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Dual function of MIPS1 as a metabolic enzyme and transcriptional regulator

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ABSTRACT

Because regulation of its activity is instrumental either to support cell proliferation and growth or to promote cell death, the universal myo-inositol phosphate synthase (MIPS), responsible for myo-inositol biosynthesis, is a critical enzyme of primary metabolism. Surprisingly, we found this enzyme to be imported in the nucleus and to interact with the histone methyltransferases ATXR5 and ATXR6, raising the question of whether MIPS1 has a function in transcriptional regulation. Here, we demonstrate that MIPS1 binds directly to its promoter to stimulate its own expression by locally inhibiting the spreading of ATXR5/6-dependent heterochromatin marks coming from a transposable element. Furthermore, on activation of pathogen response, MIPS1 expression is reduced epigenetically, providing evidence for a complex regulatory mechanism acting at the transcriptional level. Thus, in plants, MIPS1 appears to have evolved as a protein that connects cellular metabolism, pathogen response and chromatin remodeling.

INTRODUCTION

Although it was first isolated from muscles, myo-inositol (MI) is a ubiquitous compound present in all living organisms. MI is required for the biosynthesis of a huge variety of cellular components, and thereby plays a crucial role in growth and development. In plants, products of MI metabolism are involved in diverse processes such as signal transduction, second messenger signaling, stress response, cell wall biogenesis and chromatin remodeling (1). The rate-limiting step for MI biosynthesis is catalyzed by the MI phosphate synthase (MIPS, E.C.5.5.1.4), and its function has been investigated in various plant species. Loss-of-function studies highlighted the diversity of crucial cellular processes relying on MI. The Arabidopsis genome encompasses three isoforms of MIPS, but MIPS1 seems the main player in MI biosynthesis because mips1 mutants have drastically reduced MI content. mips1 mutants display pleiotropic defects, including reduced root growth, abnormal vein formation in cotyledons (2,3) and defects in auxin polar transport due to alterations in lipid metabolism (4). However, the most striking feature of mips1 mutants is the light-dependent formation of lesions on leaves, implicating MIPS1 as a repressor of programmed cell death (2,3). In plants, cellular suicide is required during many steps of development such as xylogenesis (5), plant reproduction (6), leaf and petal senescence (7,8) and root cap and endosperm cell death during germination (9,10). Hence, cell fate can be influenced by differential MIPS regulation: its sustained expression is linked to cell proliferation and differentiation, whereas its down-regulation may be involved in the controlled cell death of specific tissues.

Several studies have provided evidence for a role of MIPS in biotic stresses. Indeed, mips1 mutants display improved resistance toward Hyaloperonospora arabidopsis, while mips3 mutants are more susceptible to a broad range of pathogens, including viruses, virulent and avirulent bacterial strains and the fungus Botrytis cinerea (11). Plant defense mechanisms induced by pathogen-associated molecular pattern (PAMP) recognition include hormone signaling via salicylic acid, jasmonic acid and ethylene, regulation of gene expression, strengthening of cell wall reactive oxygen species production and, in some cases, programmed cell death in the case of hypersensitive response (12). Early events of
PAMP-induced signaling have been dissected and in the case of bacterial flagellin (flg22), recognition involves two antagonistic MAPK signaling cascades: flagellin recognition by surface receptors triggers the activation of MEKK1, which in turn activates two MAPK modules. One consisting of MKK4/MKK5 and MPK3/MPK6 appears to activate defense genes, whereas the other comprising MKK1/MKK2 and MPK4 would repress them (13). Both mpk4 and mkk1/mkk2 mutants display a dwarf phenotype, spontaneous programmed cell death and constitutive activation of pathogen response. Interestingly, the transcriptome of mips1 mutants is similar to the one of mpk4 and mkk1/2 mutants, and expression of MIPS1 is reduced in these mutants according to publicly available micro-array data, suggesting that MIPS1 down-regulation may be induced by MAPKs to promote programmed cell death and pathogen resistance. Tight control of MIPS expression seems crucial to regulate MI accumulation and localized cell death on biotic stress.

