four arabidopsis MOB-like proteins have been described (Vitulo *et al*., 2007; Pinosa *et al*., 2013), two of which were classified as MOB1-like (AtMOB1A/B) and two as MOB2-like (AtMOB2A/B), while eight proteins belonging to group VII of the plant AGC kinase superfamily were classified as NDR kinases (AtNDR1–8) (Bögre *et al*., 2003). We could not identify any putative arabidopsis protein for the RAM leucine rich repeat protein SOG2 or for the RAM RNA-binding protein SSD1. To confirm conservation of the RAM/MOR pathway in plants, we identified the orthologues also from *Oryza sativa* subspecies *japonica*, *Populus trichocarpa*, *Medicago truncatula* and *Vitis vinifera* (Supplementary Information – Table S1).

To further test whether the identified putative components of the arabidopsis RAM/MOR belonged to a common signalling network, we studied their transcriptional regulation by data mining of about 1800 microarray hybridizations (Menges *et al*., 2008). Previous works (Månsson *et al*., 2004; Hirai *et al*., 2007; Vandepoele *et al*., 2009; Murgia *et al*., 2011) have detailed the use of co-regulation analysis as a tool to suggest shared functions or a signalling pathway(s) (Menges *et al*., 2008), and/or to identify additional components located at different tiers within the same signalling module through a 'guilty-by-association' approach, thus defining bona fide transcriptional networks (Morandini *et al*., unpubl. res.). Therefore, we have analysed the transcriptional profile of the genes encoding the identified core components of the putative arabidopsis RAM/MOR in several Affymetrix microarray expression data available for arabidopsis. Probes measuring *AtFRY*, *AtSIK1*, *AtMOB1A/1B*, *AtMOB2A/2B*, *AtNDR1–8* kinases and *AtMO25-1/4* are present in the ATH1 Affymetrix array. Data for *AtMOB2A* and *AtMOB2B* are overlapping, and therefore *AtMOB2A* and *AtMOB2B* are referred to as *AtMOB2* below. Pearson correlation values for each gene pair were obtained for the raw expression values and hierarchical clusterings, by using expression

TABLE 3. *Genes previously assigned to chromatin remodelling and floral transition and positively co-regulated with a minimum cutoff value ≥0·55 (on a logarithmic base) with* AtFRY *and* AtSIK1 *are reported*

Gene name	AGI	Correlation coefficient		Known function	Ref.
		AtSIK1	*AtFRY*		
JMJ14 (Jumonji 14)	At4g20400	*0·45*	**0·55**	Histone H3K4 demethylase on FT and TSF chromatin repressing floral transition	Yang *et al.* (2010)
ELF6 (Early Flowering 6)	At5g04240	0·33	**0·58**	Histone H3K4 demethylase on FT chromatin preventing early flowering	Jeong *et al.* (2009)
ELF7 (Early Flowering 7)	At1g79730	0·26	**0·56**	Recruiting SET1 H3K4 methyl transferase on FLC chromatin: positive regulator of FLC expression	He *et al.* (2004)
ATXR7 (arabidopsis Trithorax-Related7)	At5g42400	0·41	**0·57**	Putative H3K4 methyl transferase on FLC chromatin: positive regulator of FLC expression	Tamada *et al.* (2009)
ATRX, CHR20	At1g08600	**0·59**	**0·66**	ATRX-like protein	Kanno *et al.* (2004)
HUB1 (Histone Mono-Ubiquitination 1)	At2g44950	**0·59**	**0·60**	H2B monoubiquitination on FLC chromatin. Positive regulator of FLC expression.	Xu *et al.* (2009)
SPT16	At4g10710	0·38	**0·61**	Partner of SSRP1 complex interaction with HUB1	Van Lijsebettens and Grasser (2010)
SSRP1	At3g28730	**0·67**	**0·61**	Facilitator of chromatin transcription (FACT) complex binding to HUB1 in FLC promoter.	Van Lijsebettens and Grasser (2010)
SWN (Swinger)	At4g02020	**0·57**	*0·54*	STM methylation	Shen and Xu (2009)
FRIGIDA-like protein	At4g14900	*0·45*	**0·58**		
SWI2 (Switch 2) ; CHR9 (Chromatin Remodeling 9); SNF2 (Sucrose Non-Fermenting 2)	At1g03750	**0·57**	0·42	Helicase	
ARP4 (Actin-Related Protein 4)	At1g18450	**0·58**	*0·54*	Responsible for deposition of H2A.Z histone variant. Positive regulator of FLC expression	Kandasamy *et al.* (2005)
EMB1507 (Embryo Defective 1507)	At1g20960	0·33	**0·66**	DNA/RNA helicase	
ATP-dependent RNA helicase DDX35	At4g18465	**0·59**	**0·59**		
ESP3 (Enhanced Silencing Phenotype 3)	At1g32490	**0·59**	**0·56**	DEAH RNA helicase. Early flowering	Herr *et al.* (2006)
RAD3-like DNA-binding helicase protein	At1g79950	**0·56**	*0·48*		
CHR5 (Chromatin Remodeling 5)	At2g13370	*0·52*	**0·60**	Chromodomain-helicase-DNA-binding family protein	
DRD1 (Defective In RNA-Directed Dna Methylation 1)	At2g16390	0·44	**0·60**	SNF2-like protein mediating non-CpG methylation.	Kanno *et al.* (2004)
HAC13 (Histone Acetyltransferase of the Cbp Family 13), TAF1 (Tbp-Associated Factor 1)	At1g32750	**0·68**	*0·53*	Histone acetyltransferase	Earley *et al.* (2007)
HTA9 (Histone H2A Protein 9)	At1g52740	**0·58**	0·35	Histone H2A.Z. Regulation of FLC and MAF4	March-Díaz *et al.* (2008)
CHR11 (Chromatin-Remodeling Protein 11)	At3g06400	**0·67**	**0·64**	SWI2/SNF2 chromatin remodeling protein	Li *et al.* (2012)
BSH (Bushy Growth)	At3g17590	**0·61**	0·43	Homolog of SNF5: interacts with flowering regulator FCA	Sarnowski *et al.* (2005)
SAP18 (SIN3 Associated Polypeptide P18)	At2g45640	**0·58**	0·37	Sin3/histone deacetylase (HDAC) complex, timing of floral transition and organ patterning.	Liu *et al.* (2009)
DRM3 (DOMAINS REARRANGED METHYLTRANSFERASE 3)	At3g17310	*0·47*	**0·58**	Small RNA–directed DNA methylation	Henderson *et al.* (2010)
SPY (SPINDLY)	At3g11540	*0·52*	**0·64**	N-acetyl glucosamine transferase. Upstream of CO	Tseng *et al.* (2004)
HOS15 (HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 15)	At5g67320	*0·54*	**0·60**	Histone deacetylation.	Zhu *et al.* (2008)
MBD2 (METHYL-CPG-BINDING DOMAIN PROTEIN 2)	AT5G35330	**0·56**	0·42	Cytosine C-5 DNA demethylase	

Known players of the flowering transition by regulating transcription of the flowering genes *FLC*, *FT* and *TSF* are indicated with their respective references. To highlight genes showing highly significant co-expression levels with *AtFRY* and/or *AtSIK1*, correlation coefficients >0·55 are highlighted in bold, while coefficients between 0·45 and 0·55 are in italics.

TABLE 4. *Genes positively co-regulated on a logarithmic correlation with a minimum cutoff value 0·55 with* AtFRY *and* AtSIK1 *and assigned to post-transcriptional gene silencing (PTGS), mRNA processing and RNA binding*

Gene name	AGI	Correlation coefficient		Known function	Ref.
		AtSIK1	*AtFRY*		
AGO1 (Argonaute 1)	At1g48410	*0·47*	**0·59**	miRNA and siRNA pathways for PTGS	Chandler (2012)
XRN3	At1g75660	**0·56**	**0·59**	5′–3′ exoribonuclease suppressor of PTGS	Gy *et al*. (2007)
DCL2 (Dicer-Like 2)	At3g03300	*0·50*	**0·58**	Dicer-like: production of endogenous tasiRNAs	Mallory and Vaucheret (2009)
DCL4 (Dicer-Like 4)	At5g20320	0·36	**0·58**	Dicer-like: production of endogenous tasiRNAs	Mallory and Vaucheret (2009)
KTF1 (KOW domain-containing transcription Factor 1)	At5g04290	*0·51*	**0·65**	Recruiting AGO4 and AGO4-bound siRNAs	He *et al*. (2009)
DDL (Dawdle)	At3g2055	**0·62**	*0·49*	DCL1 interactor: miRNAs and siRNAs biogenesis	Yu *et al*. (2008)
DRB4 (Double-Stranded-Rna-Binding Protein 4)	At3g62800	**0·64**	*0·51*	DCL4 interactor: : miRNA and siRNAs biogenesis	Fukudome *et al*. (2011)
THO5	At5g42920	*0·53*	**0·59**	Member of THO/TREX complexes: transport of siRNA precursor	Yelina *et al*. (2010)
EMB3011 (Embryo Defective 3011)	At5g13010	**0·68**	**0·69**	RNA helicase	
SPL7 (Squamosa Promoter Binding Protein-Like 7)	At5g18830	**0·55**	**0·66**	Expression of microRNAs in copper deficiency	Yamasaki *et al*. (2009)
Splicing factor PWI domain/RNA recognition motif (RRM)-containing protein	At1g60200	**0·58**	**0·64**	mRNA processing	
SUA (Suppressor of Abi3)	At3g54230	**0·57**	**0·58**	Splicing Factor.	
RNA-dependent RNA polymerase family protein	At2G19930	**0·59**	**0·58**	PTGS	
SMU2 (Suppressors of MEC-8 and UNC-52 2)	At2g26460	**0·56**	**0·55**	Splicing of pre-mRNAs in actively dividing tissues.	Chung *et al*. (2009)
SMU1 (Suppressors Of MEC-8 AND UNC-52 1)	At1g73720	**0·58**	*0·46*	Splicing of pre-mRNAs in actively dividing tissues.	Chung *et al*. (2009)
ML3 (MEI2-like 3)	At4g18120	**0·57**	*0·50*	RNA binding protein.	Kaur *et al*. (2006)
RNA-binding (RRM/RBD/RNP motifs) family protein	At5g46840	**0·66**	*0·45*	DNA Damage response	Shaked *et al*. (2006)
U11 snRNP-specifc protein 35K	At2g43370	**0·62**	*0·45*	U12-type or AT-AC introns splicing	Lorkovic *et al*. (2005)
PRP39	At1g04080	*0·49*	**0·55**	Floral transition. Tetratricopeptide repeat-HAT helix RNA-processing protein	Wang *et al*. (2007)
LIF2 (Lhp1-Interacting Factor 2)	At4g00830	**0·66**	**0·63**	RNA binding partner of the polycomb complex component LHP1. Control of flowering and cell fate	Latrasse *et al*. (2011)
DCP5 (Decapping 5)	At1g26110	0·34	**0·61**	mRNA decapping.	Xu and Chua (2009)

To highlight genes showing highly significant co-expression levels with *AtFRY* and *AtSIK1*, correlation coefficients >0·55 are highlighted in bold, while coefficients lower than the cutoff between 0·45 and 0·55 are in italics.

