Mutations in MYB3R1 and MYB3R4 Cause Pleiotropic Developmental Defects and Preferential Down-Regulation of Multiple G2/M-Specific Genes in Arabidopsis

Nozomi Haga, Kosuke Kobayashi, Takamasa Suzuki, Kenichiro Maeo, Minoru Kubo, Misato Ohtani, Nobutaka Mitsuda, Taku Demura, Kenzo Nakamura, Gerd Jürgens, and Masaki Ito*

Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya 464–8601, Japan (N.H., K.K., T.S., K.M., K.N., M.I.); RIKEN Plant Science Center, Yokohama, Kanagawa 230–0045, Japan (M.K., M.O., T.D.); Biomass Engineering Program, RIKEN, Yokohama 230–0045, Japan (M.O., T.D.); Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology, Tsukuba 305–8562, Japan (N.M); Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara 630–0192, Japan (T.D.); and Zentrum für Molekularbiologie der Pflanzen, Entwicklungsgenetik, Universität Tübingen, 72076 Tübingen, Germany (G.J.)

R1R2R3-Myb proteins represent an evolutionarily conserved class of Myb family proteins important for cell cycle regulation and differentiation in eukaryotic cells. In plants, this class of Myb proteins are believed to regulate the transcription of G2/M phase-specific genes by binding to common cis-elements, called mitosis-specific activator (MSA) elements. In Arabidopsis (Arabidopsis thaliana), MYB3R1 and MYB3R4 act as transcriptional activators and positively regulate cytokinesis by activating the transcription of KNOLLE, which encodes a cytokinesis-specific syntaxin. Here, we show that the double mutation myb3r1 myb3r4 causes pleiotropic developmental defects, some of which are due to deficiency of KNOLLE whereas other are not, suggesting that multiple target genes are involved. Consistently, microarray analysis of the double mutant revealed altered expression of many genes, among which G2/M-specific genes showed significant overrepresentation of the MSA motif and a strong tendency to be down-regulated by the double mutation. Our results demonstrate, on a genome-wide level, the importance of the MYB3R-MSA pathway for regulating G2/M-specific transcription. In addition, MYB3R1 and MYB3R4 may have diverse roles during plant development by regulating G2/M-specific genes with various functions as well as genes possibly unrelated to the cell cycle.

During plant development, the spatial and temporal regulation of cell division is primarily important for the formation of multicellular structures of appropriate sizes and defined shapes. Such regulation is achieved by controlling the cell cycle in each plant cell through evolutionarily conserved regulators such as cyclins and cyclin-dependent kinases (CDKs; Inzé and De Veylder, 2006). Among them, cyclins of the CYCB1, CYCB2, and CYCA1 classes and plant-specific CDKB2 are expressed specifically during late G2 and M phases (Menges and Murray, 2002). These genes, together with many other genes showing similar patterns of G2/M phase-specific expression, contain common cis-acting elements called mitosis-specific activator (MSA) elements (Ito et al., 1998; Ito, 2000). In tobacco (Nicotiana tabacum) cells, MSA elements are sufficient for G2/M phase-specific promoter activation and bind R1R2R3-Myb transcription factors, NtmybA1, NtmybA2, and NtmybB (Ito et al., 2001). Overexpression of NtmybA1 and NtmybA2 increase the activity of MSA-containing promoters in BY2 protoplasts, suggesting that these Myb proteins may act as transcriptional activators (Ito et al., 2001; Kato et al., 2009).

Arabidopsis (Arabidopsis thaliana) contains five genes encoding R1R2R3-Myb transcription factors, of which MYB3R1 and MYB3R4 are structurally similar to each other and to NtmybA1 and NtmybA2 (Ito, 2005). The
Roles of MYB3R1 and MYB3R4 in Plant Development

RESULTS

Macroscopic Defects in Postembryonic Development of myb3r1 myb3r4

A fraction of myb3r1 myb3r4 seedlings (approximately 5%) showed various morphological abnormalities. For example, the seedlings often had one or three instead of two cotyledons (Fig. 1, A, C, and D) or, rarely, appeared to lack cotyledons altogether (Fig. 1H). In other cases, the cotyledons were fused, sometimes forming a cup-shaped structure (Fig. 1, B and G). Also, roots and hypocotyls were sometimes very short or lacking (Fig. 1, E and F). The severe defects of body organization occasionally resulted in seedling lethality. However, the majority of seedlings grew and produced rosette leaves that were short and wrinkled (Fig. 1, I and J) and eventually developed into mature albeit abnormal plants. After bolting, the mutant plants were reduced in stature (Fig. 1K; Supplemental Table S1). The severity of this phenotype varied between individuals and was also influenced by the growth conditions, where light conditions might be most influential. The short plant stature resulted from reduced elongation of internodes, both between lateral branches of the main inflorescences (Supplemental Table S1) and between successive siliques, which correlated with severely reduced elongation of epidermal cells (Fig. 1, L–Q; Supplemental Table S1). These cells did not show symptoms of incomplete cytokinesis such as supernumerary nuclei, gapped cell walls, or cell wall stubs. The developing siliques of the double mutant contained ball-shaped immature seeds that were clearly distinguishable from the oblong wild-type seeds (Fig. 1, R and S) and were more often aborted than wild-type seeds (7.72% versus 0.55%). Both abnormal seed development and abnormal seedling morphology suggest that embryo development is impaired in the double mutant.

