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R1R2R3-Myb proteins positively regulate cytokinesis through activation of *KNOLLE* transcription in *Arabidopsis thaliana*

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G2/M phase-specific gene transcription in tobacco cells is mediated by R1R2R3-Myb transcriptional activators, NtmybA1 and NtmybA2, which bind to mitosis-specific activator (MSA) elements. We show here that two structurally related genes, *MYB3R1* and *MYB3R4*, which encode homologs of NtmybA1 and NtmybA2, play a partially redundant role in positively regulating cytokinesis in *Arabidopsis thaliana*. The *myb3r1 myb3r4* double mutant often fails to complete cytokinesis, resulting in multinucleate cells with gapped walls and cell wall stubs in diverse tissues. These defects correlate with the selective reduction of transcript levels of several G2/M phase-specific genes, which include B2-type cyclin (*CYCB2*), *CDC20.1* and *KNOLLE* (*KN*). These genes contain MSA-like motifs in their promoters and were activated by MYB3R4 in transient expression assays in tobacco cells. The *KN* gene encodes a cytokinesis-specific syntaxin that is essential for cell plate formation. The cytokinesis defects of *myb3r1 myb3r4* double mutants were partially rescued by *KN* gene expression from heterologous promoters. In addition, a *kn* heterozygous mutation enhanced cytokinesis defects resulting from heterozygous or homozygous mutations in the *MYB3R1* and *MYB3R4* genes. Our results suggest that a pair of structurally related R1R2R3-Myb transcription factors may positively regulate cytokinesis mainly through transcriptional activation of the *KN* gene.

KEY WORDS: Cell plate, Cyclin, Cytokinesis, Guard cell, Myb, KNOLLE, *Arabidopsis thaliana*

INTRODUCTION

Morphogenesis in plants is controlled by the rate and orientation of cell division and by cell expansion. Plant somatic cell division and in particular cytokinesis differ in some aspects from their counterparts in other organisms (Field et al., 1999). One major difference is that plant cells lack the contractile ring that assists in partitioning the cytoplasm from the surface in most animal cells, and instead construct a partitioning cell plate inside the cell. Two plant-specific microtubule structures, the preprophase band (PPB) and the phragmoplast, play central roles in the orientation and expansion of the cell plate as well as in the execution of cytokinesis (Otegui and Staehelin, 2000; Jürgens, 2005). The cortical position of the transient PPB correlates with the site at which the outward growing cell plate will fuse with the parental plasma membrane at the end of cytokinesis. The phragmoplast assembles in the center of the division plane during anaphase-to-telophase transition. During cytokinesis, secretory vesicles carrying membrane and cell wall components are guided along the phragmoplast to the division plane and form, by fusion, a membraneous compartment enclosing the immature wall, the cell plate. Phragmoplast and cell plate expand laterally until the cell plate fuses with the parental plasma membrane and cell wall.

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Relatively few genes involved in cytokinesis have been identified by mutation in plants, and they fall into two classes. Genes in the first class are required for proper orientation of the division plane, and mutants include *fass/tonneau* in *Arabidopsis*, and *discordia* and *tangled* in maize (Sylvester, 2000; Smith, 2001). Genes in the second class are required for the execution of cytokinesis. Mutations in these genes cause defects in cell plate formation, leading to a common phenotype that is characterized by the formation of multinucleate cells with gapped cell walls or cell wall stubs (Assaad et al., 1996; Yang et al., 1999; Strompen et al., 2002; Lukowitz et al., 1996; Falbel et al., 2003). Genes identified from these mutations encode proteins that are involved in cell plate membrane fusion (Assaad et al., 2001; Lukowitz et al., 1996), biogenesis of primary cell wall (Zuo et al., 2000; Lukowitz et al., 2001) or microtubule array formation or dynamics (Strompen et al., 2002; Soyano et al., 2003; Müller et al., 2004). *KNOLLE* (*KN*) and *KEULE* (*KEU*) were initially identified as mutants that are lethal at the seedling stage and which have an abnormal seedling body organization (Mayer et al., 1991). *KN* encodes a cytokinesis-specific, plant-specific syntaxin, whereas *KEU* encodes a Sec1/Munc18 protein that may regulate syntaxin function (Lukowitz et al., 1996; Assaad et al., 2001). *HINKEL* (*HIK*) is required for expansion of the phragmoplast during cell plate formation, and encodes a plant-specific kinesin-like protein (Strompen et al., 2002). *HIK* is the *Arabidopsis* ortholog of tobacco *NACK1*, which binds to the cell plate-associated mitogen-activated kinase (MAPK) kinase kinase, NPK1, and activates a MAPK pathway regulating phragmoplast expansion (Nishihama et al., 2002).

Transcripts of *NACK1* and mitotic B1-type cyclin (*CYCB1*) accumulate specifically during late G2- and M-phases in synchronized cultures of tobacco BY-2 cells (Ito et al., 2001). Transcription of these genes is regulated by a common upstream *cis*-acting element, called MSA (mitosis-specific activator) (Ito et al., 1998; Ito, 2000). A group of Myb transcription factors in tobacco, NtmybA1, NtmybA2 and

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NtmybB, bind to the MSA motif in vitro and in yeast. NtmybA1 and NtmybA2 are structurally closely related transcriptional activators, whereas NtmybB acts as a competitive repressor in tobacco cells (Ito et al., 2001; Araki et al., 2004). Plants have a large number of Myb genes, most of which encode R2R3-Myb proteins containing two tandemly repeated sequences of ~50 amino acids in the Myb domain and control diverse developmental processes (Stracke et al., 2001). By contrast, NtmybA1, NtmybA2 and NtmybB are R1R2R3-Myb proteins containing three tandemly repeated sequences and thus are structurally similar to Myb proteins in vertebrates and *Drosophila* (Ito, 2005). Mammalian Myb proteins were generally believed to play a role in cell-cycle regulation, particularly at the G1/S transition (Lipsick, 1996). According to more recent studies, however, Myb proteins may also play a role in the transcription of the *cyclin B* gene in *Drosophila* (Okada et al., 2002), zebrafish (Shepard et al., 2005), and human cells (Zhu et al., 2004). Thus, G2/M phase-specific transcription appears to be mediated by R1R2R3-Myb proteins in a wide range of evolutionarily distant organisms.

The *Arabidopsis* genome contains five genes that encode R1R2R3-Myb proteins (Stracke et al., 2001). Genome-wide expression analysis of synchronized *Arabdidopsis* cells revealed G2/M phase-specific expression of 82 genes, which include *CYCB1*, *CYCB2*, *CDC20.1*, *KN* and *AtNACK1/HIK* (Menges et al., 2005). All but 17 of these genes contain at least one MSA element, suggesting that MSA-binding R1R2R3-Myb proteins may regulate many G2/M phase-specific genes and thereby control progression of mitosis and cytokinesis. Here, we analyze the in vivo function of two closely related *Arabidopsis* R1R2R3-Myb proteins, MYB3R1 and MYB3R4 (also known as MYB3R-1 and MYB3R-4), which are homologous to tobacco transcriptional activators NtmybA1 and NtmybA2. We isolated plants with mutations in the *MYB3R1* and *MYB3R4* genes and characterized the phenotype of single and double mutants. The *myb3r1 myb3r4* double mutant showed characteristic cytokinesis defects during embryogenesis and post-embryonic development. Our genetic and molecular studies suggest that *MYB3R1* and *MYB3R4* have partially overlapping function and quantitatively promote cytokinesis, mainly through transcriptional activation of the *KN* gene.

MATERIALS AND METHODS

Plant materials and growth conditions

The *Arabidopsis* ecotype Columbia (Col) was used as wild type. Seeds with T-DNA insertion mutations in *MYB3R1* (At4g32730) and *MYB3R4* (At5g11510) from the SALK collection (Alonso et al., 2003) were obtained from the *Arabidopsis* Biological Resource Center (ABRC): SALK_018482 (*myb3r1-1*), SALK_059819 (*myb3r4-1*) and SALK_034806 (*myb3r4-2*). The *kn* allele X37-2 (Ler background) (Lukowitz et al., 1996) was used for genetic crosses and for transformation. Plants were grown either on soil or agar medium at 22°C under continuous illumination (70-120 μmol/m^2/second). Standard agar growth medium contained 0.5×MS salts, 2% w/v sucrose and 0.7% w/v agar.