TABLE 5. *Genes positively co-regulated on a linear correlation with a minimum cutoff value of 0·9 with* MO25-1, MO25-4, NDR2 *and* NDR4 *and involved in cytoskeleton organization and remodelling, vesicle trafficking and in calcium signalling*

Gene name	AGI	Correlation coefficient				Known function	Reference
		AtMO25-1	*AtMO25-4*	*AtNDR2*	*AtNDR4*		
Cytoskeleton organization and remodelling							
Myosin heavy chain-related	At1g64320	**0·95**	**0·93**	**0·91**	0·83		
	At5g41780	**0·96**	**0·95**	**0·9**	**0·93**		
P-loop containing nucleoside triphosphate hydrolases superfamily protein	At1g18410	0·85	0·85	**0·92**	**0·90**	Microtubule motor activity	
	At1g73860	**0·94**	**0·93**	**0·92**	0·81		
	At5g41310	**0·91**	**0·90**	**0·91**	0·85		
ATP binding microtubule motor family protein	At5g42490	**0·97**	**0·95**	0·82	0·82	Microtubule motor activity	
ROPGEF8	At3g24620	**0·95**	**0·94**	**0·92**	**0·90**	Rop activation	Berken *et al.* (2005)
ROPGEF9	At4g13240	0·88	0·87	**0·91**	0·84	Rop activation	Berken *et al.* (2005)
ROPGEF11	At1g52240	**0·96**	**0·96**	**0·93**	0·85	Rop activation	Berken *et al.* (2005)
ROPGEF12	At1g79860	**0·94**	**0·94**	**0·92**	0·87	Rop activation	Berken *et al.* (2005)
Rop1	At3g51300	**0·92**	**0·92**	**0·93**	0·87	Control of polarized pollen growth	Lee *et al.* (2008)
RIC1 (Rop-Interactive Crib Motif-Containing Protein 1)	At2g33460	**0·95**	**0·95**	**0·91**	0·87	ROP1 interactor, localization	Lee *et al.* (2008)
ADF7 (Actin Depolymerizing Factor 7)	At4g25590	0·88	0·87	**0·91**	0·89	Pollen specific actin dynamics	Bou-Daher *et al.* (2011)
ARO1 (Armadillo Repeat Only 1)	At4g34940	**0·90**	0·88	**0·90**	0·84	Egg Cells and Pollen specific. Organization of actin filaments	Gebert *et al.* (2008)
Pleckstrin homology (PH) and lipid-binding START domains-containing protein	At3g54800	**0·96**	**0·95**	**0·91**	0·87		
PLIM2C	At3g61230	**0·90**	0·89	**0·92**	0·87	Predominantly expressed in pollen. Regulates actin cytoskeleton organization	Papuga *et al.* (2010)
PLIM2B	At1g01780	**0·93**	**0·93**	**0·91**	0·83		Papuga *et al.* (2010)
SP1L4 (Spiral1-Like4)	At5g15600	**0·95**	**0·96**	**0·91**	0·85	Regulates cortical microtubule organization	Nakajima *et al.* (2006)
VLN5 (Villin5)	At5g57320	**0·90**	0·87	**0·90**	0·87	Actin filament stabilizing factor. Regulates pollen tube growth	Zhang *et al.* (2010)
ACT4 (Actin 4)	At5g59370	**0·92**	**0·90**	**0·92**	0·88	Predominantly expressed in reproductive tissues.	Huang *et al.* (1996)
Vesicle trafficking							
SNARE associated Golgi protein family	At1g12450	**0·92**	0·89	0·84	0·81	N-terminal protein	
ENTH/VHS/GAT family protein	At1g03050	**0·94**	**0·94**	0·88	**0·91**	Myristoylation, clathrin coat assembly	
	At1g25240	**0·97**	**0·96**	0·86	0·85		
	At1g68110	**0·96**	**0·95**	**0·90**	0·88		
	At4g02650	**0·93**	**0·92**	0·83	0·75		
	At3g59290	**0·93**	**0·91**	0·88	0·83		
SYP131 (Syntaxin of Plants 131)	At3g03800	**0·98**	**0·96**	0·86	0·84	Vesicle-mediated transport/fusion	
SYP72 (Syntaxin of Plants 72)	At3g45280	**0·93**	**0·91**	**0·91**	0·87	Vesicle-mediated transport/fusion	
SYP124 (Syntaxin of Plants 124)	At1g61290	**0·95**	**0·93**	0·87	0·85	Polarized vesicle secretion in pollen	Silva *et al.* (2010)
SNAP30 (Soluble N-Ethylmaleimide-Sensitive Factor Adaptor Protein 30)	At1g13890	**0·95**	**0·94**	**0·95**	**0·92**	Vesicle-mediated transport/fusion	
ATEXO70H3 (Exocyst Subunit Exo70 Family Protein H3)	At3g09530	**0·95**	**0·94**	0·82	0·86	Exocyst complex. Pollen specific.	Li *et al.* (2010)
ATEXO70H5 (Exocyst Subunit Exo70 Family Protein H5)	At2g28640	**0·97**	**0·95**	0·87	0·84	Exocyst complex. Pollen specific.	Li *et al.* (2010)
*ArRABA*1h	At2g33870	**0·94**	**0·92**	**0·93**	0·88		
AtRABA1i	At1g28550	**0·90**	**0·90**	**0·92**	0·88		
AtGYPB1d (RabGAP)	At5g54780	**0·95**	**0·94**	0·85	0·82	RAB GTPase activator	
Calcium signalling							
CML25 (calmodulin-like protein 25)	At1g24620	**0·97**	**0·94**	0·88	0·86	Pollen-specific calcium sensor	Zhou *et al.* (2009)
CML28 (calmodulin-like protein 28)	At3g03430	0·88	0·87	**0·91**	0·87	Pollen-specific calcium sensor	Zhou *et al.* (2009)
CML6 (calmodulin-like protein 6)	At4g03290	**0·92**	**0·92**	0·81	0·88	Pollen-specific calcium sensor	Zhou *et al.* (2009)
CDPK24 (Calcium Dependent Protein Kinase 24)	At2g31500	**0·96**	**0·96**	**0·91**	0·89	Pollen-specific calcium sensor	Zhou *et al.* (2009)

Correlation coefficients >0·9 are highlighted in bold.

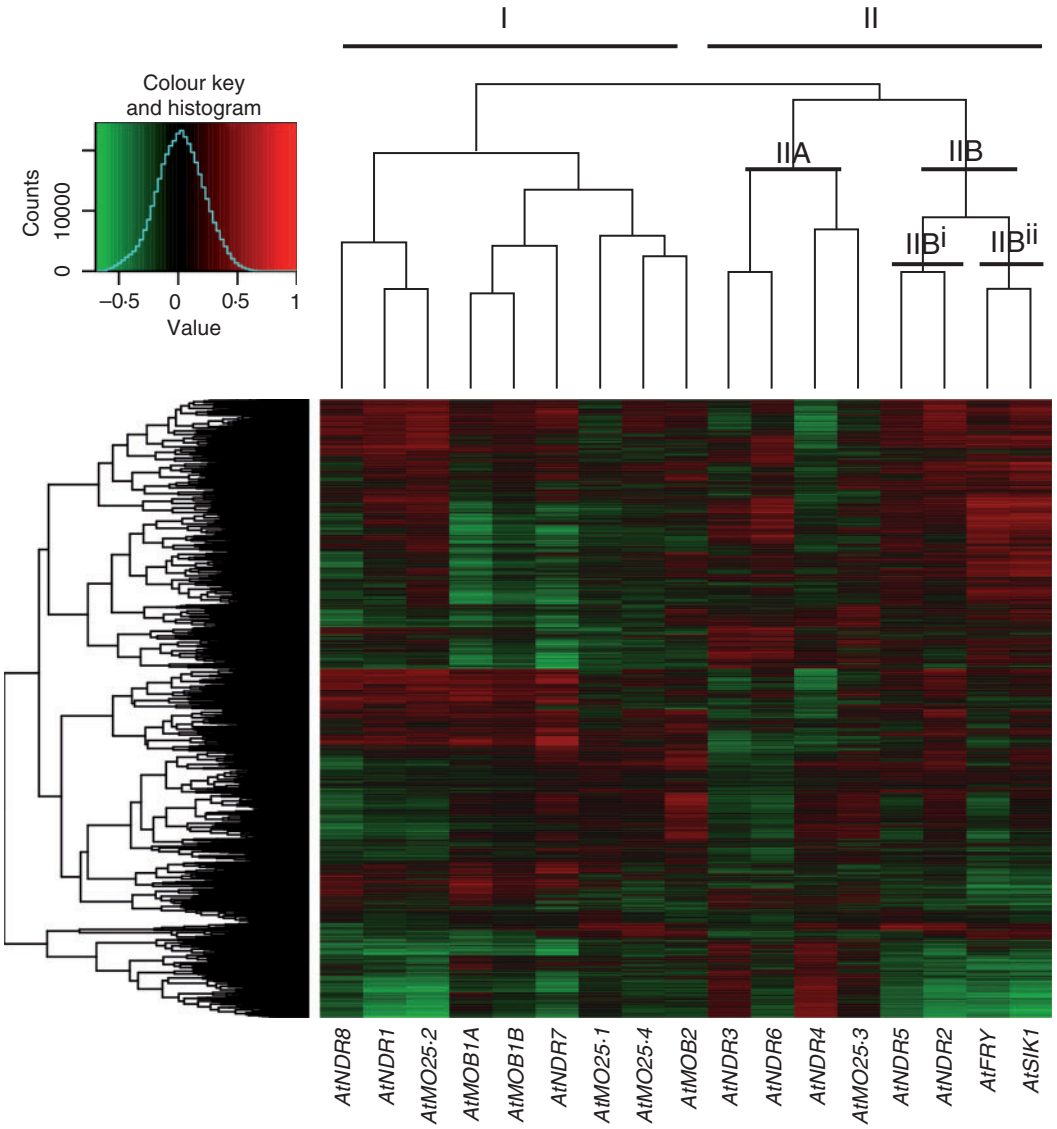

FIG. 2. Hierarchical clustering based on Pearson correlation coefficient values of gene pairs. Values were calculated using log-transformed expression values from the 17 RAM-like core genes and approx. 22 500 arabidopsis genes in 1730 experiments performed with the Affymetrix ATH1 GeneChip array and available in the public domain (Menges *et al*., 2008). The heatmap represents a compressed picture of all 21 692 unique genes represented by probes on the ATH1 array (shown on left side of the heat map), with the shading representing the degree of correlation with each of the putative RAM-like core genes: red indicates positive correlation, green negative correlation. The cluster tree on the upper part of the figure represents the similarity of expression of each RAM-like component across all probe sets.

values of genes as such or after logarithmic transformation. All data were analysed adopting both scales, based on the notion that the logarithmic scale enables one to better highlight correlations between gene expression values extending over a wide range of expression values (i.e. several orders of magnitude), while the linear scale is more effective when highlighting condition-dependent co-regulated expression such as that taking place in highly specific contexts or conditions (e.g. tissue or treatment).