Abnormal Development of myb3r1 myb3r4 Embryos

In contrast to the small minority of abnormal seedlings, the vast majority (approximately 90%) of myb3r1 myb3r4 double mutant embryos were morphologically abnormal, with severe defects often occurring at very early stages such as the first or second division. The wild-type zygote divides asymmetrically, generating a small cytoplasmically dense apical cell and an elongated vacuolate basal cell (Fig. 2A). The apical cell produces, through a regular pattern of cell divisions, most of the embryo, whereas the basal cell divides transversely several times, producing the suspensor (Fig. 2, B and C). Some double mutant embryos were rod or club shaped, containing multiple nuclei that were not separated by cell walls (Fig. 2F). This phenotype may have resulted from multiple rounds of nuclear division without cytokinesis (Haga et al., 2007).

In most double mutant embryos, cytokinesis eventually occurred, but the pattern of division was irregular. In the wild-type embryo, the apical daughter cell of the zygote divides vertically (Fig. 2B). This division, however, was horizontal in approximately 50% of the double mutant embryos (Fig. 2C). As wild-type development progresses, the regular and reproducible pattern of division generates globular and heart-shaped embryos (Fig. 2, C–E). Double mutant embryos did not undergo the stereotyped division pattern and instead displayed unusual division planes and misaligned or disorganized cell patterns (Fig. 2, H–J).
MYB3R1 and MYB3R4 Are Required for the Maintenance of Diploidy

Rosette leaves of the myb3r1 myb3r4 double mutant occasionally had extraordinarily large stomata and trichomes with supernumerary branches (Supplemental Fig. S1), which are characteristic of polyploid plants (Yu et al., 2009). When analyzed by flow cytometry, wild-type diploid leaves showed peaks at 2C, 4C, 8C, 16C, 32C, and sometimes 64C levels, with DNA levels higher than 2C generated by endoreduplication during leaf development (Fig. 3A). In the double mutant, however, 17 of 54 individuals tested showed a tetraploid pattern of DNA content, which is characterized by loss of the 2C peak (Fig. 3A; Supplemental Table S2). We confirmed that nuclei of these plants had actually twice the number of chromosomes as those of diploid plants, as judged by the number of heterochromatic chromocenters observed as bright dots in 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei (Fransz et al., 2002; Fig. 3, B and C). Less frequently, plants with hexaploid or octaploid patterns of DNA

Figure 2. Abnormal development of the myb3r1 myb3r4 embryo. Embryos were cleared and visualized by DIC microscopy. A to E, Wild-type embryo at the one-cell stage (A), two-cell stage (B), eight-cell stage (C), triangular stage (D), and heart stage (E). F to J, myb3r1 myb3r4 double mutant embryos. F shows a rod-shaped embryo with multiple nuclei that were not separated by internal cell walls, and G shows the two-cell stage with horizontal division of the apical cell. The developmental stage of each mutant embryo approximately corresponds with that of the wild-type embryo shown above. [See online article for color version of this figure.]
One explanation is that the polyploidization may be due to increased ploidy of gametes in myb3r1 myb3r4 plants. However, in such cases, the progeny of self-fertilized myb3r1 myb3r4 plants should be frequently triploid, which has not been observed in 65 polyploid individuals (Supplemental Table S2). Furthermore, reciprocal test crosses between the myb3r1 myb3r4 double mutant and the wild type yielded only diploid F1 plants, regardless of whether the double mutant was the pollen donor or acceptor (Supplemental Table S2). Alternatively, polyploidization events may occur in somatic cells during embryogenesis or postembryonic development in myb3r1 myb3r4 plants. To identify the origin of increased ploidy levels, we tested whether the plants were chimeric with regard to their ploidy levels. When ploidy levels were compared between roots and leaves of the same plant, three of 17 plants were chimeric; one plant had diploid leaves and a tetraploid root, whereas two had diploid leaves and a hexaploid root. The cells in roots and leaves originate from initial cells in the primary root and shoot meristem, respectively, which in turn derive from different cell groups of the eight-cell-stage embryo, suggesting that most polyploidization events in the double mutant might have occurred in early embryogenesis. That polyploidization events indeed occurred after fertilization was confirmed by examining the progeny of self-fertilized myb3r1/myb3r1 MYB3R4/myb3r4 and MYB3R1/myb3r1 myb3r4/myb3r4 plants. Most polyploid individuals were homozygous for both myb3r1 and myb3r4. Less frequently, polyploidy was also observed in MYB3R1/myb3r1 myb3r4/myb3r4 individuals (Supplemental Table S2). Plants with all other genotypes showed the diploid pattern of DNA content. Thus, the frequency of polyploidization strongly depends on the genotype of the embryo. The relationship between genotypes and the frequency of polyploidization are summarized in Figure 3E.

myb3r1 myb3r4 Plants Are Hypersensitive to Caffeine, an Inhibitor of Plant Cytokinesis

In addition to the abnormal development in normal growth conditions, myb3r1 myb3r4 double mutants showed higher sensitivity to caffeine, a drug that inhibits cytokinesis in plants. Caffeine induces the frequent occurrence of incomplete cytokinesis, as is observed in cytokinesis-defective mutants (Falbel et al., 2003; Yasuhara, 2005). When myb3r1 myb3r4 seedlings were grown in the absence of caffeine for 12 d, no clear difference of growth was observed compared with the wild type (Fig. 4). However, when seedlings were grown in 0.5 mM caffeine, the growth of the myb3r1 myb3r4 seedlings was severely impaired whereas that of the wild type was only slightly affected. In the medium containing 1.2 mM caffeine, where wild-type seedlings could still produce leaves, growth of the myb3r1 myb3r4 seedlings was almost completely inhibited, and these seedlings died without producing leaves.