Arabidopsis cells and synchronization

The MM2d cell line (*Arabidopsis* Ler ecotype) (Menges and Murray, 2002) was obtained from Bayer BioScience N.V. (Gent, Belgium). Maintenance and synchronization were performed essentially as described for tobacco BY-2 cells (Nagata et al., 1992; Ito et al., 1997). Briefly, cells were grown in modified Linsmaier and Skoog medium at 27°C in the dark, with rotation at 130 rpm. For subculturing, 3 ml of saturated culture were transferred into 95 ml fresh medium. For synchronization, 15 ml of 7-day-old culture were transferred into 95 ml fresh medium containing 5 mg/l aphidicolin (Wako, Japan). Cells were treated with aphidicolin for 24 hours, washed with fresh medium and then cultured in 100 ml medium. The mitotic index was determined as described (Ito et al., 1997).

Generation of transgenic lines

Promoter regions of *CDKA;1* (1.9 kb) and *RPS5A* (1.6 kb) were amplified by PCR from Col genomic DNA and cloned in the binary vector pPZP211 (Hajdukiewicz et al., 1994). Primers used for PCR were 5′-AAG-AGCTCAATTCCTGAATAATAAAGCTGAAG-3′ and 5′-AACTGC-AGTTACAACTGATAACCGTATAGCTC-3′ for *CDKA;1*, and 5′-AA-CTGCAGTTGATTCGCTATTTGCAGTGCAC-3′ and 5′-AAACAG-AGCGTGAGCTCAAATAC-3′ for *RPS5A*. For construction of the *CDKA;1::KN* and *RPS5A::KN* vectors, *KN* full-length cDNA sequence was placed downstream of the promoters. These constructs were introduced by floral-dip transformation (Clough and Bent, 1998) into *Arabidopsis* lines that were homozygous for *myb3r4-1* and heterozygous for *myb3r1-1*. We selected T2 plants that were homozygous for *myb3r1-1*, *myb3r4-1*, and also for the transgene. Similarly, we prepared *CDKA;1::MYB3R1* and *CDKA;1::MYB3R4* vectors and obtained transgenic plants that were homozygous for the transgene in the *myb3r1 myb3r4* background.

For constructing β-glucuronidase (GUS) reporter constructs, promoter regions of *MYB3R1* (1.1 kb) and *MYB3R4* (1.2 kb) were amplified by PCR and cloned upstream of the *GUS* gene in pPZP211 binary vector. Primers used for PCR were 5′-AACTGCAGTATTAGCCAATGAAGGTGGAC-TAGC-3′ and 5′-AAGTCGACAATTAAGACGCTGAGAATCCAGATG-3′ for *MYB3R1*, and 5′-AACTGCAGAGTCGGTAACATTCTGCC-AGAGATG-3′ and 5′-AAGTCGACGAGCTTCAGAAATGGAAGTGG-TTC-3′ for *MYB3R4*. The resulting constructs, *MYB3R1::GUS* and *MYB3R4::GUS*, were transformed into Col plants. Multiple lines were analyzed and had similar staining patterns.

Two *KN* promoter-deletion constructs were generated. *KNΔMSA1* was amplified by PCR from the *Sac*I/*Eco*RI *KN* expression construct (Völker et al., 2001) with the primer pair 5′-GTAATACGACTCACTATAGGGC-3′ and 5′-AAAGAATTCAAATATAGCCGTTGGGGCG-3′ and cloned in pGII BASTA. To generate the *KNΔMSA2* construct, the two regions flanking the two proximal MSA elements in the *KN* promoter were amplified by PCR with the following primer pairs: (1) 5′-TCTAGACCCGGGTTTC-TCCTTTTTCTTATATTAGAAAGAAAGC-3′ and 5′-GAATTCCCTG-CTCCCATATCCTTCATCG-3′ and (2) 5′-GAATTCTCAAGAAGAG-CTGAAACTGGTAATG-3′ and 5′-CACTGCGATTCTCTCTGATTC-3′. The PCR fragments were digested with *Xba*I and *Xba*I/*Eco*RI, respectively, and cloned in the *KN* genomic rescue fragment (Müller et al., 2003). Floral-dip transformation of *kn-X37-2* heterozygous plants and selection for BASTA resistance were performed as described (Müller et al., 2003). Transgenic plants were analyzed for rescue of the *kn* seedling phenotype.

Western-blot analysis

Western blots were performed as described (Lauber et al., 1997). The anti-KN serum (Lauber et al., 1997) was used at 1:6000. Myc-tagged KN protein expressed from the transgenes *KNΔMSA1* and *KNΔMSA2* was detected with the mouse anti-c-myc monoclonal antibody 9E10 (Santa Cruz Biotechnology) at 1:250.

Protoplast transfection assay

For construction of luciferase (LUC) reporter plasmids, promoter regions of *CYCB1;1* (1.1 kb), *CYCB1;2* (0.7 kb), *CDC20.1* (0.9 kb), *AtNACK1/HIK* (1.4 kb) and *KN* (2.2 kb) were amplified by PCR. Primers used for PCR were 5′-AACTGCAGAAGCTTACAATTGTGTGGGAACCATAGC-3′ and 5′-AAGTCGACTCTCTCAGACTAAAATCTCAGG-3′ for *CYCB1;1*, 5′-CACCTTCAGATGATAGTGTACTCAC-3′ and 5′-TTCTCTTTCG-TAAAGAGTCTCTGCG-3′ for *CYCB1;2*, 5′-AACTGCAGTGAAGA-ACATGCTTATCACACGTC-3′ and 5′-AAGTCGACAAGCTAGCGA-AGAGGGAATCGTTC-3′ for *CDC20.1*, 5′-CACCACAGACTGAAA-GCGACTTGATAGT-3′ and 5′-TCAGGCAGCTAAGAATGTAGAATC-3′ for *AtNACK1/HIK*, and 5′-CACCAGGAAAAATTAGCTTCACGAG-3′ and 5′-CATAAACGATTTCGTCATCAAGTC-3′ for *KN*. The amplified fragments were inserted upstream of the *LUC* reporter gene in pUC18. For mutagenesis of the MSA elements, a PCR fragment of the *KN* promoter was cloned in pENTR-/D-TOPO (Invitrogen, Carlsbad, CA). Using this plasmid as a template, PCR was performed with the primer pair 5′-CCCTGC-AGCTATATTTTGGCGATGTATCCAATGTCGTC-3′ and 5′-AGCTGC-

AGGGGCGAAGAAATCACAGTATTGGCTAATTTC-3′. The amplified fragment, containing the entire vector sequence, was cut by *Pst*I and then circularized by end-end ligation. The resulting plasmid contained *KN* promoter fragments with all three MSA elements being mutated (*KN*Δ*MSA*), which was then subcloned upstream of the *LUC* reporter gene in pUC18. Expression plasmids, 35S::MYB3R1 and 35S::MYB3R4, were constructed by inserting full-length cDNA sequences downstream of the double cauliflower mosaic virus (CAMV) 35S promoter in pJIT60 (Guerineau and Mullineaux, 1993). The 35S::CYCB1 expression plasmid, isolation of tobacco BY-2 protoplasts, transfections and LUC activity assays were described previously (Araki et al., 2004).

Microscopic analysis

Cells were observed with differential interference contrast (DIC) and fluorescence optics (Olympus BX54 microscope). Seedlings and ovules were fixed in an ethanol:acetic acid (9:1) solution, cleared overnight in Hoyer's solution (a mixture of 20 g of chloral hydrate, 1 ml of glycerol, 6 ml of water and 1.5 g of gum arabic) and viewed with DIC. Epidermal peels were obtained from silique valves using tweezers, mounted in Hoyer's solution and imaged by DIC. For Toluidine Blue staining, epidermal peels were immersed in a solution containing 0.1% w/v Toluidine Blue O and 50 mM sodium phosphate (pH 7.0), rinsed with water and mounted in Hoyer's solution. For DAPI staining, epidermal peels were fixed in 2% glutaraldehyde, mounted in a solution containing 1 μg/ml DAPI, 1% Triton X-100 and 2% glutaraldehyde, and viewed with fluorescence optics.