The hierarchical clustering, driven by the degree of correlation between log-transformed expression profiles of the arabidopsis transcriptome and the RAM core, led to the identification of two major clusters within the RAM core (Fig. 2). Cluster I comprised all the *AtMOB* genes (*AtMOB1A*/*B* and *AtMOB2*), three NDRs (*AtNDR1*, *AtNDR7* and *AtNDR8*) and three *AtMO25* genes *(AtMO25-1*, *AtMO25-2* and *AtMO25-4*), while *AtSIK1*, *AtFRY*, *AtNDR2*, *AtNDR3*, *AtNDR4*, *AtNDR5*, *AtNDR6* and *AtMO25-3* grouped together in a separate cluster (cluster II). Within cluster II, *AtSIK1* and *AtFRY* appeared more closely associated by sharing a common set of genes with higher degree of co-expression (Fig. 2, subcluster IIB^{II}).

When expression data were analysed without log transformation, two main clusters could again be identified. Cluster I comprised three NDRs (*AtNDR1*, *AtNDR7* and *AtNDR8*), the two *MOB1* genes and *AtMO25-2*, while cluster II grouped together *AtSIK1*, *AtFRY*, *AtMOB2*, *AtNDR2*/*3*/*4*/*5*/*6* and *AtMO25-1-3-4* (Fig. 3). Within cluster II, *AtNDR2*, *AtNDR4*, *AtNDR5*, *AtMO25-1* and *AtMO25-4* showed high global expression correlation values for a clearly distinguishable and defined set of genes (evidenced in cluster α^{I} of Fig. 3), leading to a distinct

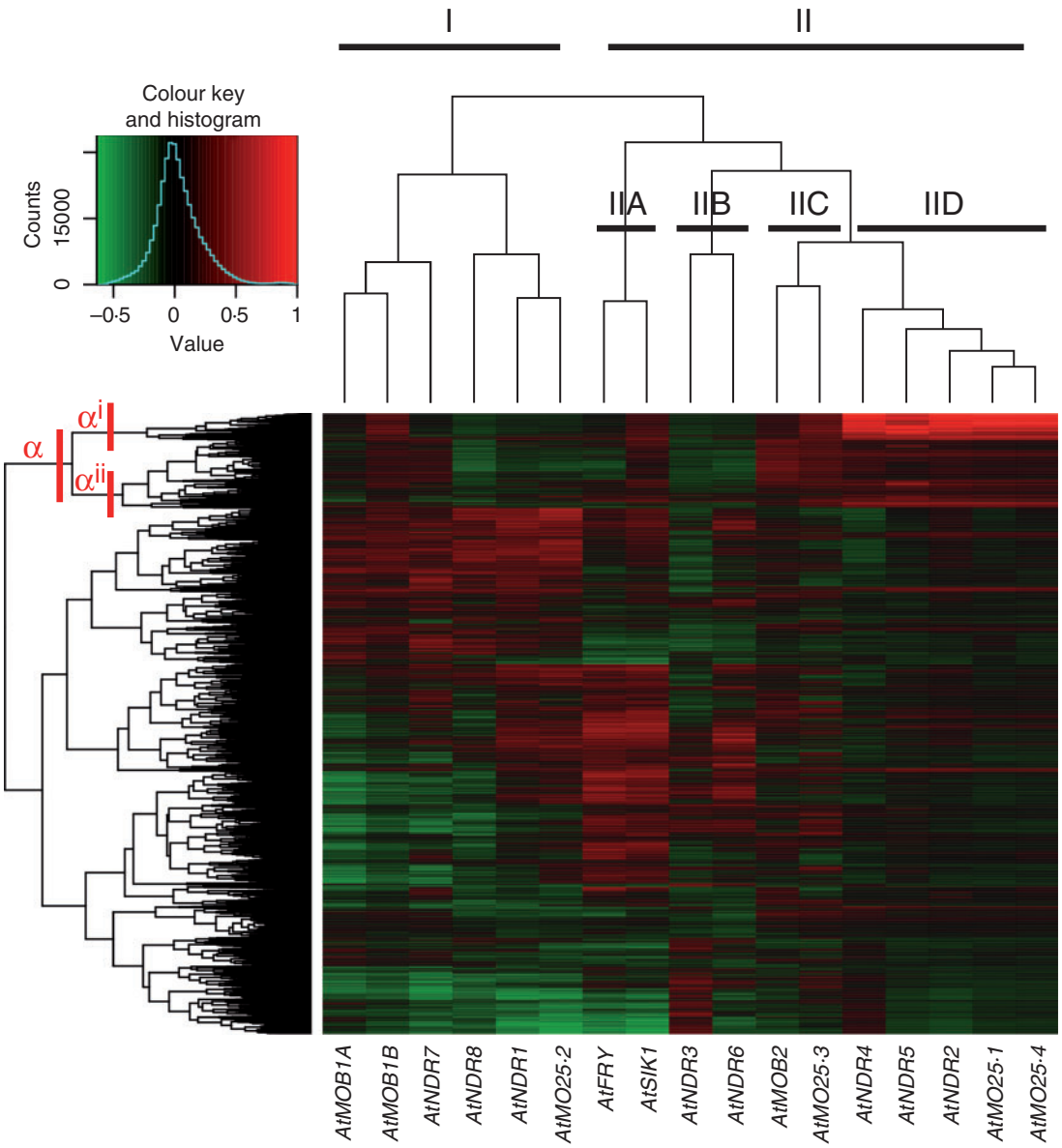

FIG. 3. Hierarchical clustering of correlation coefficient values calculated using linearly scaled expression. Values for genes were paired between the 17 arabidopsis RAM/MOR-like genes and approx. 22 500 arabidopsis genes. The shading represents the degree of correlation with each of the putative RAM-like core genes: red indicates positive correlation and green indicates negative correlation. For further details on the experimental conditions and heatmap refer to the legend to Fig. 2.

subclustering (IID) and indicating that these five genes may probably act together in a common signalling pathway functioning in a specific condition-dependent (tissue or developmental) context. *AtSIK1* and *AtFRY*, as for the logarithmic analysis, appeared closely associated in terms of global transcriptional correlations and clustered separately in a specific sub-group (IIA) within cluster II.

Overall, data from both logarithmic and linear analyses suggested the existence of separate transcriptional pathways between the putative identified core components of the arabidopsis RAM/MOR pathway. In fact, in all cases *AtFRY* and *AtSIK1* shared significant global transcriptional correlations and clustered separately from all the other components. This suggested that these two proteins may indeed act within the frame of a separate and common transcriptional network. In logarithmic analysis, few of the co-regulated genes shared between *AtSIK1* and *AtFRY* clustered together with those of AtNDRs, the kinase immediately downstream of the *AtFRY*–*AtSIK1* complex in all other eukaryotic organisms from yeast to human. This separation was particularly clear in the linear analysis, suggesting the existence of a tissue- or developmental-specific context in which *AtNDR2*, *AtNDR4* and *NDR5* may be involved in a signalling pathway together with *AtMO25-1* and *AtMO25-4*. Clusters II from both linear and logarithmic analyses included a component from almost all of the RAM representatives (with the only exception being *MOB2*, missing in logarithmic analysis) and for this reason they were evaluated further. Clusters I from both analyses, by including the two *AtMOB1A/B* genes together with *NDR1*, *7* and *8*, are likely to represent the SIN/MEN pathway and were not considered for