KN-Dependent and KN-Independent Abnormalities in myb3r1 myb3r4 Plants

We have previously shown that the double mutation myb3r1 myb3r4 results in decreased KN mRNA levels (Haga et al., 2007). To reveal spatial aspects of the activation of KN transcription mediated by the MSA-MYB pathway, we examined the expression of a KN...
promoter-GUS fusion construct (KN::GUS) in the wild type and the double mutants. In wild-type plants, strong GUS activity was observed in the root tip containing rapidly dividing cells (Fig. 5A). GUS activity was also detected in young leaves, where dividing stomatal lineage cells, such as meristemoids and guard mother cells, were densely stained (Fig. 5B). The GUS activity in these cells was markedly decreased in the myb3r1 myb3r4 double mutant (Fig. 5C and D). We also observed that the KN promoter had very weak activity in the meristematic region of the root and dividing stomatal lineage cells when all the MSA motifs were mutated (Fig. 5E and F). These data support the view that KN is transcriptionally activated by the binding of MYB3R1 and MYB3R4 to its MSA elements.

In our previous study, incomplete cytokinesis in myb3r1 myb3r4 was rescued by transgenes expressing KN under the promoters of CDKA;1 and RPS5A genes, which are active in proliferating tissues (Haga et al., 2007). To test if increased KN gene expression could also rescue other abnormalities of myb3r1 myb3r4 plants, we examined the double mutants with or without the transgene expressing KN. The frequency of polyploid plants was significantly decreased when parents homozygous for myb3r1 and myb3r4 carried either CDKA;1::KN or RPS5A::KN (Table I). This suggests that the frequent polyploidization observed in myb3r1 myb3r4 double mutants may be caused, at least partially, by the defective cytokinesis that results from a deficiency of KN protein. Similarly, the frequency of seedlings with abnormal body organization was largely reduced. However, the apical daughter cell of the zygote still divided abnormally, as CDKA;1::KN or RPS5A::KN only slightly reduced the frequency of horizontal divisions (Table I). Likewise, the introduction of CDKA;1::KN or RPS5A::KN did not rescue the semidwarf phenotype and caffeine hypersensitivity of the myb3r1 myb3r4 double mutant (Table I).

**Microarray Analysis of myb3r1 myb3r4 Seedlings**

The observed KN-dependent and KN-independent phenotypes in the double mutant suggested that multiple target genes are critical for the abnormalities. To get more insight into the transcriptional regulation by MYB3R1 and MYB3R4, we pursued a genome-wide approach to compare gene expression profiles between wild-type and double mutant seedlings using the Affymetrix GeneChip (Arabidopsis ATH1 genome array chip), which covers 22,810 genes in Arabidopsis. Microarray hybridization was performed with three biological replicates for the wild type and the double mutant. We selected 16,034 genes for further analysis that are expressed at statistically high levels (detection call in the Affymetrix analysis is present) in at least two biological replicates of wild-type sample.

To evaluate the effects of the myb3r1 myb3r4 double mutation on the expression of G2/M-specific genes, we first defined 185 genes with G2/M-specific expression by a combination of coexpression analysis using ATTED-II (Obayashi et al., 2009) and gene clustering analysis with the microarray data of synchronized MM2d cells (Menges et al., 2003; see “Materials and Methods”). This newly defined G2/M-specific class contained 79 out of 82 genes that had been previously regarded as mitosis-specific genes (Menges et al., 2005) and 134 out of 352 genes that had been classified as M-associated genes (Menges et al., 2003; Supplemental Table S3). When applied to our microarray data, we noticed a strong tendency for this new class of genes to be frequently and strongly down-regulated in the double mutant. Figure 6 displays a frequency distribution of genes against the fold change in the expression levels between the double mutant and the wild type. It was obvious that the frequency distribution of G2/M-specific genes was strongly biased toward down-regulation in the double mutant when compared with the distribution patterns of all other genes on the microarray. Approximately 11.6% of G2/M-specific genes were down-regulated more than 2-fold in the double mutant, whereas only 0.38% of all 16,034 genes analyzed showed such down-regulation. Table II shows the top 30 genes that are most down-regulated in the double mutant (P < 0.05, paired t test), which included 16 genes (53.3%) of the G2/M-specific class, although this class represents only 0.83% of all genes on the microarray. These results supported the idea that MYB3R1...
and MYB3R4 may transcriptionally activate multiple G2/M phase-specific genes in Arabidopsis. However, this analysis also revealed that not all the G2/M-specific genes were affected by the double mutation. In fact, the majority of G2/M-specific genes were unaffected or even up-regulated in the myb3r1 myb3r4 double mutant (Fig. 6). In addition, many genes whose expression is not related to the cell cycle were also significantly down-regulated in the double mutant, although roughly as many genes were up-regulated to a similar extent (Fig. 6, black bars; Table II).