Real-time RT-PCR

RNA was extracted from inflorescences containing young flower buds or MM2d cells using Trizol reagent according to the manufacturer's instructions (Invitrogen). Poly(dT) cDNA synthesis was performed using a SuperScript First-Strand Synthesis System for RT-PCR according to the manufacturer's instructions (Invitrogen). Quantification was performed on a Light Cycler 1.5 system (Roche, Mannheim, Germany) using the SYBR Premix EX Taq (Takara Biochemicals, Japan). Thermal cycling conditions were as follows: 10 seconds at 95°C, and then 40 cycles of 5 seconds at 95°C, 20 seconds at 60°C. For gene expression analysis in wild type and mutants, three replicate assays were performed with RNA isolated from distinct individuals. Results were normalized to the expression of *ACT2* mRNA. Information on the primer sets used for real-time RT-PCR is available upon request.

RESULTS

Arabidopsis R1R2R3-Myb genes

Three tobacco genes, *NtmybA1*, *NtmybA2* and *NtmybB*, encode R1R2R3-Myb transcription factors that bind to MSA elements (Ito et al., 2001). The *Arabidopsis* genome contains five related but functionally uncharacterized genes, *MYB3R1-MYB3R5* (Stracke et al., 2001), and there are four genes encoding R1R2R3-Myb proteins in the rice genome (The TIGR rice database, http://rice.tigr.org/). By phylogenetic analysis of the conserved DNA-binding domains, *Arabidopsis* MYB3R1 and MYB3R4 form a clade together with NtmybA1, NtmybA2 and two rice proteins (Fig. 1A). That these Myb proteins might perform an identical biological function is also suggested by two stretches of conserved amino acid sequences in their C-terminal half (Fig. 1B). Another clade comprised MYB3R3, MYB3R5 and two rice proteins but no previously characterized Myb protein, whereas *Arabidopsis* and rice appear to lack orthologs of the tobacco NtmybB transcriptional repressor (Fig. 1A).

Mutations affecting *MYB3R1* and *MYB3R4* genes

To examine the role of the *Arabidopsis R1R2R3-Myb* genes *MYB3R1* and *MYB3R4* in cell cycle and development, we characterized T-DNA insertion mutants from the SALK collection (Alonso et al., 2003). The T-DNA insertions of *myb3r4-1* and *myb3r4-2* were close to each other in the second exon (Fig. 1C). The *myb3r4-1* insertion occurs within the open reading frame N-terminal

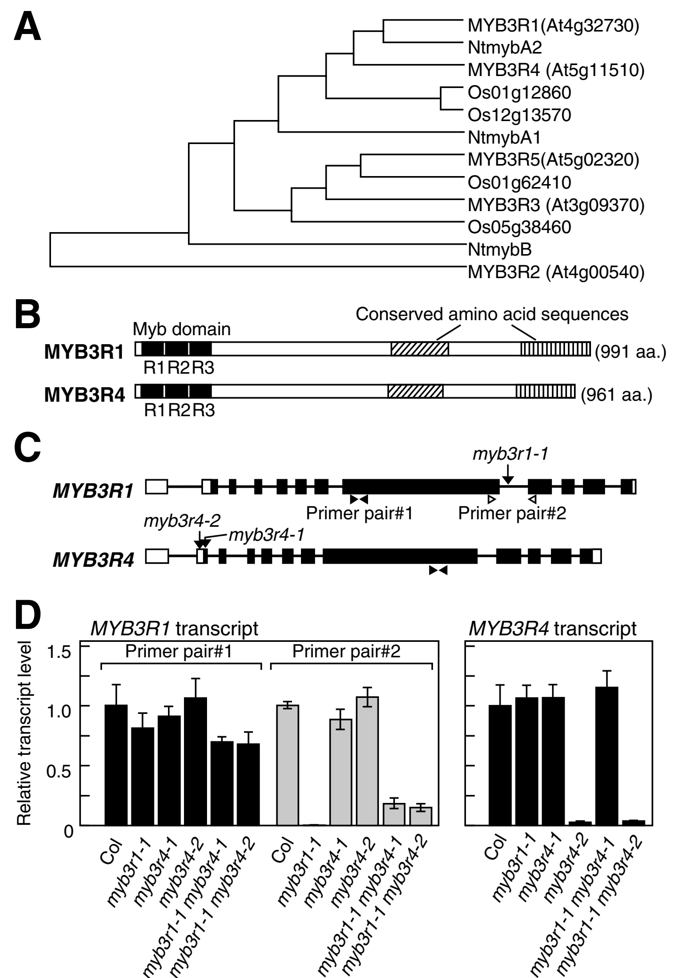

Fig. 1. Molecular characterization of MYB3R1 and MYB3R4. (**A**) Phylogenetic relationship of R1R2R3-Myb proteins in plants. The phylogenetic tree was constructed based on the amino acid sequence similarities in the Myb domains of R1R2R3-Myb proteins from tobacco (NtmybA1, NtmybA2 and NtmybB), *Arabidopsis* (MYB3R1-MYB3R5) and rice (Os01g12860, Os12g13570, Os01g62410 and Os05g38460). (**B**) Protein structure of MYB3R1 and MYB3R4. The Myb domain is located in the N-terminus and contains tandemly repeated sequences that are designated R1, R2, and R3 (black boxes). Two separate regions in the C-terminal half show sequence similarity to their homologs in tobacco and rice (hatched boxes). (**C**) Position of T-DNA insertions in *MYB3R1* and *MYB3R4*. Boxes and lines represent exons and introns, respectively. Arrows above the boxes indicate positions of T-DNA insertions. Black and white boxes correspond to coding and non-coding regions, respectively. Arrowheads indicate the positions of primers used for quantitative RT-PCR. (**D**) Transcript levels of *MYB3R1* and *MYB3R4* in wild-type and T-DNA insertion mutants. Levels of each transcript were determined by real-time quantitative RT-PCR using the primer pairs shown in C.

to the Myb domain and causes an 18-bp deletion, impairing protein function (see below). The *myb3r4-2* insertion is located 35 bp upstream of the initiation codon. However, this mutation reduced the level of *MYB3R4* transcript to less than 3% of the wild-type level (Fig. 1D). The T-DNA of *myb3r1-1* is inserted in the eighth intron of *MYB3R1*, which is flanked by exons encoding conserved amino acid sequences (Fig. 1C). Quantitative RT-PCR analyses with two different primer pairs indicated a normal level of *MYB3R1* mRNA 5′ to the insertion site [Fig. 1C, primer pair (1)] but no mRNA with

Table 1. Ectopic expression of *KN* partially rescues cytokinesis defects in *myb3r1 myb3r4* plants

		Genotype (transgene)				
		myb3r1 myb3r4 (no transgene)	*myb3r1 myb3r4* (*CDKA;1::KN*)		*myb3r1 myb3r4* (*RPS5A::KN*)	*myb3r1 myb3r4* (*CDKA;1::MYB3R4*)
	n		Line 3	Line 10		
Cytokinesis-defective stomata (%)*	5	19.3±5.4	6.1±1.4	7.5±2.7	14.3±2.3	0±0
Epidermal cells with gapped cell wall (%)†	5	4.4±1.6	0.11±0.15	0±0	0±0	0±0
Single-celled embryo with multiple nuclei (%)	⩾184	25.1	0.92	1.09	0	0

myb3r1 myb3r4 plants that are homozygous for each transgene were measured.
*Frequency of cytokinesis-defective stomata was determined in outer epidermis of silique valves. More than 200 stomata were analyzed in each plant.
†Frequency of epidermal cells with gapped wall was determined in outer epidermis of silique valves. More than 200 epidermal cells were analyzed in each plant.

proper splicing around the insertion site [Fig. 1C, primer pair (2)], suggesting that *myb3r1-1* transcript encodes a truncated protein that lacks conserved sequences (Fig. 1D). The *myb3r1-1 myb3r4-1* and *myb3r1-1 myb3r4-2* double mutants, however, expressed low levels (~20% of wild-type level) of properly spliced *MYB3R1* transcript, which may encode wild-type protein (Fig. 1D). In summary, the T-DNA insertions appear to represent loss-of-function alleles of *MYB3R1* and *MYB3R4* genes, although they may still allow for some residual activity, especially of MYB3R1 in the double mutant.

myb3r1 and *myb3r4* single mutants displayed no macroscopic defect, although *myb3r4* plants had very weak cellular defects of cytokinesis, to be described later. The *myb3r1-1 myb3r4-1* and *myb3r1-1 myb3r4-2* double mutants showed essentially identical phenotypes. Most of these plants were viable and developed into mature, fertile plants, although some macroscopic abnormalities were found in a subpopulation, which include abnormal seedling morphology and impaired shoot elongation. In addition, we found that all the double mutant individuals had cellular defects in cytokinesis. The defective cytokinesis was fully or partially complemented by transgenes that expressed MYB3R1 or MYB3R4 from the *CDKA;1* promoter (data not shown, see Table 1), supporting that the T-DNA insertions inactivate the *MYB3R1* and *MYB3R4* genes at least partially. All experiments reported below were done with the *myb3r1-1 myb3r4-1* double mutant, whose phenotype was slightly stronger than that of the *myb3r1-1 myb3r4-2* double mutant.