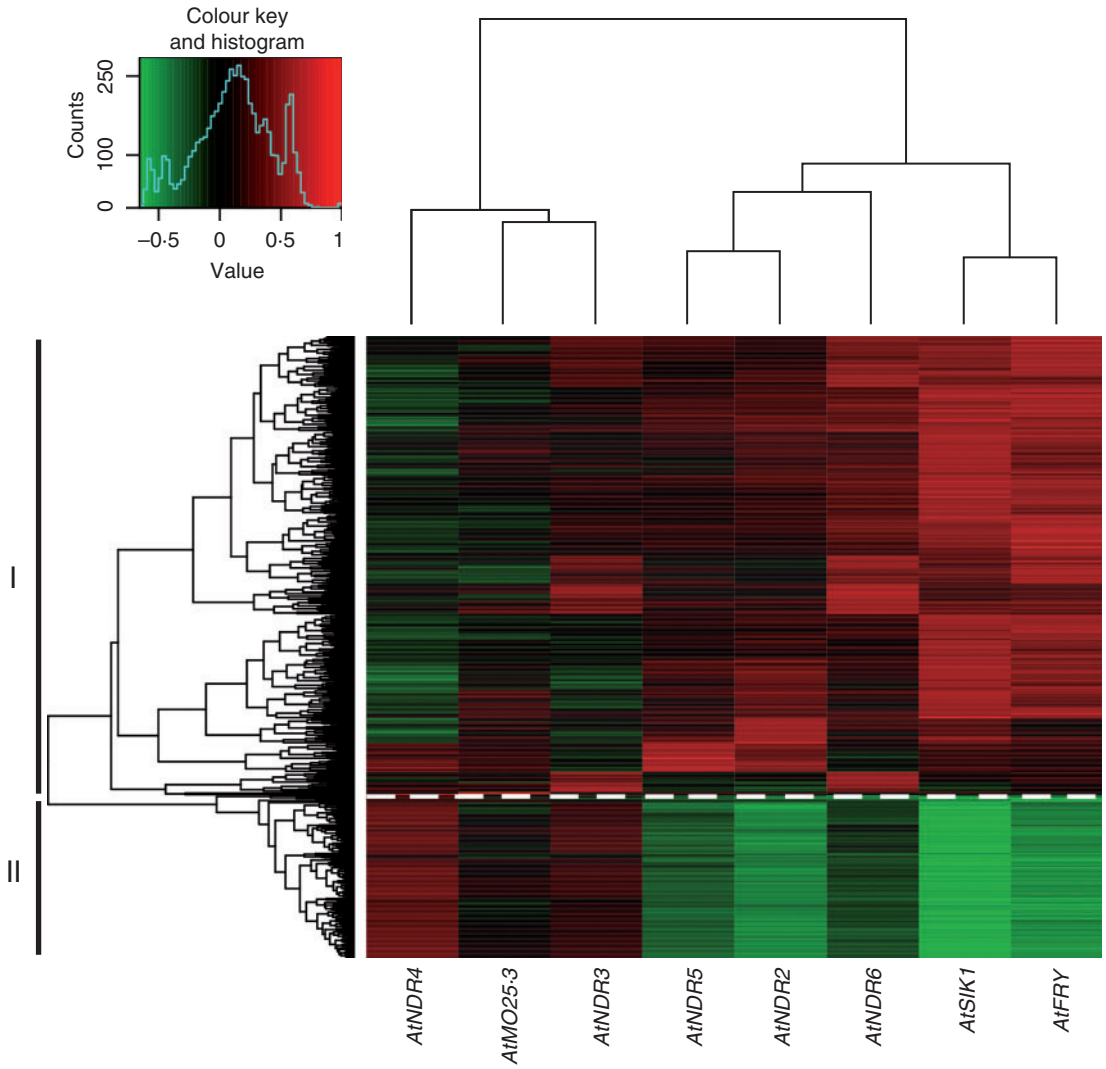

FIG. 4. Arabidopsis RAM/MOR putative core in relation to the whole genome transcriptomic data (0.55 threshold). Correlators reaching the threshold for at least one of the RAM/MOR putative core genes were kept. The shading represents the degree of correlation with each of these probe sets (red positive, green negative). A set of 718 correlated unique genes was evidenced and grouped into two main clusters. For additional details on the probe set, refer to the legend to Fig. 2.

further characterization. Thus, because *AtFRY* and *AtSIK1* genes represent the distinctive elements of the RAM network with respect to other signalling networks (such as SIN and MEN) in yeast (Nelson *et al*., 2003; Bedhomme *et al*., 2008) we conducted an in-depth study of their associated transcriptional network. In addition, because *AtNDR2*, *AtNDR4*, *AtNDR5*, *AtMO25-1* and *AtMO25-4*, grouped strongly together in the linear analysis, pointing to a supposed signalling pathway that may act in specific conditions, these genes were also selected for further analyses to characterize the putative RAM/MOR transcriptional network(s) in arabidopsis.

Identification of the putative RAM transcriptional network(s) in arabidopsis

To better define the hypothetical arabidopsis RAM pathway(s) and to identify relevant transcriptional module(s), hierarchical clusterings were produced from absolute expression values of co-expressed genes derived from the two clusters II obtained from linear and from logarithmic analyses (Figs 2 and 3), separately, keeping correlators having at least one correlation value higher than a stringent threshold (0·55 for logarithmic and 0·9 for linear analysis; Menges *et al*., 2008). In other words, we weeded out all the genes showing poor correlation. Hierarchical clustering, based on stringent logarithmic parameters, further highlighted the high degree of commonality between *AtFRY*- and *AtSIK1*-associated transcriptional signatures (Fig. 4). Overall, 164 positive correlators could be identified for *AtSIK1* and 213 for *AtFRY*, of which a set of approx. 61 genes appeared to be transcriptionally co-regulated with both genes with values above the threshold level (Fig. 4, cluster I), while approx. 40 genes shared anti-correlation (Fig. 4, cluster II). A full list of the co-expressed genes is provided in Supplementary Information – Table S2.

Interestingly, while cluster II comprised negatively associated genes mostly coding for chloroplastic proteins

(Supplementary Information – Table S2), cluster I included genes that could be associated with closely related functional processes that can be overall referred to the specification and maintenance of stem cell identity at the shoot apical meristem (Table 2) and to chromatin remodelling and post-transcriptional gene silencing, especially in relation to floral transition (Tables 3 and 4). Among co-expressed factors involved in SAM meristem maintenance, we identified genes encoding proteins putatively involved in the control of asymmetric cell divisions, such as *POL* (*POLTERGEIST*), in meristematic cell proliferation, such as *LUG* (*LEUNIG*) and its interactors *SEU* (*SEUSS*) and *SWP* (*STRUWWELPETER*), *PAS1* (*PASTICCINO 1*) and *GIF2*, or in stem cell maintenance, such as *SYD* (*SPLAYED*) (Table 2). In *H. sapiens*, NDR kinases recognize and phosphorylate the consensus motif HX(R/H/K)XX(S/T) in their substrates (Hao *et al*., 2008). POL contains a consensus motif recognized by NDR kinases, while SYD comprised two. Among genes involved in the transition from vegetative to floral meristem a significant number appeared to be remarkably connected to the regulation of *FLC* and *FT* and related to chromatin remodelling through methylation/demethylation (*JMJ14*, *EF6/7*, *ATXR7*) and histone ubiquitination (*HUB1*) (Table 3). A group of genes encoding proteins involved in post-transcriptional gene silencing (Table 4) were also identified, most notably players involved in the control of the expression or signal transduction of stem cell identity, such as *AGO1* (*ARGONAUTE 1*) and/or genes for the transition between vegetative and floral meristem. Besides, a series of genes were identified that could be related to auxin and cell polarity, such as the kinase *D6PKL2* (*D6 PROTEIN KINASE LIKE 2*), the ARF-like GTPase *TTN5* (*TITAN 5*), *BIG1/3* and the cullin *ATCUL1* (*ARABIDOPSIS THALIANA CULLIN 1*) or to signal transduction (kinases or TF) (Supplementary Information – Table S3). Among these, interestingly, two kinases were found (*D6PKL2*) and *KIPK* (*KCBP-interacting protein kinase*), both belonging to the group VIIIa of AGC kinases.

Hierarchical clustering based on correlation of linear expression data of the 367 genes with high correlation values (>0·9) identified two main clusters (Fig. 5) (a full list of the co-expressed genes is reported in Supplementary Information – Table S4). All the positively co-regulated genes displayed very high correlation values with *AtNDR2*, *AtNDR4*, *AtMO25-1*, *AtMO25-4* and, to a lesser extent, with *AtNDR5* (grouped in cluster I of Fig. 5), while no genes displaying anticorrelation could be identified below the threshold (–0·9). Cluster II comprised the six genes *AtFRY*, *AtSIK1*, *AtMO25-3*, *AtNDR3*, *AtNDR6* and *AtMOB2*. Within cluster I, genes could be identified whose action could overall be related to cytoskeleton organization and regulation of cell polarity, vesicular trafficking and calcium signalling (Table 5) or cell-wall remodelling and sugar metabolism (Supplementary Information – Table S5). Among genes involved in cytoskeleton organization and regulation of cell polarity, interestingly the Rho-like GTPase *ROP1* and its regulatory proteins ROP-GEFs 8, 9, 11 and 12 were found to have a high degree of co-regulation along with LIM proteins. ROP-GEF11/12 contains the consensus recognized by NDR kinases (data not shown). Concerning vesicle trafficking, *RAB1h/1i* and its regulatory protein RAB-GEF appeared co-regulated. From sugar metabolism, a relevant gene was *STP11* (*SUGAR TRANSPORTER 11*), which is supposedly involved in the supply of monosaccharides to growing pollen tubes (Schneidereit *et al*., 2005). Several RLKs or RLCKs, mostly reported to be expressed in pollen, were identified, two of which (*PRK2A* and *CDPK34*) (Zhang and McCormick, 2007; Zhou *et al*., 2009) have been demonstrated to be involved in polarized pollen tube growth (Supplementary Data – Table S6).

Developmental- and condition-dependent regulation of arabidopsis RAM/MOR signalling genes

The expression data of arabidopsis RAM/MOR genes from publicly available microarray datasets from various organs, developmental stages, response to hormone treatments and biotic/abiotic stresses were analysed. First, an atlas was obtained exclusively based on the tissue-specific expression of the 11 arabidopsis RAM/MOR core genes. *AtNDR2*, *AtMO25-1*, *AtMO25-4*, *AtNDR4* and, to a lesser extent, *AtNDR5* were highly expressed in pollen (Fig. 6 and Supplementary Information – Fig. S1). Thus, pollen represents one highly specific context where these genes are co-expressed, explaining, at least in part, their high linear correlation values. Logarithmic correlation analysis allowed us to highlight the coordinate expression of *AtMO25-3*, *AtFRY* and *AtSIK1* in the shoot apex (Supplementary Information – Fig. S1). Neither linear nor logarithmic analysis of transcriptional regulation of RAM-like signalling components in response to abiotic (cold, osmotic, salt, drought, genotoxic, oxidative stress, UV-B stress, wounding, heat stress), biotic (*Pseudomonas syringae*, *Phytophthora infestans*, *Botrytis cinerea*) stress, or to hormonal stimuli (ABA, MJ, BL, ACC, ET inhibitor, IAA, auxin inhibitor, cytokinin, CHX), or to light highlighted obvious differences in expression levels (Supplementary Information – Figs S2 and S3).

Expression data from the whole set of RAM/MOR co-regulated genes taken as such (linear values, Supplementary Information – Table S3) or after log-transformation (Supplementary Information – Table S1) were examined from various organs and developmental stages in the same way as for the RAM-like core genes. In the logarithmic case, positively and negatively co-regulated genes relative to *AtSIK1* and *AtFRY* were considered (genes co-expressed with *AtNDR2*, *AtNDR3*, *AtNDR4*, *AtNDR5*, *AtNDR6* and *AtMO25-3* were neglected) but this analysis did not lead to identification of evident tissue-specific clusters (Supplementary Information – Fig. S4). For linear analysis, the 367 positively co-regulated genes (composing cluster I reported in Fig. 5) were highly expressed in pollen (Supplementary Information – Fig. S5). A similar co-regulation and the same expression pattern strengthen the possibility that *AtNDR2*, *AtNDR4*, *AtMO25-1*, *AtMO25-4* and *AtNDR5* and these 367 genes may act together at different tiers of a common pollen-specific pathway.