Complex regulation of MIPS genes has been found in eukaryotes. In yeast, expression of the INO1 gene, encoding MIPS, is regulated depending on MI availability by three members of the SWI2/SNF2 class of chromatin remodeling complexes: it is activated in the absence of MI by SWI1/SNF, and INO80, whereas it is repressed on MI addition by ISW1 (14). In addition, the MI derivatives inositol polyphosphates (IPs) modulate the activity of several ATP-dependent chromatin remodeling complexes. In vitro data have shown that NURF-, ISW2- and INO80-stimulated nucleosome mobilization is inhibited by inositol hexakisphosphate (IP6). On the contrary, inositol tetrakisphosphate (IP4) and inositol pentakisphosphate (IP5) stimulate nucleosome mobilization catalyzed by SWI/SNF complex (15). In mammals, the gene encoding MIPS is regulated by DNA methylation (16). Hence, chromatin remodeling appears to be a widespread mechanism regulating MIPS expression in many eukaryotes, and similar mechanisms may operate in plants. Interestingly, we showed that the Arabidopsis MIPS1 protein interacts with ATXR5 and ATXR6 (2), two histone methyltransferases (HMTs) involved in the methylation of the lysine 27 of histone H3 (17). These observations led us to ask whether MIPS1 itself may be involved in the regulation of its own expression as a part of the plant response to exogenous cues such as pathogen attacks. Indeed, rapid changes in transcript levels, including down-regulation of a gene subset, are triggered by the bacterial elicitor flagellin (18). Pathogen-induced programmed cell death may therefore rely on the repression of MIPS1 expression via transcriptional mechanisms.

Here we show that in addition to its function as a key enzyme of MI metabolism, MIPS1 controls its own transcription through chromatin changes.

Additionally, regulation of MIPS1 on flagellin treatment and in mpk4 mutants points to a role of this mechanism in cell death. This dual function of MIPS1 may ensure MIPS1 expression under normal growth conditions and its down-regulation on a pathogen attack to induce programmed cell death.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds were surface sterilized by treatment with Bayrochlore (Bayrol) for 20 min, washed and imbibed in sterile water for 2–4 days at 4°C to obtain homogeneous germination. Seeds were sown on commercially available 0.5× Murashige and Skoog medium (Basalt Salt Mixture M0221, Duchefa) solidified with 0.8% agar (Phyto-Agar HP696, Kalys) with the suitable antibiotic if needed and grown in a long-day (16 h light, 8 h night, 21°C) growth chamber. After 2 weeks, the plants were transferred to soil in a greenhouse or in a growth chamber under short-day conditions (8 h light at 20°C, 16 h night at 18°C) for 2 weeks before being transferred to long-day conditions.

RNA extraction and real-time quantitative PCR analysis

Total RNA was extracted from seedlings using the RNasy MiniPrep kit (Qiagen) according to the manufacturer’s instructions. First-strand cDNA was synthesized from 2 μg of total RNA using Imprrom-II reverse transcriptase (A3802, Promega) according to the manufacturer’s instructions. 1/25th of the synthesized cDNA was mixed with 100 nM solution of each primer and LightCycler® 480 Sybr Green I master mix (Roche Applied Science) for quantitative PCR analysis. Products were amplified and fluorescent signals acquired with a LightCycler® 480 detection system. The specificity of amplification products was determined by melting curves. AtPTF2 was used as internal control for signal normalization. Exor4 relative quantification software (Roche Applied Science) automatically calculates relative expression level of the selected genes with algorithms based on ΔΔCt method. Data were from duplicates of at least two biological replicates. Primers used are described in Supplementary Table S1.

Histochemical β-glucuronidase assays

After 15-min fixation in 80% cold acetone, complete seedlings were stained in Eppendorf tube, and β-glucuronidase (GUS) activity was assayed using 5 mM ferri/ferrocyanide as described (19). After 2 h at 37°C, samples were washed in 70% ethanol for 10 min, and then cleared using chloral hydrate solution (8 g of chloralhydrate, 1 ml of glycerol and 2 ml of water). Images were captured on a microscope Axioskop (Zeiss) with a camera Spot RT slider (Diagnostic instrument) and enhanced using Adobe Photoshop software.