Defective cytokinesis in *myb3r1 myb3r4* plants

Defective cytokinesis was found in the *myb3r1 myb3r4* embryo, most frequently during the first or second division after fertilization. These embryos were rod- or club-shaped and contained multiple nuclei that were not separated by cell walls (Fig. 2B, compare with wild type shown in Fig. 2A). This phenotype may have resulted from multiple rounds of nuclear division without cytokinesis. In support of this interpretation, we frequently observed embryos in which all nuclei progressed synchronously through metaphase (Fig. 2C). A similar defective embryo phenotype has been reported for the *kn keule* double mutant in which somatic cytokinesis is completely blocked (Waizenegger et al., 2000). The most notable defects of cytokinesis were found in stomata, as reported for other cytokinesis-defective mutants (Falbel et al., 2003; Söllner et al., 2002; Yang et al., 1999). In normal stoma development, a guard mother cell divides symmetrically to produce two guard cells whose closely apposed common cell walls then partially detach from one another to form the pore (Fig. 2D). Stomata were irregularly shaped in the double mutant and thus similar to those of other cytokinesis-defective mutants, lacking the ventral wall or containing cell wall stubs (Fig. 2E-G). The surrounding guard cell was binucleate, which suggests that these abnormal stomata resulted from a failure of the guard mother cell to undergo cytokinesis (Fig. 2I, compare with the wild-type stoma shown in Fig. 2H). Cytokinesis-defective stomata were detected in most organs at similar frequencies of 20-30%, including cotyledons, cauline leaves, inflorescence stems, silique valves and sepals, but were almost absent from rosette leaves (Fig. 2N). Gapped cell walls and cell wall stubs were also observed in epidermal cells of most organs, except rosette leaves (Fig. 2J-M). Thus, the requirement of the *MYB3R1* and *MYB3R4* genes for cytokinesis may vary between organs and cell types.

The gene dosage of *MYB3R1* had a quantitative effect on the severity of the cytokinesis defect in *myb3r4* homozygous plants. Cytokinesis-defective stomata were much less frequent in the *myb3r4* single mutant than in the *myb3r1 myb3r4* double mutant (1.1% versus 22.1% in the outer epidermis of silique valve), whereas *myb3r1/MYB3R1 myb3r4/myb3r4* plants, with just one copy of *MYB3R1*, showed an intermediate frequency of 8.7% defective stomata. The observed severity of the cytokinesis defects in each genotype was inversely correlated with the abundance of properly spliced *MYB3R1* transcript (data not shown). By contrast, neither the *myb3r1* single mutant nor *myb3r1/myb3r1 myb3r4/MYB3R4* plants displayed abnormal stomata, suggesting that *MYB3R4* makes a larger contribution to cytokinesis than does *MYB3R1*, although cytokinesis is promoted by the combined level of expression of the two functionally redundant *Myb* genes, *MYB3R1* and *MYB3R4*.

myb3r1 myb3r4 plants have decreased expression of G2/M phase-specific genes

MYB3R1 and MYB3R4 are homologous to transcriptional activators NtmybA1 and NtmybA2 in tobacco, which activate G2/M phase-specific genes *CYCB1* and *NACK1* through binding to their MSA elements (Ito et al., 2001). To examine whether MYB3R1 and MYB3R4 have similar function, we compared mRNA levels of several cell cycle-related genes between *myb3r1 myb3r4* and wild-type plants, analyzing RNA from inflorescences and young flower buds by quantitative RT-PCR (Fig. 3A). No changes in abundance were detected for *CDKA;1*, *histone H4*, *CYCD3;1*, *CYCA2;2* and *CYCA3;1* genes, which lack MSA motifs and are expressed constitutively or before G2/M in synchronized *Arabidopsis* MM2d cells (Menges et al., 2003). Potential target genes of MYB3R1 and MYB3R4 should contain MSA motifs and show G2/M phase-specific expression in synchronized cells. The transcript levels of five such genes, *CYCA1;1*, *CYCB2;1*, *CYCB1;2*, *CYCB1;4* and *CDC20.1*, were differentially reduced, whereas the mRNA level of *CYCB1;1* was slightly increased (Fig. 3A).

The severe cytokinesis defects in *myb3r1 myb3r4* double mutants suggested some genes that have essential functions in cytokinesis and are upregulated during G2/M phase, such as *AtNACK1/HIK*,

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PLEIADE (*PLE*)*/MAP65-3*, *ANQ1* and *KN*, might be downregulated (Menges et al., 2003). Although all these genes contain MSA motifs in their promoter regions, transcript abundance for *AtNACK1/HIK*, *PLE/MAP65-3* and *ANQ1* was unchanged or even increased in the double mutant (Fig. 3A). By contrast, the level of *KN* mRNA was decreased dramatically to approximately 30% of the wild-type level.