In situ *analysis of the putative RAM/MOR transcriptional core network*

To corroborate the data obtained by hierarchical clustering analyses, the putative arabidopsis RAM/MOR transcriptional core network was investigated by *in situ* experiments to confirm expression in stem cells of the shoot apical meristem.

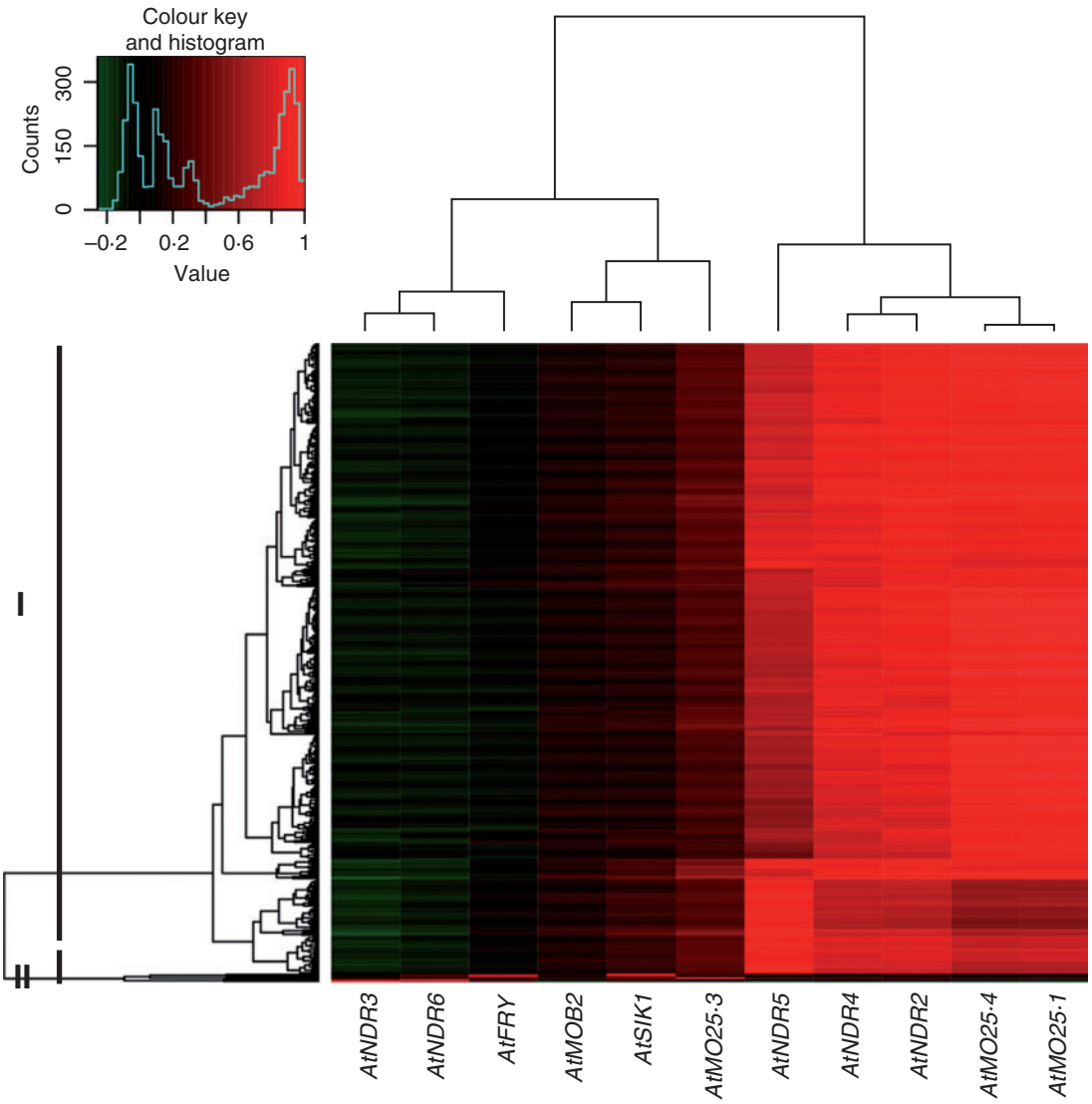

FIG. 5. Expression linear correlation of the arabidopsis transcriptome with components of the arabidopsis RAM/MOR pathway. A minimum cutoff threshold of 0.9 (correlators reaching the threshold for at least one of the RAM/MOR putative core genes were kept) was applied to correlation values for the selection of co-regulated genes, allowing the identification of 372 unique genes. The shading represents the degree of correlation with each of these probe sets (red positive, green negative). Correlated genes were grouped into two main clusters (I and II, reported on the left). Details are given in the text.

Five of the eight RAM-like core genes (*AtFRY*, *AtSIK1*, *AtMO25-3*, *AtNDR3* and *AtNDR4*) highlighted by logarithmic analysis, and belonging to groups IIA and IIB (Fig. 2), were localized in floral and embryo tissues of arabidopsis plants (Figs 7–9).

Sections of SAM in the transition phase and young embryos were chosen because the *AtFRY*- and *AtSIK1*-associated transcriptional signatures pointed predominantly to their putative involvement in the processes of maintenance of stem cell identity and, in particular, of floral transition (*in silico* data, Tables 2–4).

In the primary inflorescence apical meristem (IM) *AtFRY*, *AtSIK1*, *AtMO25-3* and *AtNDR4* signals were seen in all the tunica layers and in the corpus, thus including the proximal flower meristems (FMs) (Fig. 7A–C, E). Expression of *AtSIK1* and *NDR4* during the early stages of development of the distal FMs appeared to be absent from sepal primordia but present in the developing stamens and gynoecium (i.e. Fig. 7B, E). *AtNDR3* expression, differently from the other genes, appeared concentrated within few inner cells of the FM (Fig. 7D). When later stages of flower development were considered all five genes displayed almost fully overlapping expression domains: in the gynoecium (ovules, embryos) and stamens (Fig. 7F–O).

Expression was further investigated during flower development, and, because the expression pattern was the same for all genes considered (with exception of *AtNDR3* in the early stages, Fig. 8A, B), here we have reported as an example the time course expression of *AtFRY*. In the gynoecium *AtFRY* signals seemed to be linked to ovule development (Fig. 8A–F). In fact they were first present in the placenta (Fig. 8C), then in the small ovular bulges along the placenta (ovule primordium, op) (Fig. 8D) and in their expansion and extension until the nucellus was apparent (Fig. 8E), in the ovule itself and in the embryo (Fig. 8F). In the stamens *AtFRY* expression also appeared to follow pollen development given that it was highlighted in all the four developing anther lobes in the sporogenous cells (Sp)

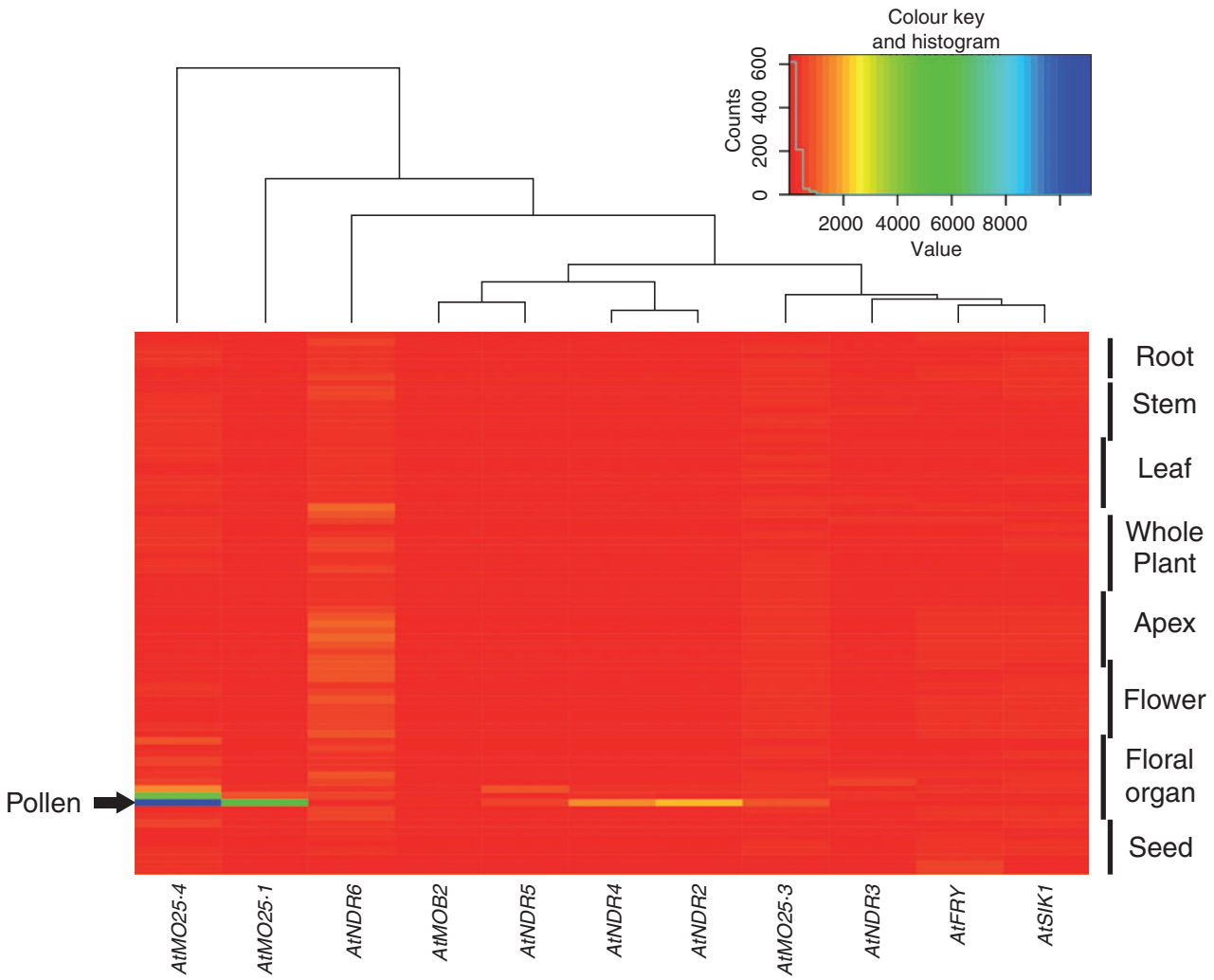

FIG. 6. Atlas of the arabidopsis RAM/MOR-like core genes during organ development. Linear expression data for RAM-like signalling genes obtained from different stages of development in roots, stems, leaves, seedlings, shoots, flowers, pollen, siliques and seeds were normalized for fold changes and hierarchical clustering was applied to group genes based on similarities in their expression, represented as a heat map.