Presence of MSA Elements in G2/M-Specific Genes

We then analyzed the occurrence of the MSA elements in 149 G2/M-specific genes with annotated transcription start sites. The 1.0-kb upstream regions of these genes were searched for overrepresented six- or eight-base motifs using the motif-finding software tool Weeder (Pavesi et al., 2004). The output of this search showed that all the top-scoring motifs except one contained AACGG or its complementary sequence CCGTT, which is identical to the core motif of the MSA consensus sequence, TCYACCGGYYA (Supplemental Table S4; Ito, 2000). In one exceptional case, the six-base motif sequence contained four bases (CGTT) of the CCGTT core motif. The AACGG motifs were significantly enriched in the upstream regions of G2/M-specific genes, especially in the proximal region up to position −200 (Fig. 7). For example, we found 5.2-fold enrichment of the AACGG motif in G2/M-specific genes compared with all genes when promoter regions up to −100 were analyzed. To reevaluate the MSA consensus sequence from a large number of motifs, all the AACGG-containing motifs from the proximal region up to −200 were used to determine the frequency of each base at each position of the MSA motif. This analysis revealed a conserved sequence of 15 nucleotides, HNYAACGGYYANWWT (H = A, C, or T; Y = C or T; W = A or T) that is similar to, but has a four-base extension at the 3’ end of, the previously defined consensus sequence (Ito, 2000; Supplemental Fig. S2A). We examined the occurrence of the MSA consensus motifs in upstream regions of G2/M-specific genes, allowing a fixed number of mismatches outside of the AACCG core sequence, and found that the motif with higher identity showed a more significant enrichment in G2/M-specific genes (Fig. 8A) and a stronger tendency to locate proximally to the transcription start site (Fig. 8B).

Our microarray analysis showed that a fraction of G2/M-phase-specific genes were significantly down-regulated in the double mutant but other genes in this class were not. One possibility is that the unaffected genes may not contain the MSA motif. When the occurrence of MSA motifs (identity score higher than 80%) was examined in the G2/M-specific genes, we found a significant enrichment of the motif in the down-regulated genes (Fig. 9, gray bars). This may suggest that target genes of MYB3R1/4, which represent a subset of G2/M-specific genes, are enriched in the down-regulated genes. However, even when the unaffected or up-regulated genes were surveyed, there was still a significant overrepresentation of the MSA motifs in G2/M-specific genes compared with all other genes, suggesting that different effects of the double mutation may not simply be due to the absence of the MSA motif (Fig. 9, compare gray and black bars). To test for the possibility that different effects of the myb3r1 myb3r4 double mutation may be due to variation of the MSA sequences, we analyzed 57 motifs from the 16 G2/M-specific genes that were down-regulated more than 2-fold. The results showed that the consensus motif in the down-regulated genes was indistinguishable from that derived from 218 motifs found in all the G2/M-specific genes (Supplemental Fig. S2, A and B).

Figure 5. GUS reporter analysis of the KN promoter. The GUS reporter construct fused to the wild-type KN promoter (KN::GUS) and the KN promoter with all the MSA element mutated (KNΔMSA::GUS) were constructed. GUS activities in the transgenic seedlings were analyzed by staining with X-gluc. A and B, Wild-type seedlings with KN::GUS. C and D, myb3r1 myb3r4 seedlings with KN::GUS. E and F, Wild-type seedlings with KNΔMSA::GUS. GUS expression in root tips (A, C, and E) and leaf epidermis (B, D, and F) are shown.
The double mutation also affected the expression of multiple genes that are not obviously related to the cell cycle. If such genes were directly regulated by MYB3R1/4, they may contain MSA motifs in the promoter regions. As in Figure 9 (black bars), the MSA motifs (identity score higher than 80%) are significantly enriched in the down-regulated genes with fold change levels (log2 value) below $-0.4$ compared with other genes (0.50 versus 0.42; $P = 0.0035$, Fisher’s exact test), even when all the G2/M-specific genes were excluded from the analysis. The observed over-representation indicates that there may be some cell cycle-unrelated target genes that are transcriptionally activated by MYB3R1 and MYB3R4.

**MYB3R4 Binds to MSA-Like Motifs in Vitro**

Finally, we tested if MYB3R4 binds directly to MSA motifs in vitro using the cycle amplification of targets (CASTing) method (Wright et al., 1991). The glutathione S-transferase (GST)-MYB3R4 fusion protein coupled to glutathione-Sepharose beads was incubated with random oligonucleotides, and the bound oligonucleotides were recovered and amplified by PCR. After this cycle had been repeated six times, the selected oligonucleotides were subcloned and sequenced. Motif-finding analysis with Weeder showed significant enrichment of the AACGG motif in 94 oligonucleotides obtained in the CASTing experiment (Supplemental Table S5). At least one AACGG motif was contained in 74 out of 94 sequences, among which 35 contained multiple such motifs. Figure 10 shows a sequence logo that was created using 39 sequences in which the AACGG motif occurred only once (as listed in Supplemental Fig. S3). The MYB3R4 recognition sequence in vitro was very similar, if not identical, to the consensus sequence of MSA motifs that occurred in promoters of G2/M-specific genes (Fig. 10; compare with Supplemental Fig. S2A). This suggest that MYB3R4, and possibly MYB3R1 as well, may bind directly to promoters of G2/M-specific genes by recognizing MSA motifs.