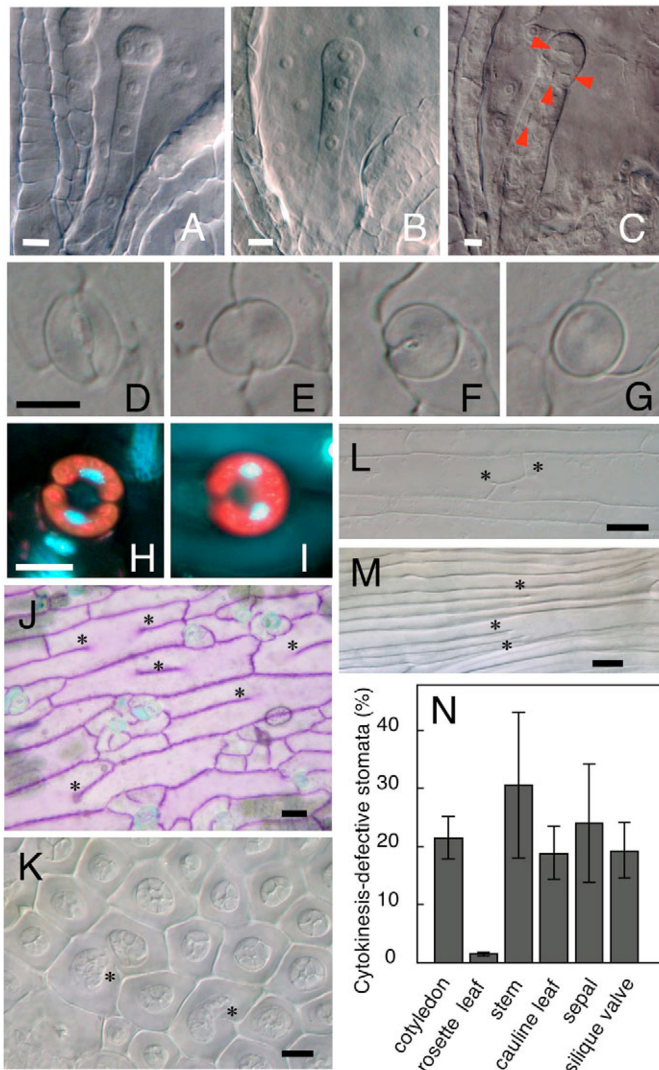

Fig. 2. Defective cytokinesis in *myb3r1 myb3r4* plants. (**A**) Wild-type proembryos at the two-cell stage. (**B**) Rod-shaped *myb3r1 myb3r4* embryo with multiple nuclei that were not separated by internal cell walls. (**C**) *myb3r1 myb3r4* embryo without internal cross walls, containing multiple metaphase chromosomes (arrowheads). (**D**) Normally developed stoma in wild-type silique valve. (**E-G**) Cytokinesis-defective single-celled stomata in *myb3r1 myb3r4* silique valves. (E) Stoma consists of single guard cell with two internal cell wall stubs, lacking a detectable pore. (F) Stoma with incomplete internal cell wall that forms a small pore. (G) Stoma without internal pore-forming cell wall. (**H**,**I**) DAPI-stained stomatal nuclei in wild-type (J) and *myb3r1 myb3r4* (K) silique valve. Note two nuclei in single-celled stoma of *myb3r1 myb3r4*. Red, autofluorescence of chlorophyll. (**J-M**) Frequent occurrence of cytokinesis-defective cells with gapped cell walls and cell wall stubs (asterisks) in various organs of *myb3r1 myb3r4* plants. (J) Toluidine Blue-stained outer epidermis of silique valve. (K) Outer integument of ovule. (L) Epidermis of filament in stamen. (M) A longitudinally elongated layer of chlorenchyma in valve of silique. (**N**) Frequency of cytokinesis-defective stomata in various organs of *myb3r1 myb3r4* plants, as determined by counting normal and defective stomata in the epidermis of each organ. A-G,K-M are DIC images. Scale bars: 10 μm in A-D,H; 20 μm in J-M.

Since the gene dosage of *MYB3R1* in the *myb3r4* mutant background influenced the severity of cytokinesis defects, we examined whether it also affected the expression of potential target genes *KN* and *CYCB2;1* (Fig. 3B). No changes were detected in the *myb3r1* single mutant, whereas transcript levels of both *KN* and *CYCB2;1* were significantly reduced in the *myb3r4* single mutant, even more strongly in the *myb3r1 myb3r4* double mutant, and to intermediate levels in *MYB3R1/myb3r1 myb3r4/myb3r4* plants. Thus, the two Myb proteins MYB3R1 and MYB3R4 may quantitatively activate transcription of G2/M phase-specific genes, with MYB3R4 making the larger contribution.

Genetic interaction between *MYB3R1*, *MYB3R4* and *KN*

To analyze the genetic relationship between KN and the two regulators, we crossed the *myb3r1 myb3r4* double mutant with the *kn* heterozygote to generate F2 progeny with different genotypes at those three loci. An initial survey of 48 F2 plants with cytokinesis defects in the epidermis of silique valves revealed that all these plants were homozygous for *myb3r4*, indicating that a single copy each of the

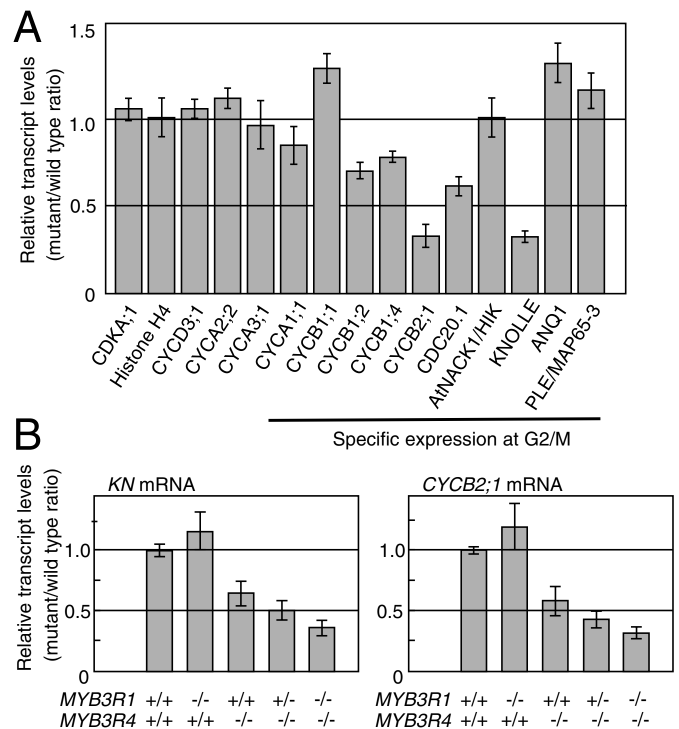

Fig. 3. Mutations in *MYB3R1* and *MYB3R4* genes affect the abundance of G2/M phase-specific transcripts. (**A**) Relative transcript levels of genes associated with cell-cycle regulation and cytokinesis in the *myb3r1 myb3r4* plants. G2/M phase-specific genes that contain MSA elements are indicated by the black bar. (**B**) Relative transcript levels of *KN* and *CYCB2;1* genes in plants with various allele combinations of *MYB3R1* and *MYB3R4*: +/+, homozygous for wild-type allele; +/–, heterozygous; –/–, homozygous for mutant allele. All measurements in A and B were performed on inflorescences containing young flower buds. Expression levels were determined by real-time quantitative RT-PCR and normalized relative to those in wild-type plants. Columns represent mean values; error bars represent s.d.

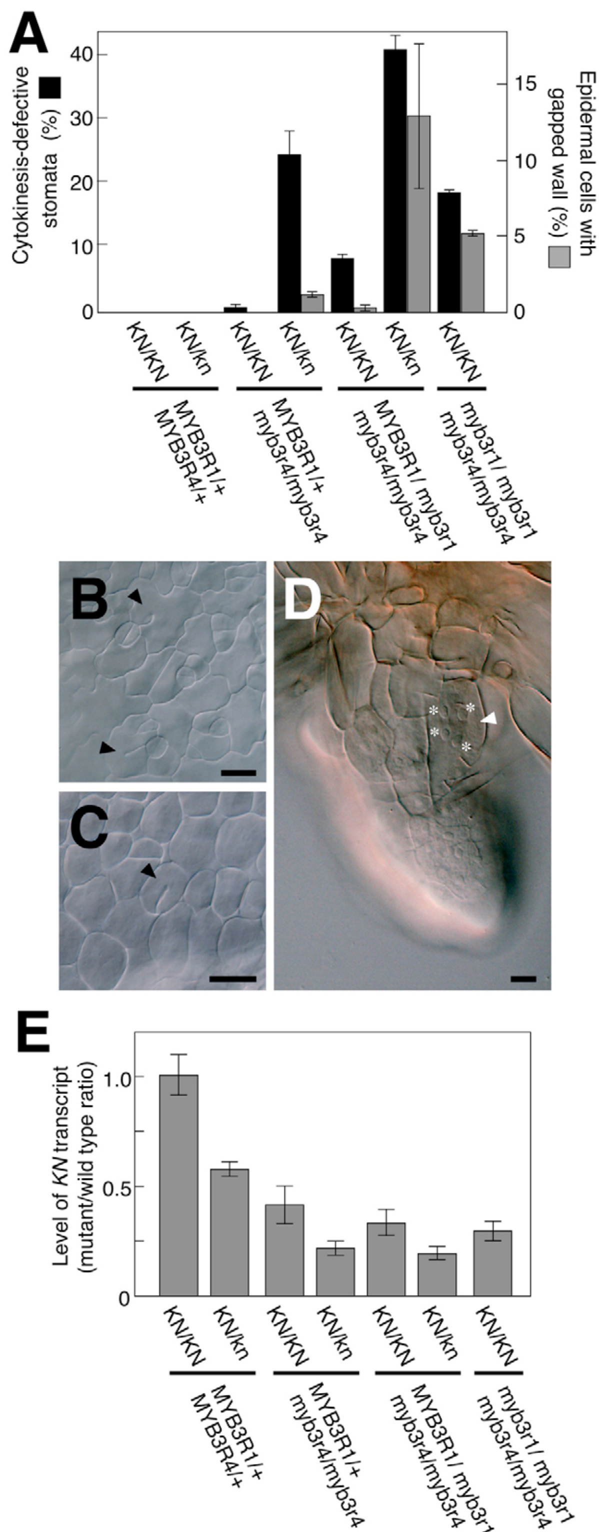

Fig. 4. Genetic interaction between *myb3r1*, *myb3r4* and *kn*. (**A**) Effects of heterozygous mutation of *kn* on the severity of cytokinesis defects. Frequency of cytokinesis-defective stomata and epidermal cells were determined in the silique valve of the plants with the indicated genotypes. Data for *myb3r1/myb3r1 myb3r4/myb3r4 KN/kn* plants are not available because these plants do not produce siliques. Columns represent mean values; error bars represent s.d. (**B-D**) Defective cytokinesis in *myb3r1/myb3r1 myb3r4/myb3r4 KN/kn* plants. (B) Pavement epidermal cells and (C) palisade cells in rosette leaves, showing cell wall stubs (arrowheads). (D) Enlarged cells (arrowhead) with multiple nuclei (asterisks) in root epidermal cells. Scale bars in B-D, 20 μm. (**E**) Transcript levels of *KN* in plants with various allele combinations of *MYB3R1*, *MYB3R4* and *KN* genes. All measurements were performed on inflorescences containing young flower buds in mature plants. Expression levels were determined by real-time quantitative RT-PCR and normalized relative to those in wild-type plants. Columns represent mean values; error bars represent s.d.