(Fig. 8G), in the meiotic cell (MC) (Fig. 8H), in microspores (MSp) (Fig. 8I) and in the tapetum (t) (Figs 7F–J and 8J).

To further assess the expression of the selected genes in the vegetative SAM of mature embryos, ISH was also carried out on siliques. *AtFRY*, *AtSIK1*, *AtMO25-3* and *AtNDR4* transcripts could not be detected in embryos (Fig. 9), while *AtNDR3* expression appeared highly localized and restricted to a few cells within SAM at the late torpedo stage (Fig. 9). Negative controls of ISH are shown in Supplementary Data – Fig. S6.

Overall, ISH data corroborate *in silico* analyses and confirm the involvement of this putative core gene set in the maintenance of stem cell identity within the SAM and FM.

DISCUSSION

Conserved RAM/MOR pathway in A. thaliana

The RAM/MOR network of proteins has been established as a central signalling module regulating asymmetric cell division and cell polarity in a number of uni- and multi-cellular eukaryotic organisms including *S. cerevisiae*, *D. melanogaster* and *H. sapiens* (Maerz and Seiler, 2010). Despite the importance of RAM/MOR proteins in co-ordinating cell polarization and differentiation with cell division (Maerz and Seiler, 2010) and, in multicellular organisms, organ polarity and organ size (Halder and Johnson, 2011), this network has not been studied in plants so far. In this paper we have performed a bioinformatic investigation on the model plant *Arabidopsis thaliana* to uncover the existence of a RAM network in plants and to pinpoint putatively conserved and divergent elements of the plant RAM module with respect to other eukaryotic systems. To do so, we have first identified the conserved putative 'core elements' of the arabidopsis RAM/MOR with high degree of similarity with the 'core' elements from the yeast–metazoan pathway, namely TAO3, CBK1, KIC1, MOB, HYM1 and SOG2. On the basis of sequence similarity (BLASTp and PSI-BLAST) searches we identified a single STE20-like kinase (AtSIK1), one scaffold protein TAO3 (AtFRY), four HYM1 (AtMO25 1–4) and eight NDR (AGCVII) kinases, and four homologues of the NDR co-

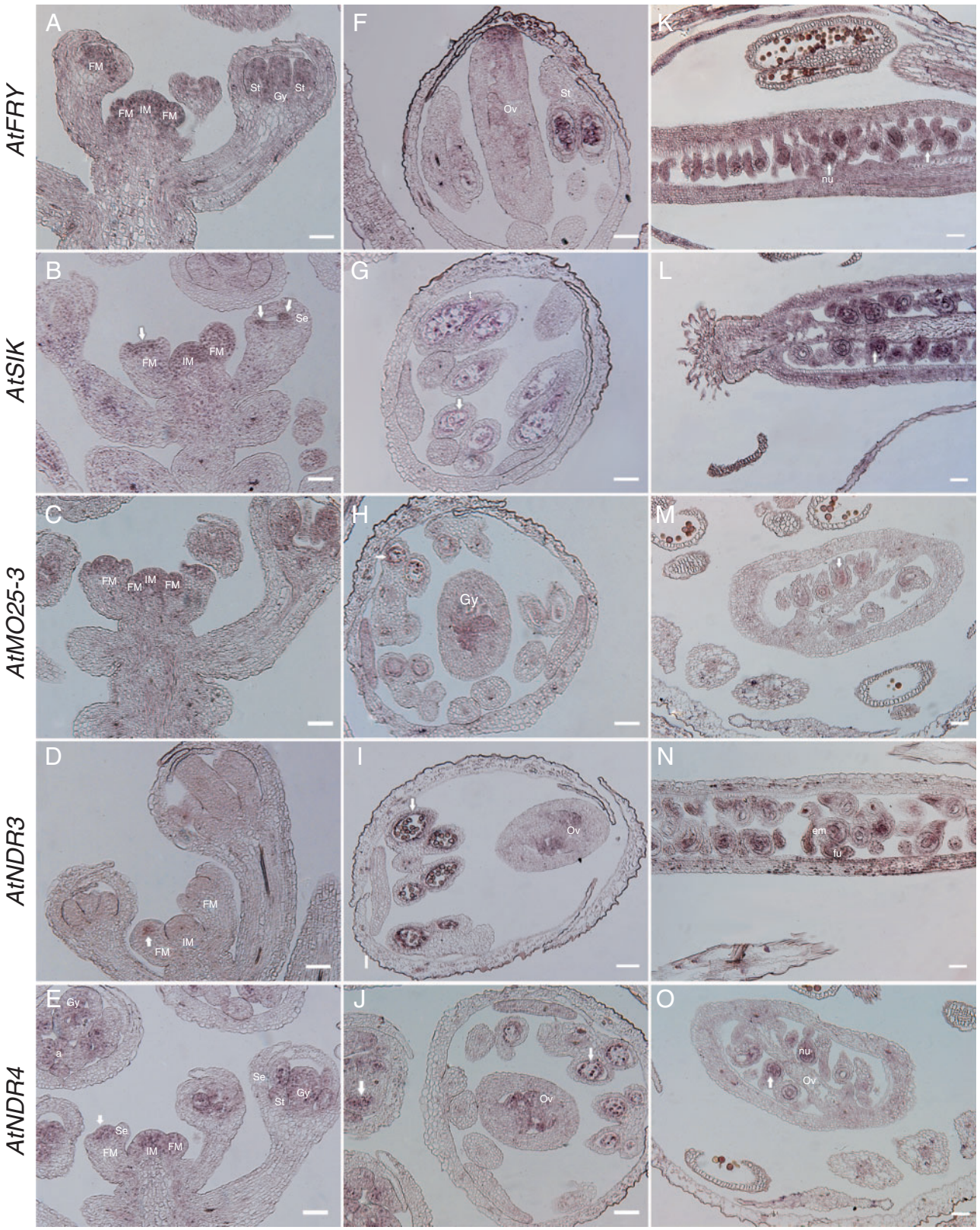

FIG. 7. *In situ* hybridization on sections of arabidopsis flowers. Antisense *AtSIK1*, *AtFRY*, *AtMO25-3*, *AtNDR3* and *AtNDR4* riboprobes were used to hybridize sections of the shoot apical meristem at the early stages of flowering (A–E) and flower organs during later stages (F–O). Panels K–O show close-ups of localizations of mRNAs in developing ovules. Arrows indicate localization of signals. em, embryo; FM, flower meristem; fu, funiculus; Gy, gynoecium; IM, inflorescence meristem; nu, nucellus; Ov, ovule; Se, sepal; Sg, stigma; St, stamen; t, tapetum. Scale bar = 50 µm.

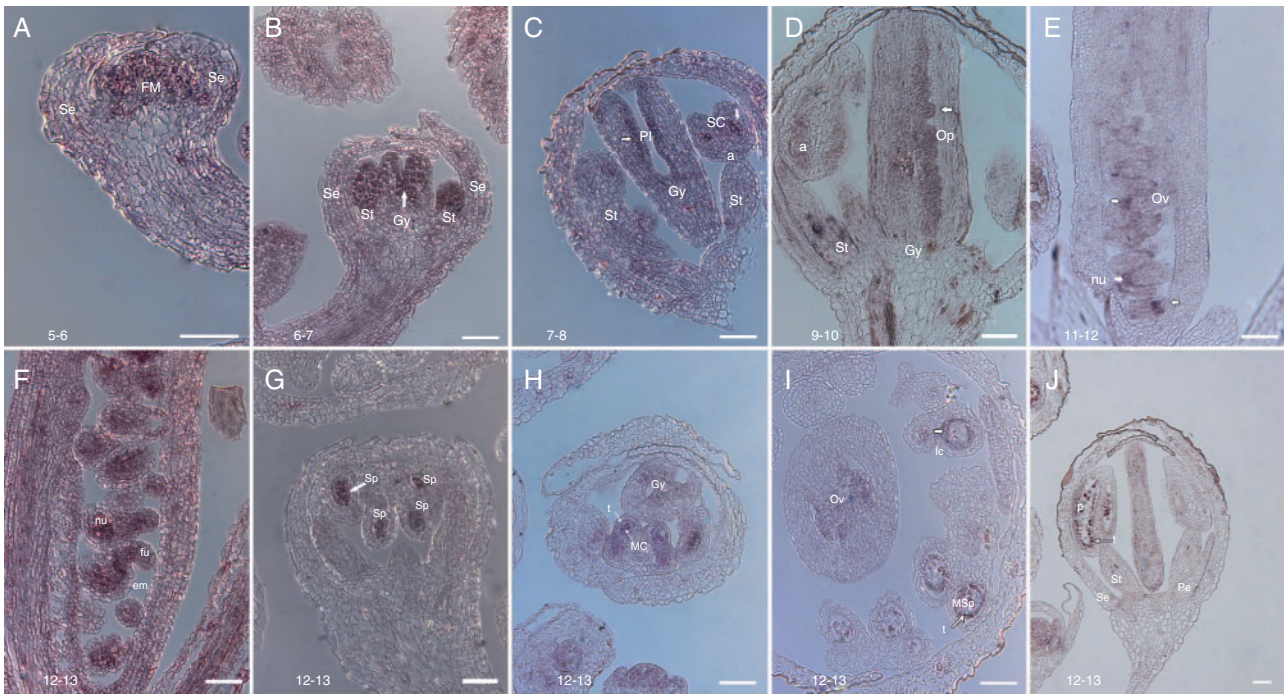

FIG. 8. Time course analysis of *AtFRY* expression by ISH. Antisense *AtFRY* riboprobe was hybridized on sections of flowers at different stages of development. Floral developmental stages were defined according to Alvarez-Buylla *et al.* (2010) and are indicated by numbers reported on the bottom left corner of each panel. Panels E–F: close-up of female organs, G–J: male organs. Arrows indicate where signals are present. a, anther; em, embryo; FM, flower meristem; fu, funiculus; Gy, gynoecium; lc, locule; nu, nucellus; Op, ovule primordium; Ov, ovule; Pl, placenta; p, pollen; Se, sepal; Sp, sporogenous cells; St, stamen; t, tapetum; SC, sporogenous cells; MC, meiotic cell; Msp, microspores. Scale bar = 30 μm.