**DISCUSSION**

**Multiple Developmental Defects in the myb3r1 myb3r4 Double Mutant**

The myb3r1 myb3r4 double mutant showed various developmental abnormalities, such as dwarfism, irregular morphology of seedling and embryo, and production of polyploid offspring. These macroscopic abnormalities may be consequent to primary cellular defects of cytokinesis or caused separately. Since MYB3R1/4-independent expression of KN from CDKA;1::KN or RPS5A::KN transgenes rescued the cytokinesis defects in the double mutant, the developmental abnormalities of the double mutant can be classified as secondary consequences of cytokinesis defects if they are KN dependent or as unrelated defects if they are not resuable by MYB3R1/4-independent KN expression. By these criteria, abnormal

---

**Table 1. Effects of the introduction of a transgene containing KN in the myb3r1 myb3r4 background**

<table>
<thead>
<tr>
<th>Effect</th>
<th>Genotype (Transgene)</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>myb3r1 myb3r4 (No Transgene)</td>
<td></td>
</tr>
<tr>
<td>Horizontally divided apical cell (%)</td>
<td>≥90</td>
<td>47.8</td>
</tr>
<tr>
<td>Seedlings with abnormal body organization (%)</td>
<td>≥54</td>
<td>4.9</td>
</tr>
<tr>
<td>Internode length between siliques (% of the wild type)</td>
<td>≥9</td>
<td>61.6 ± 34.8</td>
</tr>
<tr>
<td>Plant height (% of the wild type)</td>
<td>≥9</td>
<td>85.3 ± 20.2</td>
</tr>
<tr>
<td>Frequency of polyploid progeny (polyploid plants per total plants examined [%])</td>
<td>24 of 54 (44.4)</td>
<td></td>
</tr>
<tr>
<td>Fresh weight of seedlings grown in 0.5 mM caffeine (% of the wild type)</td>
<td>17.6 ± 0.04</td>
<td></td>
</tr>
</tbody>
</table>

*Number of apical cells examined. *The average length of five successive internodes from the lowest siliques was determined for each plant.
seeding morphology and polyploidization likely result from defective cytokinesis, which is consistent with the abnormal seeding body organization of cytokinesis-defective mutants such as \( kn \), \( keu \), and \( hik \) (Assaad et al., 1996; Lukowitz et al., 1996; Strompen et al., 2002). Likewise, enlarged nuclei with supernumerary chromosomes are a characteristic feature of cytokinesis-defective embryos (Lukowitz et al., 1996). Our genetic and ploidy analyses suggest that polyploid \( myb3r1 \) \( myb3r4 \) double mutants originate very early in embryogenesis, probably during the first or second cell division. Absence of cytokinesis at that early stage would generate multiple nuclei in the single proembryo cell, which might eventually generate a polyploid nucleus.

The semidwarf phenotype of the \( myb3r1 \) \( myb3r4 \) double mutant results from reduced growth of internodes, which is caused, at least partially, by reduced cell elongation. This defect was not rescued by transgene expression of \( KN \), suggesting that other, cytokinesis-unrelated target genes of \( MYB3R1 \) and \( MYB3R4 \) may be critical for reduced cell elongation. This view was further supported by our observation that the semidwarf phenotype was not apparent when the \( myb3r4 \) single mutant also had a heterozygous mutation of \( KN \), which enhanced cytokinesis defects to a similar extent to that in \( myb3r1 \) \( myb3r4 \) double mutant (Haga et al., 2007).

We also found \( KN \)-independent cellular abnormalities that might be related to defective cytokinesis. Abnormal division patterns of \( myb3r1 \) \( myb3r4 \) embryos, such as the horizontal division of the apical cells, were only partially rescued by the \( KN \) transgene, which completely eliminated multinucleate embryo cells (Haga et al., 2007). Similarly, transgene expression of \( KN \) could not rescue the hypersensitivity to caffeine that inhibits cell plate formation during cytokinesis. It could be speculated that the decreased expression of some cytokinesis-related genes other than \( KN \) might cause the abnormal division plane and enhanced sensitivity to caffeine. Actually, our microarray data showed decreased expression of \( PHRAGMOPLAST-ORIENTING KINESIN1 \), a mutation of which causes misplaced cell walls (Müller et al., 2006). Furthermore, multiple genes encoding previously uncharacterized kinesins (AT3G51150, AT2G47500, and AT5G66310) were also significantly down-regulated in \( myb3r1 \) \( myb3r4 \) seedlings, and these kinesins could potentially be involved in cytokinetic microtubule structures and