wild-type alleles of *MYB3R4* and *KN* are sufficient for normal cytokinesis. For a detailed analysis, we genotyped 96 F3 individuals derived from self-fertilized F2 plants that were homozygous for *myb3r4*, but heterozygous for *myb3r1* and *kn*. The severity of cytokinesis defects as represented by the frequency of abnormal stomata in the outer epidermis of silique valve was dramatically increased in both *myb3r4* single mutant and *MYB3R1/myb3r1* *myb3r4/myb3r4* background by the heterozygous mutation of *kn* (Fig. 4A). There was also a comparable increase in the frequency of epidermal cells of silique valves that had gapped cell walls or cell wall stubs (Fig. 4A), suggesting that KN becomes limiting when the level of activating Myb transcription factor is reduced.

Genotyping of 96 F3 plants suggested that the heterozygous mutation of *kn* may dramatically increase the frequency of embryonic lethality of *myb3r1 myb3r4*. One surviving plant of this genotype was tiny and produced a few small curly leaves, and subsequently stopped growing entirely. We identified a further eight such plants among ~500 F3 plants, and examined their phenotype. In the rosette leaves of these plants, the frequency of cytokinesis-defective stomata was much higher than that observed in the *myb3r1 myb3r4* plants (45.2% versus 1.5%, *n*=5). In addition, gapped cell walls were frequently found in epidermal pavement cells and palisade cells of rosette leaves when plants had the heterozygous mutation of *kn* in the *myb3r1 myb3r4* background (Fig. 4B,C). Enlarged cells with multiple nuclei were also observed in roots of this genotype but not in the absence of the heterozygous mutation of *kn* (Fig. 4D).

kn heterozygotes on their own did not show any phenotype of defective cytokinesis (Fig. 4A). Thus, the observed genetic interaction between *KN* and the two *Myb* genes was not simply because of additive effects of each mutation. Rather, the reduced copy number of functional *KN* gene might have further decreased the already reduced level of *KN* gene expression in the *myb3r1 myb3r4* double mutant, leading to enhanced defects of cytokinesis. To test this idea, we analyzed transcript abundance of *KN* in the F3 individuals (Fig. 4E). The relative decrease in *KN* mRNA level in each genotype was roughly correlated with the frequency of cytokinesis-defective stomata and epidermal cells (compare Fig. 4E with Fig. 4A), supporting the notion that inactivation of *MYB3R1* and *MYB3R4* genes causes reduced *KN* gene expression, which, in turn, leads to defective cytokinesis.

Ectopic expression of *KN* rescued the cytokinesis defects of *myb3r1 my3r4* plants

To test whether enhanced *KN* gene expression could rescue the cytokinesis defects of *myb3r1 myb3r4* plants, we generated transgenic plants that expressed KN protein from heterologous promoters. Expression of KN protein from the *CaMV 35S* promoter failed to rescue the cytokinesis-defective *kn* mutant embryo, which was probably because of weak activity of the *CaMV 35S* promoter

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in mitotically dividing cells (Völker et al., 2001). We tested the promoters of the *CDKA;1* and *RPS5A* genes, which are active in proliferating tissues (Hemerly et al., 1993; Weijers et al., 2001). We confirmed that *kn* homozygous mutant seeds were partially rescued by the *CDKA;1::KN* and *PRS5A::KN*. *CDKA;1::KN* transgenic plants that were homozygous for *kn* developed past the seedling stage and produced a few small leaves, before growth ceased at the vegetative stage. The *RPS5A::KN* transgene rescued *kn* mutant plants more effectively, resulting in the formation of fertile flowers. Introduction of these transgenes into the *myb3r1 myb3r4* double mutant also partially rescued their cytokinesis defects, significantly reducing the frequency of cytokinesis-defective cells, which include abnormal stomata, epidermal cells with gapped cell walls and the multinucleate single-celled embryos without cross walls (Table 1).

MYB3R1 and MYB3R4 activate transcription of *KN*

The *KN* gene contains three MSA motifs within 59 bp of its promoter region (Fig. 5A). To evaluate the function of these MSA motifs in *KN* gene expression, we analysed different promoter deletion constructs of a genomic rescue clone of *KN* (Müller et al., 2003). The promoter deletion construct *KNΔMSA1* (Fig. 5A) retaining a fragment up to –286 bp from the *KN* start codon was sufficient for normal expression (Fig. 5B) and rescuing activity of Myc-tagged KN protein (data not shown). Because this promoter fragment still contained two MSA motifs with high homology to the consensus sequence TC(T/C)AACGG(T/C)(T/C)A (Ito, 2000), we deleted these two MSA motifs from the Myc-KN rescue construct by replacing them with two additional restriction sites (Fig. 5A). This mutant construct, *KNΔMSA2*, did not rescue *kn* mutant plants (data not shown). The *KNΔMSA2* construct encoded a Myc-tagged KN protein (Fig. 5A), which can be distinguished from protein encoded by endogenous *KN* gene. In transgenic plants carrying the *KNΔMSA2*, Myc-KN expression was reduced below detection level, indicating that MSA-dependent activation of *KN* expression is essential for KN activity in cytokinesis (Fig. 5C).

To directly demonstrate that MYB3R1 and MYB3R4 activate the *KN* gene promoter, we performed co-transfection assays in which the Myb transcription factor genes and a fusion between the *KN* promoter and the *LUC* reporter gene were introduced into tobacco BY-2 protoplasts. We have used this system previously to demonstrate that transfection of an NtmybA2 expression plasmid resulted in increased activity of reporter constructs such as CYCB1::LUC and NACK1::LUC fusions (Ito et al., 2001). We have further shown that this transactivation activity of NtmybA2 was dramatically increased by co-transfection of 35S::CYCB1, possibly because the activity of NtmybA2 was enhanced upon phosphorylation by the cyclin/CDK complex (Araki et al., 2004). Expression of the KN::LUC reporter construct was stimulated approximately twofold by co-transfection of 35S::MYB3R4, and this activation was further increased by the additional transfection of a 35S::CYCB1 expression plasmid (Fig. 6A). In comparison, KN::LUC reporter activity was unchanged when 35S::MYB3R1 was transfected, but was activated approximately twofold when 35S::CYCB1 was additionally co-transfected (Fig. 6A). Thus, MYB3R1 and MYB3R4 act as transcriptional activators of the *KN* gene promoter, with MYB3R4 being more effective than MYB3R1. Activation of the *KN* promoter by MYB3R4 is dependent on the presence of MSA elements, because a mutant *KN* promoter that lacks all three MSA elements (KNΔMSA) was no longer activated by 35S::MYB3R4 alone or in combination with 35S::CYCB1 (Fig. 6B).