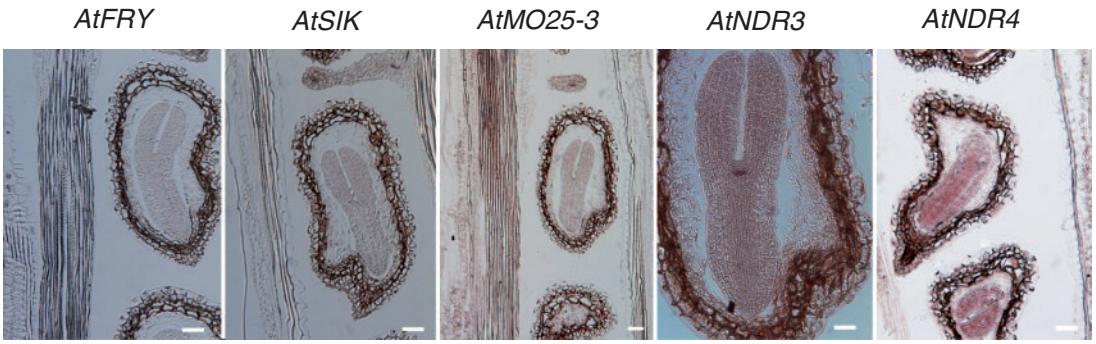

FIG. 9. *In situ* hybridization (ISH) on sections of developing arabidopsis siliques. Embryos at the late torpedo stage are visualized after ISH with *AtSIK1*, *AtFRY*, *AtMO25-3*, *AtNDR3* and *AtNDR4* probes. Signals of *AtSIK1*, *AtFRY*, *AtMO25-3* and *AtNDR4* were not detectable. AtNDR3 expression appears to be restricted to the stem cells niche of SAM (indicated by arrow). Scale bar = 50 μm.

activator MOB, as RAM-like core components encoded in the arabidopsis genome. AtNDR kinases 1–8 and AtMOB1A-1B and AtMOB2A-2B were also previously described (Bögre *et al.*, 2003; Vitulo *et al.*, 2007). AtSIK1 was identified (Karpov *et al.*, 2009) but was not assigned to a common signalling pathway. Using different BLAST algorithms it was not possible to identify bona fide SOG2 homologues. To further characterize the transcriptional network of the plant's RAM/MOR an in-depth search of available microarray data was performed to pinpoint shared and/or divergent transcriptional signatures between the identified components of the arabidopsis RAM/MOR pathway through a guilty-by-association approach. This analysis was carried out with complementary approaches. Linear co-expression was exploited to highlight co-expression in specific (condition-dependent) contexts, while logarithmic analysis pointed to co-expression in a more general condition-independent context and on a broader range of expression values. With both analyses the candidate arabidopsis RAM/MOR homologues grouped into two main clusters. Interestingly, *AtMOB1* genes clustered together with the three NDR kinases *AtNDR1*, *AtNDR7* and *AtNDR8* separately from all other putative components of the RAM/MOR. This suggests that these MOBs and NDRs may be involved in a separate pathway which may represent the SIN/MEN pathway rather than RAM/MOR

network. Conversely, the bona fide RAM/MOR pathway of arabidopsis may thus in general include *AtSIK1* (Ste20-like kinase) and *AtFRY* (TAO3-like, scaffold), *AtNDR2*, *AtNDR3*, *AtNDR4*, *AtNDR5*, *AtNDR6* (AGCVII NDR kinases) and *AtMO25-3* (scaffold) as seed elements (as evidenced by analysis of log-transformed expression data). Nevertheless, in specific developmental contexts, this pathway may also include *AtMOB2*, *AtMO25-1* and *AtMO25-4* (as evidenced by linear co-expression data). Within this putative regulatory module, in all cases, *AtSIK1* and *AtFRY* appeared to be more closely associated with each other than with the other hypothetical plant RAM/MOR members. These data would suggest that the arabidopsis RAM/MOR pathway may split into two sub-pathways that may be active in different developmental contexts. One sub-pathway may be composed of *AtFRY* and *AtSIK1*, showing a high degree of correlation of gene expression in both logarithmic and linear analysis, and lacking the presence of closely co-regulated NDR (AGCVII) kinases. The second sub-pathway may be composed of the scaffolds *AtMO25-1* and *AtMO25-4* and of the AGC group VII kinases *AtNDR2*, *AtNDR4* and *AtNDR5*, showing a high degree of correlation of expression data in linear analysis. Because linear analysis reflects condition-dependent co-expression, it is conceivable that the latter sub-pathway may be active in a very specific context represented by a restricted tissue/developmental/response situation. This hypothesis was further confirmed when these groups of genes were employed separately to mine array data for the identification of the putative arabidopsis RAM/MOR transcriptional network(s).

Context-specific RAM/MOR transcriptional network(s) in arabidopsis: pollen-specific expression and regulation of cell polarity

Linear co-expression data pointed to the presence of a context-specific RAM/MOR module, composed of *AtNDR2*, *AtNDR4*, *AtNDR5*, *AtMO25-1* and *AtMO25-4*, which appeared to share a set of 367 genes with a high degree of co-regulation. Remarkably, this set included several genes shown to be involved in polarized growth of pollen tubes and specifically expressed in pollen, on the basis of data mining of tissue-specific arrays (http://jsp.weigelworld.org/expviz/expviz.jsp). Our data point to a strong co-regulation between these components of the RAM/MOR pathway and the ROP machinery (specifically ROP1 and RIC1), a pivotal element in the regulation of polarized growth in pollen (Cheung and Wu, 2008; Lee *et al*., 2008). Consistently with these data, four *ROP-GEFs* (ROP activators; Molendijk *et al*., 2004; Berken *et al*., 2005; Gu *et al*., 2006), namely *ROP-GEF 8*, *9*, *11* and *12* (the latter three shown to be specifically expressed in arabidopsis pollen by Kaothien *et al*., 2005) were found to be highly co-expressed with the RAM/MOR core. These ROP-GEFs may be targets of AtNDR2, 4 and 5, in the same way as the *S. pombe* CDC42-GEF is regulated at the cell cortex by the CBK1/NDR kinase homologue ORB6 (Das *et al*., 2009). Consistently, we found that arabidopsis ROP-GEF11/12 contain the consensus motif recognized by NDR kinases (data not shown), and thus these ROP-GEFs could be downstream targets of NDR kinases 2/4/5 in the regulation of pollen tube polar growth in arabidopsis. Upstream of ROP-GEFs may also lie the highly co-regulated gene encoding the receptor kinase AtPRK2, shown to be involved in the regulation of pollen tube growth through phosphorylation of ROP-GEFs (Chang *et al*., 2013) and a close homologue of the tomato pollen-specific receptor-like kinase LePRK2, shown to interact with the pollen-specific ROP-GEF KPP (Kaothien *et al*., 2005). The co-ordination of the RAM/MOR pathway elements with the ROP1 machinery is further reinforced by evidence supporting the co-regulation of several genes involved in cell polarity through coordination of the dynamics of surface signals, cytoskeleton organization, calcium fluxes and vesicle trafficking (Cheung and Wu, 2008). In our analysis two members of the exocyst complex, the pollen-specific *ATEXO70H3* and *ATEXO70H5* (Li *et al*., 2010), were co-expressed with the RAM/MOR core, as well as members of the SNARE receptors and RAB GTPases families regulating specific vesicle docking and fusion with target membranes (Suwastika *et al*., 2008). Three SNARE members were co-expressed in pollen (*SYP72*, *SYP124*, *SYP131*) and one of them, SYP124 (syntaxin), was recently shown to be involved in polarized vesicle secretion during pollen polar growth (Silva *et al*., 2010). Similarly, two yet uncharacterized RAB family members, *RABA1h* and *RABA1i*, and one RAB activator (RAB-GEF), *GYPB1d*, displayed a high degree of co-regulation. In pollen, calcium gradients are essential for polarized tip growth, directional pollen tube elongation and growth oscillation (Zhou *et al*., 2009). Among the RAM/MOR co-regulated genes we identified several genes related to calcium sensing and transport. Among these, PIPK11, a PIP kinase, and ADF7 (actin depolymerization factor 7) may be involved in PIP2 formation that may act as a second messenger regulating ADFs for polar growth of pollen tube (Bou-Daher *et al*., 2011). Interestingly, several pollen-specific calcium sensors (*CDPK24*, *CML6*, *25* and *28*; Zhou *et al*., 2009), one of which (CDPK24) was shown to be involved in tube elongation (Zhou *et al*., 2009), appeared to be co-regulated with the RAM/MOR components, along with *ACA9*, a calcium efflux pump reported to be required for normal pollen tube growth (Schiøtt *et al*., 2004). In addition, as reported for the RAM/MOR pathway of fungi, which appears to be actively involved in the coordination of cell wall remodelling for polar growth of hyphae (Das *et al*., 2009), the arabidopsis pathway also seems to be involved in the coordination of cell-wall remodelling, as suggested by the co-regulation of a range of genes encoding cell-wall-remodelling enzymes and monosaccharide transporters (Supplementary Information – Table S5).