<table>
<thead>
<tr>
<th>Log2</th>
<th>( P^a )</th>
<th>Arabidopsis Genome Initiative Code</th>
<th>Expression</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>-3.76</td>
<td>0.001</td>
<td>AT3G02120</td>
<td>G2/M</td>
<td>Hyp-rich glycoprotein family protein</td>
</tr>
<tr>
<td>-2.91</td>
<td>0.011</td>
<td>AT3G51740</td>
<td>G2/M</td>
<td>IMK2/protein Ser/Thr kinase</td>
</tr>
<tr>
<td>-2.19</td>
<td>0.018</td>
<td>AT4G31840</td>
<td>G2/M</td>
<td>Plastocyanin-like domain-containing protein</td>
</tr>
<tr>
<td>-2.11</td>
<td>0.039</td>
<td>AT3G52110</td>
<td>G2/M</td>
<td>Unknown protein</td>
</tr>
<tr>
<td>-2.05</td>
<td>0.017</td>
<td>AT3G42725</td>
<td>G2/M</td>
<td>Unknown protein</td>
</tr>
<tr>
<td>-1.95</td>
<td>0.035</td>
<td>AT1G31335</td>
<td>G2/M</td>
<td>Unknown protein</td>
</tr>
<tr>
<td>-1.79</td>
<td>0.019</td>
<td>AT3G02640</td>
<td>G2/M</td>
<td>Unknown protein</td>
</tr>
<tr>
<td>-1.69</td>
<td>0.034</td>
<td>AT2G42110</td>
<td>G2/M</td>
<td>Unknown protein</td>
</tr>
<tr>
<td>-1.67</td>
<td>0.016</td>
<td>AT5G15720</td>
<td>G2/M</td>
<td>GLIP7; carboxylesterase/lipase</td>
</tr>
<tr>
<td>-1.60</td>
<td>0.018</td>
<td>AT5G55180</td>
<td>G2/M</td>
<td>Glycosyl hydrolase family 17 protein</td>
</tr>
<tr>
<td>-1.60</td>
<td>0.012</td>
<td>AT4G02290</td>
<td>G2/M</td>
<td>A1GH9B13 (Arabidopsis glycosyl hydrolase 9B13)</td>
</tr>
<tr>
<td>-1.58</td>
<td>0.014</td>
<td>AT1G20030</td>
<td>Pathogenesis-related thaumatin family protein</td>
<td></td>
</tr>
<tr>
<td>-1.42</td>
<td>0.027</td>
<td>AT3G51720</td>
<td>G2/M</td>
<td>Unknown protein</td>
</tr>
<tr>
<td>-1.35</td>
<td>0.012</td>
<td>AT1G69230</td>
<td>G2/M</td>
<td>Unknown protein</td>
</tr>
<tr>
<td>-1.32</td>
<td>0.013</td>
<td>AT2G01130</td>
<td>G2/M</td>
<td>Unknown protein</td>
</tr>
<tr>
<td>-1.30</td>
<td>0.025</td>
<td>AT4G20430</td>
<td>Subtilase family protein</td>
<td></td>
</tr>
<tr>
<td>-1.24</td>
<td>0.021</td>
<td>AT5G25090</td>
<td>G2/M</td>
<td>Plastocyanin-like domain-containing protein</td>
</tr>
<tr>
<td>-1.21</td>
<td>0.004</td>
<td>AT1G64470</td>
<td>Ubiquitin family protein</td>
<td></td>
</tr>
<tr>
<td>-1.19</td>
<td>0.034</td>
<td>AT2G17620</td>
<td>G2/M</td>
<td>CYCB2;1</td>
</tr>
<tr>
<td>-1.18</td>
<td>0.039</td>
<td>AT2G17850</td>
<td>Unknown protein</td>
<td></td>
</tr>
<tr>
<td>-1.16</td>
<td>0.010</td>
<td>AT5G16250</td>
<td>Unknown protein</td>
<td></td>
</tr>
<tr>
<td>-1.14</td>
<td>0.032</td>
<td>AT3G21720</td>
<td>G2/M</td>
<td>Unknown protein</td>
</tr>
<tr>
<td>-1.12</td>
<td>0.037</td>
<td>AT2G27900</td>
<td>Unknown protein</td>
<td></td>
</tr>
<tr>
<td>-1.12</td>
<td>0.039</td>
<td>AT1G49870</td>
<td>Unknown protein</td>
<td></td>
</tr>
<tr>
<td>-1.10</td>
<td>0.019</td>
<td>AT1G08560</td>
<td>G2/M</td>
<td>SYP111 (SYNTAXIN OF PLANTS111); KNOLLE</td>
</tr>
<tr>
<td>-1.10</td>
<td>0.008</td>
<td>AT3G17640</td>
<td>G2/M</td>
<td>Leu-rich repeat family protein</td>
</tr>
<tr>
<td>-1.08</td>
<td>0.031</td>
<td>AT3G26800</td>
<td>Unknown protein</td>
<td></td>
</tr>
<tr>
<td>-1.07</td>
<td>0.043</td>
<td>AT1G68640</td>
<td>G2/M</td>
<td>PAN (PERIANTHIA); DNA-binding/transcription factor</td>
</tr>
<tr>
<td>-1.04</td>
<td>0.042</td>
<td>AT3G60330</td>
<td>AHA7 (Arabidopsis H+-ATPase 7)</td>
<td></td>
</tr>
<tr>
<td>-1.00</td>
<td>0.034</td>
<td>AT1G25510</td>
<td>Aspartyl protease family protein</td>
<td></td>
</tr>
</tbody>
</table>

\( ^a \)Uncorrected \( P \) values. Multiple testing correction has not been performed due to high variance.
dynamics. Taken together, our phenotypic analysis showed that MYB3R1 and MYB3R4 might positively regulate the expression of KN, as well as some other cytokinesis-related and -unrelated target genes, thus leading to the pleiotropic phenotypes.