We also tested *LUC* reporter constructs fused to the promoters of other genes expressed during G2/M transition (Fig. 6C). Transfection of 35S::MYB3R4, either alone or in combination with 35S::CYCB1, resulted in the activation of *CYCB1;2* and *CDC20.1* promoters, consistent with the decreased expression of these genes in the *myb3r1 myb3r4* double mutant. By contrast, the promoters of *CYCB1;1* and *AtNACK1/HIK*, whose transcript levels were unchanged in the double mutant, were also nearly unchanged by co-transfection of either 35S::MYB3R4 alone or 35S::MYB3R4 plus

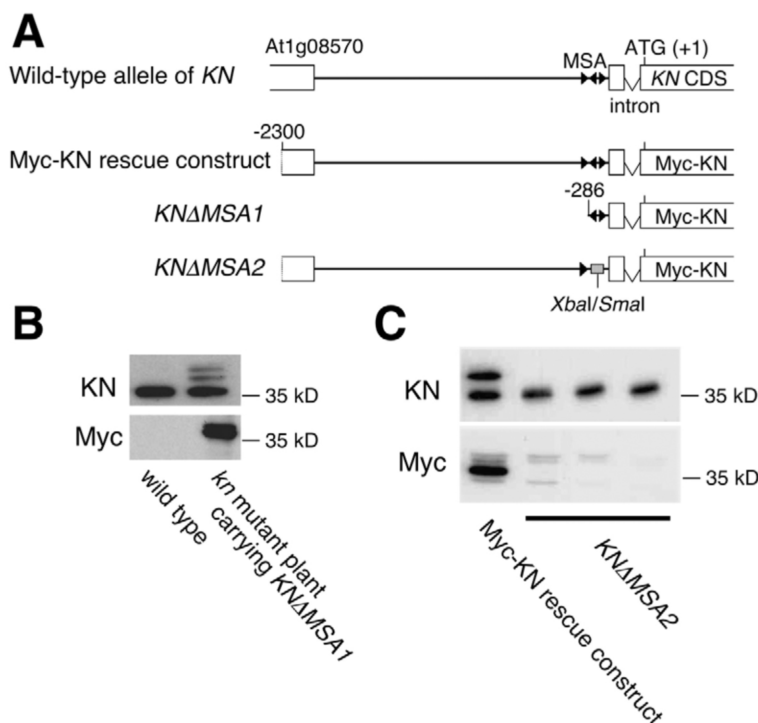

Fig. 5. Two MSA elements are required for sufficient expression of the *KN* gene. (**A**) The *KN* gene contains three MSA motifs in different orientations in its promoter region (arrowheads). The promoter-deletion construct *KNΔMSA1* only contains a promoter fragment from –286 bp to the *KN* start codon (ATG, +1). In the *KNΔMSA2* construct, two MSA motifs and the sequence between them (–278 to –250 bp) were replaced by a 12-bp sequence containing *Xba*I and *Sma*I restriction sites. (**B**) Western blots of Myc-tagged KN from transgenic *kn* mutant plants carrying *KNΔMSA1*. (**C**) Western blots of Myc-tagged KN from transgenic plants carrying Myc-KN rescue construct and *KNΔMSA2*. In all three independent *KNΔMSA2* transgenic plants, only endogenous KN without the Myc-tag was detected. KN, anti-KN antiserum; Myc, peroxidase-coupled anti-Myc monoclonal antibody.

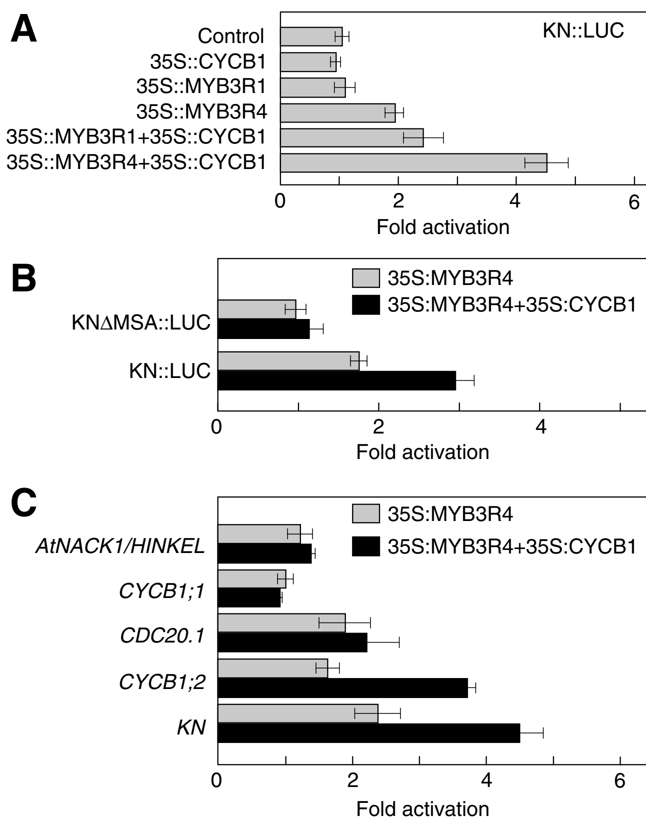

Fig. 6. MYB3R1 and MYB3R4 act as transcriptional activators. (**A**) Activation of *KN* gene promoter by MYB3R1 and MYB3R4. KN::LUC reporter plasmid and 35S::MYB3R1 or 35S::MYB3R4 expression plasmid were co-transfected into tobacco BY-2 protoplasts, either with or without 35S::CYCB1. (**B**) MSA elements are required for MYB3R4-induced activation of the *KN* promoter. The KNΔMSA::LUC construct contains the *KN* promoter in which all three MSA elements are mutated. KN::LUC and KNΔMSA::LUC were transfected into tobacco BY-2 protoplasts together with 35S::MYB3R4 expression plasmids, either with or without 35S::CYCB1. (**C**) Effects of *MYB3R4* on the activity of G2/M phase-specific promoters. Each promoter was fused to *LUC* and transfected into tobacco BY-2 protoplasts together with 35S::MYB3R4 expression plasmids, either with or without 35S::CYCB1. All LUC activities in A, B and C are normalized relative to the control transfection without expression plasmid. Bars represent mean values in each of five independent transfections; error bars represent s.d.

35S::CYCB1 (Fig. 6C). These results again suggest that MYB3R4 acts as a transcriptional activator for various G2/M phase-specific genes, and that its transactivation varies between potential target genes.

Expression of *MYB3R1* and *MYB3R4*

We examined the expression patterns of *MYB3R1* and *MYB3R4* during the cell cycle in cultures of *Arabidopsis* MM2d cells synchronized by aphidicolin block and release. Progression of the cell cycle was monitored by mitotic index and transcript abundance of *histone H4*, an S phase-specific gene. *Histone H4* mRNA level and mitotic index peaked at 2 and 12 hours, respectively, after aphidicolin release (Fig. 7A,B). Transcript levels of *MYB3R4* and *KN* changed in a similar pattern during the cell cycle, and their peak was recorded 2 hours before the peak of mitotic index (Fig. 7A,B). By contrast, the level of *MYB3R1* mRNA was nearly unchanged (Fig. 7A).

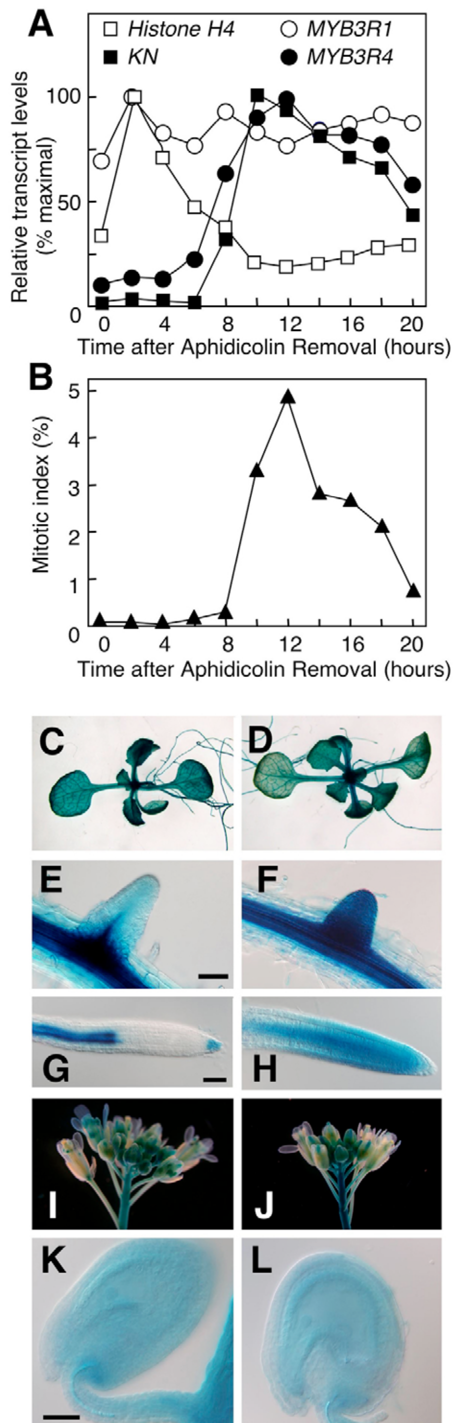

Fig. 7. Expression of *MYB3R1* and *MYB3R4*. (**A**,**B**) Expression during the cell cycle. (A) Change in transcript abundance during the cell cycle in synchronized MM2d cells. Transcript levels of each gene were analyzed by quantitative RT-PCR. (B) Change in mitotic index of the synchronized MM2d cells. (**C-L**) Expression domains of *MYB3R1::GUS* (C,E,G,I,K) and *MYB3R4::GUS* fusions (D,F,H,J,L). (C,D) 12-day-old seedling. (E,F) Emerging lateral roots. (G,H) Root tips. (I,J) Flowers. (K,L) Ovules with developing embryos. Scale bars: 50 μm.