RAM-like core genes in the SAM and IM

Logarithmic analysis underlined the general context in which the RAM/MOR group of genes *AtSIK1*, *AtFRY*, *AtNDR2*, *AtNDR3*, *AtNDR4*, *AtNDR5*, *AtNDR6* and *AtMO25-3* are co-regulated. *AtSIK1* and *AtFRY* were very closely associated with each other, sharing a consistent body of transcriptionally co-regulated genes, suggesting that these two genes may indeed belong to a common transcriptional regulatory module. These analyses have identified a set of 389 genes which may represent a RAM/MOR transcriptional network in which *AtFRY* and *AtSIK1* would represent the core element and which would be acting separately from that identified in pollen tube growth. Most of the 314 genes strongly positively co-regulated with

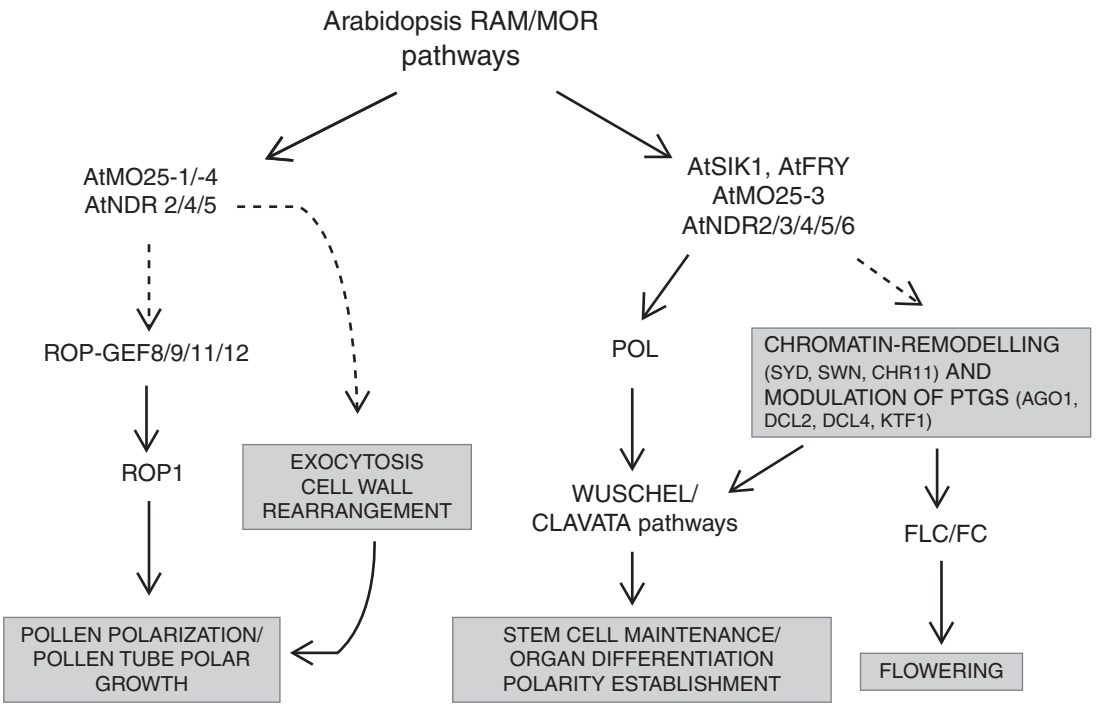

Fig. 10. Schematic representation of the putative arabidopsis RAM/MOR-like signalling cascade(s) identified in this work. On the basis of co-expression analyses the RAM/MOR-like pathway could be divided into two distinct signalling branches being active in at least two different developmental contexts: one including AtNDR2, AtNDR4, AtNDR5, AtMO25-1 and AtMO25-4, acting upstream of the Rho-like GTPase ROP1 and its regulators ROP-GEF8/9/11/12, and putatively involved in the regulation of polar growth of pollen tube. The same signalling module may be involved in parallel in the regulation of exocytosis and cell wall remodeling, during pollen tube growth. The second pathway, including AtSIK1, AtFRY, AtNDR2, AtNDR3, AtNDR4, AtNDR5, AtNDR6 and AtMO25-3, may be actively involved in the regulation of SAM maintenance and in floral transition, by influencing the CLV/WUS pathway, through chromatin remodelling and modulation of post-transcriptional gene silencing (PTGS). The identification of phosphorylation consensus motifs of NDR kinases on ROP-GEFs, on POL and on SYD proteins may suggest that the RAM/MOR pathway could represent, at least in part, a regulatory module operating upstream of these pathways.

AtSIK1 and *AtFRY*, were expressed in SAM and in IM, and implicated in stem cell maintenance and in organ polarity establishment. Remarkably, genes such as *REV*, *AGO1* and *LUG* were found (Chandler, 2012). Both REV and LUG are involved in SAM maintenance and organ polarization (Otsuga *et al*., 2001; Stahle *et al*., 2009). AGO1 regulates *REV* expression producing the miRNA that directly targets *REV* mRNA (Husbands *et al*., 2009). REV is an upstream regulator of the CLAVATA (CLV) pathway, a central network contributing to SAM maintenance (Otsuga *et al*., 2001; Carles and Fletcher, 2003). Consistently, *AtSIK1* and *AtFRY* appeared also to be co-regulated with the phosphatase *KAPP*, an upstream negative regulator of the CLV1 pathway (Carles and Fletcher, 2003) and with the phosphatase POL, downstream negatively regulated by CLV1 (Gagne *et al*., 2008). Interestingly, POL presented a consensus motif for NDR phosphorylation, hypothetically downstream of AtSIK1 and AtFRY. The link between the RAM/MOR pathway and the coordination of organ polarization by regulation of cell number during organogenesis may be further reinforced by co-regulation with, besides REV and LUG, the LUG co-regulator SEU, involved in pre-patterning and polarization of incipient floral primordia (Chandler, 2012) and organ identity determination (Franks *et al*., 2006).

Early patterning events involve chromatin-remodelling factors (Shen and Xu, 2009) and *AtSIK1* and *AtFRY* appear to be co-regulated with *SWN* (*SWINGER*), *SYD* and *CHR11*. These factors regulate the balance between stem cell renewal and cell differentiation for organ formation. SWN is involved in H3K27 methylation of the class I *KNOX* gene *STM*, causing its suppression, thus confining SAM activity and allowing cell differentiation (Shen and Xu, 2009). SYD is a member of Snf2 class chromatin remodelling ATPases and regulates meristem maintenance by positively regulating *CLV3* and *WUS* transcription (Kwon *et al*., 2005) and preventing *LFY* expression in an environmental-dependent way (Wagner and Meyerowitz, 2002). Interestingly, as shown for POL, SYD presented two potential consensus motifs for phosphorylation by NDR kinases. CHR11, another Snf2 class chromatin remodelling ATPase, is involved in the vegetative to reproductive phase transition (Li *et al*., 2012).

The co-expression patterns of the putative RAM/MOR core genes in SAM and IM are supported by our ISH analyses. The results showed that the arabidopsis RAM/MOR core genes present expression patterns that completely overlap with those of *SEU* and *REV* in inflorescence meristems, with the exception that, differently from *SEU* and *REV*, signals did not localize adaxially and/or abaxially at later stages of organ development. *SEU* and *REV* expression could be overlapped by RAM-like core genes in the first stages of ovule and stamen development. Also, *SYD* and *CHR11* expression domains (Li *et al*., 2012) in SAM inflorescence and in gametophyte development appeared to have the same localization to RAM-like components.

CONCLUSIONS

We have identified a novel putative regulatory module in arabidopsis that may correspond to the RAM/MOR pathway of higher eukaryotes, a central element for the fine tuning of cell staminality and differentiation, cell polar growth and establishment of organ polarity. Based on transcriptional co-expression data, and on ISH data for SAM/IM, the arabidopsis RAM/MOR signalling network appears to comprise two regulatory submodules, one active in pollen tube polarized growth, possibly acting upstream of ROP1, and one active in fine tuning stem cell maintenance, differentiation and organ polarity within SAM/IM in concert with the regulation of mRNA processing and chromatin remodelling elements (Fig. 10). We speculate that the novel arabidopsis RAM/MOR-like pathway may represent an upstream regulatory module for ROP-GEF11/12 and SYD and POL, displaying consensus motifs for NDR kinase-mediated phosphorylation (Fig. 10). These findings suggest intriguing hypotheses, to be tested in future work, on the putative involvement of the individual components of the identified arabidopsis RAM/MOR pathway in the regulation of ROP1 and, possibly, of WUS/CLV signalling networks.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: orthologues of the RAM/MOR components in plants. Table S2: list of co-expressed genes (logarithmic correlation). Table S3: genes coding kinases, transcriptional regulators, small-GTPases/accessory proteins and TFs positively co-regulated with *AtFRY* and *AtSIK1*. Table S4: list of co-expressed genes (linear correlation). Table S5: sugar metabolism, transport and cell-wall remodelling genes positively co-regulated with *Mo25-1*, *Mo25-4*, *NDR2* and *NDR4*. Table S6: genes coding RLK/RLCK and TFs positively co-regulated with *AtMO25-1*, *AtMO25-4*, *AtNDR2* and *AtNDR4*. Fig. S1: RAM-core gene atlas during arabidopsis development. Fig. S2: data from http://jsp.weigelworld.org/expviz/expviz.jsp showing regulation of RAM-core genes by abiotic stress, transcriptional regulation of RAM-core genes by bacterial pathogens, transcriptional regulation of RAM signalling components by treatment with hormones, various inhibitors, imbibition and temperature, and transcriptional regulation of RAM-core genes by light. Figure S3: data from http://jsp.weigelworld.org/expviz/expviz.jsp (log_{10}) showing regulation of RAM-core genes by abiotic stress, transcriptional regulation of RAM-core genes by bacterial pathogens, transcriptional regulation of RAM signalling components by treatments with hormones, various inhibitors, imbibition and temperature, and transcriptional regulation of RAM-core genes by light. Figure S4: logarithmic RAM-like core co-expressed genes, from the cluster II (Fig. 2) atlas, during arabidopsis development. Figure S5: linear RAM-like core co-expressed genes, from the cluster II (Fig. 3) atlas, during arabidopsis development. Fig. S6: *in situ* hybridization control.

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APPENDIX

List of abbreviations

RAM	Regulation of ACE2p activity and cellular morphogenesis
NDR	Nuclear Dbf2 related
Mob	Mps one binder
NLS	Nuclear localization signal
NES	Nuclear export signal
TRC	Tricornered
FRY	Furry
MOR	Morphogenesis Orb6 network
MO25	Mouse embryo scaffolding protein
SIK1	Stress induced kinase
SSD1	Suppressor of SIT4 deletion
KIC1	Kinase that interacts with CDC31
TAO3	Transcriptional activator of OCH1
CBK1	Cell wall biosynthesis kinase
TF	Transcription factor
SAM	Shoot apical meristem
IM	Inflorescence meristem
ISH	*In situ* hybridization
ABA	Abscisic acid
MJ	Methyl jasmonate
BL	Brassinolide
ACC	1-Aminocyclopropane-1-carboxylate
ET inhibitor	Ethylene inhibitor
IAA	Indole-3-acetic acid

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