Regulation of Multiple G2/M-Specific Genes by MYB3R1 and MYB3R4

Consistent with the pleiotropic defects in the double mutant, our microarray analysis revealed that a subset of G2/M-specific genes with various functions were down-regulated in the myb3r1 myb3r4 double mutant (Table II; Fig. 6). In addition, we showed that most of the G2/M-specific genes contain MSA-like motifs: 83% of G2/M-specific genes, but only 32% of other genes, contain at least one motif that shows more than 80% identity to the MSA consensus sequence. In CASTing experiments, the MSA motifs could actually bind to GST-MYB3R4 in vitro. These results support our view that MYB3R1 and MYB3R4 might activate the transcription of many G2/M-specific genes by binding to the MSA elements. On the other hand, the microarray data also showed that many G2/M-specific genes were unaffected by the double mutation. This is not due to the absence of the MSA element in the unaffected genes; instead, such genes also showed significant overrepresentation of the MSA motif. In addition, we could not find a significant difference in the base frequencies at any position in MSA motifs between down-regulated and other genes (Supplemental Fig. S2). These results led us to speculate that there may be some other MSA-binding activator or some other cis-acting elements in the promoters. The GCCCR motif that binds with the TCP20 transcription factor may be a candidate for such elements, because this motif acts as an enhancer element in the CYCB1;1 promoter (Li et al., 2005). As a next step of this study, it would be interesting to investigate how MYB3R1 and MYB3R4 differentially regulate G2/M-specific genes carrying MSA motifs with equivalent sequences and how G2/M-specific transcription is achieved independently of MYB3R1 and MYB3R4.

Are There Cell Cycle-Unrelated Target Genes?

In addition to the G2/M-specific genes, the myb3r1 myb3r4 double mutation caused significant down-

Figure 7. Overrepresentation of the AACGG core motif in the proximal promoter region of G2/M-specific genes. The number of AACGG motifs (per gene) was calculated in 100-bp intervals upstream from annotated transcription start sites in 149 G2/M-specific genes and total genes. Asterisks denote statistically significant differences (P < 0.05, Fisher’s exact test).
regulation of other genes with cell cycle-unrelated expression. The double mutation also caused up-regulation of an equivalent number of genes to a similar extent (Fig. 6), suggesting that the contribution of MYB3R1 and MYB3R4 to the regulation of cell cycle-unrelated target genes, if present at all, is relatively small. However, even in the genes outside of the G2/M-specific class, a significant overrepresentation of the MSA motifs was observed in the down-regulated genes (log2 fold change value of less than −0.4) compared with all genes (P = 0.0044, Fisher’s exact test; Fig. 9). This suggests that some cell cycle-unrelated genes may be transcriptionally activated by MYB3R1/4 by binding to the MSA elements. There is additional evidence that supports the presence of such cell cycle-unrelated target genes. Such evidence comes from a recent report on the cold-resistant phenotype of rice (*Oryza sativa*) plants overexpressing OsMYB3R-2, the expression of which was greatly induced during cold stress (Ma et al., 2009). We also showed in this study that some of the phenotypes of the *myb3r1 myb3r4* double mutant are not dependent on KN and hence may be caused separately by cell cycle-unrelated target genes. To clarify this possibility, we are currently in the process of genome-wide identification of direct target genes regulated by MYB3R1 and MYB3R4. Identification of such cell cycle-unrelated target genes might reveal an additional role of R1R2R3-Myb proteins beyond cell cycle regulation and also uncover a possible link between the cell cycle and some other cellular and whole-plant processes.

### MATERIALS AND METHODS

#### Plant Materials and Growth Conditions

The Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia was used as the wild type. The *myb3r1 myb3r4* double mutant was constructed previously by crossing T-DNA insertion mutants from the SALK collection (Alonso et al., 2003; Haga et al., 2007). Plants were grown on soil or agar medium at 22°C under continuous illumination (70–120 μmol m⁻² s⁻¹). Standard agar growth medium contained 0.5× Murashige and Skoog salts, 2% (w/v) Suc, and 0.2% (w/v) agar. Caffeine (Wako Pure Chemical) was dissolved in water, filter sterilized, and added to the agar medium.

#### Microscopic Observation

An Olympus BX54 microscope was used for observation with differential interference contrast (DIC) and fluorescence optics. For observation of embryos, oocytes were fixed in an ethanolacetic 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 inflorescence stems using tweezers, mounted in Hoyer’s solution, and imaged by DIC. GUS histochemical staining was performed using whole seedlings stained in a 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc) solution containing 1 mg mL⁻¹ X-gluc, 50 mM NaPO₄, pH 7.2, 0.5 mM K₃Fe(CN)₆, and 0.5 mM K₄Fe(CN)₆ at 37°C and cleared with 70% ethanol. DAPI staining was done as described previously (Haga et al., 2007).