Confocal imaging

Plantlets were mounted in 5% glycerol and directly imaged on a TCS-SP2 upright microscope (Leica Microsystems) with 488-/543-nm excitation, 488-/543-nm beam-splitter filter and 515- to 615-nm (green channel) and 610- to 625-nm (red channel) detection windows. Transmitted light was also collected. All images were acquired with similar gain adjustments.
Chromatin immunoprecipitation analysis

Chromatin immunoprecipitation (ChiP) assays were performed on 12-day-old in vitro seedlings using anti-GFP Fluorescent Protein (GFP) (Santa Cruz), IgG control (Millipore), anti-H3K27mel (Millipore) or anti-H3K9ac (Millipore) antibodies, using a procedure adapted from Gendrel et al. (2005) (20). Briefly, after plant material fixation in 1% (v/v) formaldehyde, tissues were homogenized, and nuclei isolated and lysed. Cross-linked chromatin was sonicated using a water bath Bioruptor UCD-200 (Diagenode, Liège, Belgium) (30s on/30s off pulses, at high intensity for 24 min). Protein/DNA complexes were immunoprecipitated with antibodies, overnight at 4°C with gentle shaking, and incubated for 1 h at 4°C with 50 μL of Dynabeads Protein A (Invitrogen, Ref. 100-02D). Immunoprecipitated DNA was then recovered using the IPure kit (Diagenode, Liège, Belgium) and analyzed by quantitative real-time PCR. An aliquot of untreated sonicated chromatin was processed in parallel and used as the total input DNA control. Primers used as described in Supplementary Table S1.

Cell fractionation

14-day-old plantlets were ground to a fine powder in liquid nitrogen. Cells were homogenized and lysed in a buffer on ice (0.5 M sucrose, 15 M Tris at pH 7.5, 60 mM KCl, 0.25 mM ethylenediaminetetraacetic acid (EDTA), 0.125 mM ethylene glycol tetraacetic acid (EGTA), 0.5 M spermidine, 0.15 mM spermine, 1 mM dithiothreitol). Subsequently, Nonidet P-40 (Sigma) was added to a final concentration of 1%, and lysates were centrifuged to obtain a cytosolic supernatant and an nuclear pellet. Nuclei were further purified by centrifugation at 1000g through a 0.88 M sucrose cushion.

Transgenic lines generated in this study

The genotypes of the lines used in this work are as follows.

T-DNA insertion lines and their crossings: mips1-1 (Col0) and mips1-2 (2) T-DNA insertion lines.

Transgenic lines: PMIPS1::UidA, Col0; PMIPS1::UidA, Ws; PMIPS1::UidA, mips1-1, PMIPS1::UidA, mips1-2; PMIPS1::UidA, ddm1; PMIPS1::UidA, atxr5 atxr6 mips1-1.

PMIPS1::MIPS1-GFP (Col0); PMIPS1::MIPS1-GFP (atxr5 atxr6).

Strains and plasmids

Plasmid PMIPS1::UidA reporter was created by first amplifying the putative MIPS1 promoter, corresponding to 980 bp upstream of the translation start codon. PCR was performed on genomic DNA isolated from Col0 plants using the following primers: PMIPS1_promoter_Up (5′-CATAGAGAGTAGAGAGTGTAAG-3′) and PMIPS1_3’down (5′-GAGAGAGAAAGGAAATTTGGTTG-3′), and cloned in pGEM-T (Promega). The PMIPS1 fragment was then digested with Sphl and Sall and ligated into the pBI101-UidA. The HindIII–EcoRI fragment encompassing the PMIPS1::UidA fusion and the Nos terminator was then transfterred in the binary vector pPZP100 to obtain the PMIPS1::UidA plasmid. The MIPS1-GFP fusion driven by the PMIPS1 promoter was obtained as follows. First, PMIPS1-pBI101-UidA was digested with HindIII and Smal, and the resulting PMIPS1 fragment was ligated to pH7WG2 to obtain pH7WG2pMIPS1 (Plant Systems Biology, Vlaams Interuniversitair Instituut voor Biotechnologie, Ghent University) where the MIPS1 promoter replaces the 35S promoter. Then, the full-length cDNA encoding AtMIPS1 cloned between the BamHI and XhoI sites of the pENTRA1 vector (Invitrogen) was introduced in pH7WG2pMIPS1 by recombination using the Gateway technology (Invitrogen) according to manufacturer’s instructions.