To examine expression domains of *MYB3R1* and *MYB3R4* in vivo, we generated transgenic plants carrying 1.1 kb of the *MYB3R1* promoter or 1.2 kb of the *MYB3R4* promoter upstream of the *GUS*

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gene and examined *GUS* expression patterns by X-gluc staining. In both *MYB3R1::GUS* and *MYB3R4::GUS* lines, X-gluc staining was observed throughout the cotyledons and rosette leaves in 12-day-old seedlings, where vascular tissues were strongly stained (Fig. 7C,D). In the root, the vascular cylinder was darkly stained in both lines (Fig. 7E,F). The division zone of primary root tips and emerging lateral roots were stained in *MYB3R4::GUS* lines, whereas such staining was absent in *MYB3R1::GUS* lines (Fig. 7E-H). Instead, *MYB3R1::GUS* expression was observed in the columella root cap (Fig. 7G). Floral organs in young flower buds were stained in both *MYB3R1::GUS* and *MYB3R4::GUS* lines (Fig. 7I,J). In *MYB3R1::GUS* flowers, strong expression was observed in vascular tissues of filaments and anthers. In ovules of both *MYB3R1::GUS* and *MYB3R4::GUS* lines, the developing embryo and maternal tissues were weakly and uniformly stained (Fig. 7K,L).

In summary, *MYB3R4* is expressed in a cell cycle-dependent manner in synchronized cells, and its promoter is active in proliferating tissues, such as root tips and emerging lateral roots. However, the expression domains of *MYB3R1::GUS* and *MYB3R4::GUS* are not restricted to the meristematic tissues, and levels of expression do not generally correlate with cell division activity. This may suggest that MYB3R1 and MYB3R4 are post-transcriptionally regulated such that their ability for transcriptional activation is enhanced only in tissues with high cell-division activities. One such mechanism might be CDK-dependent phosphorylation, which activates the transactivation potential of NtmybA2 in tobacco cells (Araki et al., 2004). Consistently, both MYB3R1 and MYB3R4 contain multiple consensus phosphorylation sites by CDK (S/T-P-X-K/R), and their activities are enhanced by CYCB1 in tobacco cells (see Fig. 6B).

DISCUSSION

MYB3R1 and MYB3R4 are positive regulators acting on a subset of G2/M phase-specific genes

R1R2R3-Myb proteins NtmybA1 and NtmybA2 are transcriptional activators that bind to MSA elements, which are necessary and sufficient for transcription of the G2/M phase-specific genes *CYCB1* and *NACK1* in tobacco cells (Ito et al., 1998; Ito et al., 2001). In *Arabidopsis*, MSA elements are present in most G2/M phase-specific genes (Menges et al., 2005). MSA element-binding R1R2R3-Myb proteins might thus act as general positive regulators of mitotic events by inducing genes with essential mitotic functions. In this study, we have characterized T-DNA insertion mutants of *Arabidopsis MYB3R1* and *MYB3R4* genes, which are homologous to tobacco *NtmybA1* and *NtmybA2*, and observed downregulation of several G2/M phase-specific genes including *CDC20.1*, *CYCB2;1* and *KN* in the *myb3r1 myb3r4* double mutant. The primary defect was no or incomplete cytokinesis of several cell types in different organs, which correlated with a reduced transcript level of *KN*, a gene required for cytokinesis. The *KN* gene contains three MSA motifs in its promoter and is expressed in a cell cycle-dependent manner preferentially during G2/M phase in embryos and cultured cells (Lukowitz et al., 1996) (this study). As shown here, the two promoter-proximal MSA elements are essential for a functionally sufficient level of *KN* transcription, and transient expression of MYB3R4 activated the *KN* gene promoter in an MSA-dependent manner in tobacco cells. These findings strongly suggest that MYB3R4 activates *KN* transcription in vivo by binding to its MSA elements.

Our genetic analysis suggests that Myb proteins MYB3R1 and MYB3R4 may have redundant function, but MYB3R1 clearly contributes much less to *KN* and *CYCB2;1* gene activation and promotion of cytokinesis, and its contribution is only detected in the absence of MYB3R4. This difference cannot be attributed to differences between their promoters or remaining activities of T-DNA insertion alleles because (1) *CDKA;1::MYB3R1* was unable to rescue reduced *KN* expression in *myb3r4* plants (data not shown), and (2) 35S::MYB3R4, but not 35S::MYB3R1, on its own was able to activate *KN* and other MSA-containing promoters in tobacco cells. Transactivation activity of MYB3R1 was evident only when CYCB1 was co-expressed, which may enhance its transactivation potential (Araki et al., 2004). *MYB3R4* was expressed in proliferating tissues and preferentially during G2/M phase in synchronous cell cultures, whereas expression of *MYB3R1* appeared to be unrelated to the cell cycle. Taken together, MYB3R4 may be a major positive regulator for transcription of G2/M phase-specific genes, whereas MYB3R1 may assist MYB3R4 and may have other unrelated biological functions.

Arabidopsis may express other MSA-binding activators

The seedling lethality of the cytokinesis-defective *kn* mutant was not rescued by a *KN* genomic clone lacking the two promoter-proximal MSA elements, indicating that MSA-binding activator(s) are essential for *KN* expression. However, the *myb3r1 myb3r4* double mutant is viable and fertile, although it shows cytokinesis defects, and *KN* expression is not completely abolished. The apparent discrepancy might be explained by residual activity of MYB3R1 in the double mutant. However, an alternative explanation is suggested by the observation that the *myb3r1 myb3r4* double mutation did not decrease, but even slightly increased, the expression of the *CYCB1;1* gene (this study), although the presence of MSA elements is essential for its transcription (Planchais et al., 2002; Li et al., 2005). This may not be due solely to remaining activity of MYB3R1 in the double mutant, because the *myb3r1-1* single mutation, which eliminated the accumulation of the normal *MYB3R1* transcript, did not affect the *CYCB1;1* transcript level (data not shown). In addition, we showed that the *CYCB1;1* promoter was not activated by 35S::MYB3R1 (data not shown) nor 35S::MYB3R4 (see Fig. 6C) in tobacco cells. Thus, we speculate that *Arabidopsis* might express additional transcriptional activator(s) that act(s) through MSA elements redundantly with MYB3R1 and MYB3R4. This hypothesis may also explain the differential effects of the *myb3r1 myb3r4* double mutation on their potential target genes, by assuming differential contributions made by the redundant factor(s) on each target gene. *Arabidopsis* contains three other *R1R2R3-Myb* genes, *MYB3R2*, *MYB3R3* and *MYB3R5*, which, however, may not be simply functionally redundant with *MYB3R1* and *MYB3R4*, because their mutations do not enhance the cytokinesis defect of *myb3r4* single mutant and *myb3r1 myb3r4* double mutant (M.I., unpublished). Candidates for such redundant factor(s) might be a TCP family transcription factor (Li et al., 2005) and a Myb domain-containing protein (Planchais et al., 2002), both of which bind to the promoter of the *CYCB1;1* gene. Identification and functional characterization of the redundant activator(s), and possibly genetic studies on *MYB3R2*, *MYB3R3* and *MYB3R5* genes as well, would unravel the mechanisms that regulate gene expression during the G2/M phase in *Arabidopsis*.

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