#### Ploidy Analysis

Rosette leaves were used for the determination of ploidy levels, unless otherwise mentioned. A single rosette leaf was excised from each plant and chopped using a razor blade in 0.5 mL of nucleic-extraction buffer (solution A of the High Resolution Kit for Plant DNA; Partec). After filtration through a 30-μm mesh nylon sieve, 2.0 mL of the staining solution containing DAPI (solution B of the kit) was added. Ploidy levels were measured using a PAS flow cytometer (Partec). The lowest peak of wild-type plants was assumed to represent 2C nuclei (C is the haploid DNA content).

#### Generation of Transgenic Lines

The transgenic lines used in this study had been previously generated by transforming *myb3r1 myb3r4* double mutants with CDKA;1:KN and RPSSA::KN (Haga et al., 2007). For preparing GUS reporter constructs, pBGUS binary vectors were used in which the Gateway cassette (Invitrogen) was fused to the 5‘ end of GUS. Promoter fragments of *KN* (2.2 kb) were amplified from genomic DNA of the Columbia ecotype by PCR using primer sets 5‘-CACCAAGGAAAAATTAGCTTCACGAG-3’ and 5‘-CATAAAGCATTTCGCTC-ATCAAGTC-3’. The amplified fragment was cloned into pENTR/D/TOPO vector (Invitrogen) creating pENTR-KNpro, which was then used for subcloning into pBGUS binary vectors (Kubo et al., 2005) using LR Clonase (Invitrogen). PCR-based mutagenesis of MSA elements was performed using

---

**Figure 9.** Enrichment of MSA motifs in down-regulated genes in the *myb3r1 myb3r4* double mutant. The horizontal axis represents fold change levels in transcript abundance between the double mutant and the wild type (expressed as log2 value), while the vertical axis represents the average number of MSA motifs (identity score higher than 80%) within 1.0-kb upstream regions of the G2/M-specific genes (gray bars) and all other genes (black bars). Three bins (0.6–0.8, 0.8–1.0, and greater than 1.0) that held only two G2/M-specific genes maximum were excluded from the analysis of motif frequency (shown by asterisks). In all bins except these three, differences between G2/M-specific genes and other genes are statistically significant (P < 0.05, Fisher’s exact test).

**Figure 10.** Binding sequence of GST-MYB3R4 in vitro. The CASTing experiment with recombinant GST-MYB3R4 fusion protein resulted in the identification of 34 sequences with one AACGG motif. The sequence logo was created based on these sequences using WebLogo (Crooks et al., 2004). The overall height of each stack indicates the sequence conservation at each position (indicated as bits), whereas the height of symbols within the stack reflects the relative frequency of the corresponding bases at that position. (See online article for color version of this figure.)
Microarray Analysis
Three biological replicates for the wild type and the double mutant were used for microarray analysis with the Affymetrix Arabidopsis ATH1 GeneChip. Ten micrograms of total RNA, which had been extracted from 9-d-old seedlings, was used for labeling and hybridization following the manufacturer’s instructions (Affymetrix). Data analysis was performed using Microarray Suite version 5 (Affymetrix) and GeneSpring 7.1 (Agilent Technologies). Per-chip normalization was performed using the 50th percentile of all measurements to adjust total signal intensity in each chip. To verify the reliability of the data, whose detection call was present at least in the two biological replicates of wild-type samples were selected and used for further analysis (Figs. 6 and 9; Table II). After logarithmic transformation of the normalized data, two-tailed paired Student’s t test was applied comparing the wild type versus the double mutant, and only those genes with P < 0.05 were analyzed in Table II.

All microarray data have been deposited in the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/projects/geo/) under accession number GSE29433.

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure S1. Cell size effects of polyploidy in double myb3r1 myb3r4 mutants.

Supplemental Figure S2. MSA consensus sequence in promoters of G2/M-specific genes.

Supplemental Figure S3. Consensus DNA-binding motif of GST-MYB3R4.

Supplemental Table S1. Measurement of the macroscopic phenotypes in myb3r1 myb3r4 double mutants.

Supplemental Table S2. Frequency of occurrence of polyploid progeny.

Supplemental Table S3. List of 185 G2/M-specific class genes and comparison with previously defined classes.

Supplemental Table S4. Overrepresented motifs in promoters of the G2/M-specific genes.

Supplemental Table S5. Overrepresented motifs in oligonucleotides bound to GST-MYB3R4.

ACKNOWLEDGMENTS
We thank Chie Kotani, Ayana Kojima, Miki Yoshioka, Kanako Komatsu, Yuka Sako, and Hiro Iguchi for technical assistance and Yasunori Machida for helpful discussions.

Received May 26, 2011; accepted August 17, 2011; published August 23, 2011.

LITERATURE CITED
tively regulate cytokinesis through activation of KNOLLE transcription in Arabidopsis thaliana. Development 134: 1101–1110