Immunoprecipitations

First, expression and purification of Glutation S-transferase (GST)-ATXR5PhD-SET was performed according to Jacob et al. (2009) (21). Second, protein extraction of MIPS1-GFP plantlets was performed. Fourteen-day-old MIPS1-GFP plantlets were ground to a fine powder in liquid nitrogen. Cells were homogenized and lysed in Chris buffer containing 50 mM Tris (pH 8.0), 0.5% Nonidet P-40, 200 mM NaCl, 0.1 mM EDTA, 10% glycerol, 10 mM N-Ethylmaleimide (NEM) and protease inhibitors (Complete EDTA free, Roche).

Total cell lysates were then mixed with GST-tagged ATXR6 and incubated for 2 h at 4°C. MIPS1-GFP was then immunoprecipitated using a monoclonal anti-GFP antibody (Santa Cruz). Immune complexes were collected by incubation for 2 h at 4°C with Protein G plus/Protein A agarose (Pierce) and washed three times in lysis buffer.

Pulled-down proteins were detected by immunoblot using a polyclonal anti-GST antibody or the anti-GFP antibody.

Transient expression

Transient expression in tobacco leaves has been done following the protocol developed by Sparkes et al. (2006) (22). Proteins were extracted after 48 h.

Transient expression directly into 7-day-old Arabidopsis seedlings after vacuum infiltration of agrobacteria was done according to Marion et al. (2008) (23). Plantlets were observed by confocal microscopy after 48 h.

HMT activity

Measurement of HMT activity was performed following EpiQuik HMT Assay Kit instructions (Epigentek P-3005).

Supporting information

Accession numbers

The GenBank accession numbers of the genes and proteins discussed in this article are MIPS1: At4g39800; ATXR5: At5g09790; ATXR6: At5g24330; DCL1: At1g01370; MPK4: At4g01370; and DDM1: At5g66750. Lines used in this study were T-DNA lines SALK_023626 (mips1-1) and Flag_605F08 (mips1-2) for mips1, SALK_130607 and SAIL_0004263 for atxr5 and atxr6 and EMS line for ddm1 (24).
RESULTS

MIPS1 is required for its own expression

In yeast, expression of the INO1 gene encoding MIPS is induced when cellular levels of MI are low. To determine whether this regulation was conserved in Arabidopsis, we analyzed the transcription of MIPS1 in mips1 mutants, which are full knock-outs of the MIPS1 gene and have a drastic reduction of MI (2). We introduced a construct encompassing a 980-bp genomic fragment upstream of the MIPS1 start codon fused in-frame to the uidA reporter gene encoding GUS (PMIPS1::uidA). In wild-type Col0 plants, this construct had a similar expression pattern to the one reported by Donahue et al. (2010) (3), using a larger genomic fragment for the MIPS1 promoter (Figure 1A). Surprisingly, in the mips1-1 background, GUS staining was weaker than in Col0 in >45 transformed lines (Figure 1A). As silencing frequently occurs in SALK lines derived from Col0 (25), the reduced activity of the MIPS1 promoter could be an indirect consequence of the T-DNA insertion in the MIPS1 sequence. We therefore introduced the same construct in the mips1-2 mutant (2) and in the Ws control to confirm the previous observation in ten independent lines and discard any silencing effect. Quantification of uidA expression levels by qRT-PCR confirmed that in the absence of MIPS1, the MIPS1 promoter activity was reduced by >10-fold (Figure 1B). MI addition to the growth medium failed to restore normal activity of the MIPS1 promoter, demonstrating that this MIPS1-mediated regulation was not related to the cellular content in MI, which is consistent with the observation that addition of MI to the growth medium of wild-type plants does not modify MIPS1 mRNA accumulation (data not shown). To confirm that MIPS1 is required for its own expression, we transiently transformed mips1-1 mutants with a vector containing PMIPS1::MIPS1-GFP and with or without a plasmid containing 35S::MIPS1-RFP. Confocal microscopy analysis revealed that MIPS1-GFP was expressed in the mutant only when co-transformed with the 35S::MIPS1-RFP construct (Supplementary Figure S1). These results suggest that the MIPS1 promoter requires the MIPS1 protein to be active. Hence, the MIPS1 protein may have an unexpected role in the activation of its own transcription.

MIPS1 is localized both in cytoplasm and in nucleus, where it binds to its own promoter

In tobacco BY2 cells, a MIPS1-GFP fusion was localized both in the nuclear and the cytoplasmic compartment (2). This observation can be linked to the presence of a predicted nuclear export signal in the MIPS1 sequence (Supplementary Figure S2), and supports that MIPS1 could regulate its own expression in the nucleus. To confirm MIPS1 accumulation in the nucleus in planta, we analyzed Arabidopsis transgenic plants expressing a MIPS1-GFP fusion downstream of the MIPS1 promoter (PMIPS1::MIPS1-GFP). We first checked whether the promoter sequence we used conferred an expression pattern to the reporter gene that is identical to the one reported by Donahue et al. (2010) (3). To this end, we took advantage of the plasmids developed by Deal and Henikoff (2011) (26), which allow the expression of GFP targeted to the nuclear envelope under the control of a promoter of interest. Observation of plantlets by confocal microscopy revealed that all leaf cells but only some root cells (Supplementary Figure S3A) were GFP labeled. To quantify this, we next analyzed nuclei extracted from transgenic lines by flow cytometry to estimate the proportion of labeled cells in roots and leaves. As shown in Supplementary Figure S3B, almost all leaf cells (95%) but not all root cells (only 30%) express the MIPS1 gene, which is in agreement with previous observations (3). In addition, the MIPS1-GFP fusion is fully functional, as we demonstrated that it can complement the mutant phenotype (2). GFP localization in the transgenic PMIPS1::MIPS1-GFP plants can thus be considered as a good indicator of wild-type MIPS1 protein distribution. Confocal microscopy analysis revealed that the MIPS1-GFP fusion was localized not only mainly in the cytoplasm but also in the nuclei of root cells (Figure 2A). Furthermore, in a cell fractionation experiment (Figure 2B), the small RuBisCO subunit and histone H3 were used as controls for cell fractionation, MIPS1 was detected both in the nuclear and the cytoplasmic compartment, further supporting the hypothesis that MIPS1 could have a nuclear function.

To investigate whether MIPS1 can interact with its own promoter to promote transcription of the MIPS1 gene, we used ChiP analysis on 12-day-old light-grown seedlings of the PMIPS1::MIPS1-GFP plants using GFP antibodies. A marked enrichment of MIPS1 binding on the MIPS1 promoter (Figure 2C) was found using primers encompassing the whole locus. Hence, the MIPS1 protein is associated with chromatin and binds to its own promoter, suggesting that MIPS1 can directly regulate transcription of MIPS1.

MIPS1 interacts with ATXR6 and inhibit its activity in vitro

One possible mechanism accounting for the ability of MIPS1 to bind to its own promoter would be through interaction with proteins involved in chromatin re-modeling. Indeed, we initially isolated MIPS1 in a yeast two-hybrid screen as an interactor of ATXR5 and ATXR6 (2), two Arabidopsis SET [Suvar(3–9), Enhancer of zeste, Trithorax]-domain proteins involved in histone methylation and heterochromatin formation (21). To confirm this interaction, we first performed a co-immunoprecipitation. PATXR6::ATXR6-GFP and PMIPS1::MIPS1-HA constructs were transfected in tobacco leaves via Agrobacterium-mediated infiltration. After protein extraction, ATRX6 or MIPS1 was immunoprecipitated using, respectively, anti-GFP or anti-hyaluronic acid (HA) antibodies (Figure 3A). A band corresponding to GFP-tagged ATXR6 was revealed in the HA immunoprecipitate, and reciprocally a band corresponding to HA-tagged MIPS1 was revealed in the GFP immunoprecipitate (Figure 3A). When the same experiment was performed with leaves transfected...
only with MIPS1-HA or ATRX6-GFP, no band could be revealed in the GFP precipitate using an HA antibody or in the HA precipitate using the GFP antibody, confirming the specificity of the detected signal in the co-immunoprecipitation assay. This demonstrates that MIPS1 and ATXR6 interact in planta. As an independent test, we performed a pull-down experiment. A protein extract from stable transgenic plants expressing a MIPS1-GFP fusion protein was mixed with GST-tagged ATXR6 produced in a bacterial system. MIPS1-GFP was then immunoprecipitated using a monoclonal GFP antibody, and pulled-down proteins were detected by immunoblot using a polyclonal GST antibody (Supplementary Figure S3). A band corresponding to GST-tagged ATXR6 was revealed in the GFP immunoprecipitate (Supplementary Figure S4). All together, our results confirm that MIPS1 and ATXR6 interact and therefore could act in the same complex(es) in planta to regulate MIPS1 transcription.

ATXR6 is an HMT that catalyzes monomethylation of histone H3 at lysine 27 in Arabidopsis. It therefore functions as a repressor of gene expression. One function of the MIPS1 protein could thus be to antagonize the activity of ATXR5 and ATXR6 to allow MIPS1 expression. To test this hypothesis, we produced ATXR6 in vitro, and we measured its HMT activity alone or in the presence of MIPS1-GFP immunoprecipitated from transgenic plants. First, we checked that HMT activity was an increasing function of the ATXR6 quantity (Supplementary Figure S5A). Second, we mixed a constant quantity of ATXR6, which produced the maximum activity with increasing amounts of MIPS1-GFP, and we observed in this condition that HMT activity was a decreasing function of the MIPS1 amount (Supplementary Figure S5B). These results suggest that MIPS1 could inhibit ATXR6 activity in vitro.

Expression of MIPS1 is regulated by ATXR5- and ATXR6-dependent histone modification

To analyze whether MIPS1 regulates its own expression via its ability to modulate ATXR5 and ATXR6 activity, we investigated the role of ATXR5 and ATXR6 proteins in the control of MIPS1 transcription using the triple mutant atxr5 atxr6 mips1-1 transformed with PMIPS1::uidA. After analysis of GUS staining and quantification of GUS transcripts by qRT-PCR, we observed a 20- to 100-fold increase in the transcriptional activity of the MIPS1 promoter compared with the mips1-1 background (Figure 1A and B). This result suggests a repressive role of the HMT ATXR5/6 in the control of MIPS1...
promoter activity, likely through histone modifications. Surprisingly, qRT-PCR analysis revealed that MIPS1 mRNA accumulation is reduced in atxr5 atxr6 mutants (Supplementary Figure S6), suggesting an additional level of regulation of MIPS1 expression at the post-transcriptional level.

To explore more deeply the molecular mechanism of this ATXR5/6-dependent transcriptional repression, we analyzed histone modifications at the MIPS1 promoter to identify epigenetic marks associated with ATXR5 and ATXR6 activity. In vitro characterization of the enzymatic activity of ATXR5 and ATXR6 has previously revealed that these enzymes are H3K27 monomethyltransferases (21). We therefore performed ChIP assays using antibodies raised against monomethylated histone H3K27 on DNA fragments isolated from seedlings of the wild type and mips1-1. The enrichment of fragments distributed on the whole locus was compared between mips1-1 and the wild type (Figure 3B). We observed that monomethylation of H3K27 on the MIPS1 promoter was significantly increased on the whole locus in the mips1-1 mutant. This relative increase was particularly marked in the proximal promoter (insert of Figure 3B): indeed, H3K27me1 was increased 1.5- and 1.8-fold in fragments 2 and 3, whereas it was increased 1.8- and 3.5-fold in fragments 5 and 6, respectively. Fragments 2 and 3 are distant from the transcription start site (TSS) of the MIPS1 gene, but fragments 5 and 6 are in the immediate vicinity of the TSS, which is situated just downstream of fragment 6 (Figure 3B, top): increase of H3K27me1 in the proximal MIPS1 promoter therefore provides a potential mechanism for the observed reduction of MIPS1 expression. These data suggested that MIPS1 affects H3K27 methylation of its own promoter by antagonizing ATX5/6 activity, further demonstrating that ATXR5/6 activity at the MIPS1 locus is enhanced in the absence of MIPS1.
Taken together, our results demonstrate that transcriptional regulation of MIPS1 relies on the recruitment of the MIPS1 protein to reduce the deposition of inhibitory histone marks on its own promoter.

Activity of the MIPS1 promoter is regulated by DNA methylation

The combinatorial analysis of chromatin marks published by Roudier et al. (2011) (27) showed that H3K27me1 was often associated with DNA methylation. We then tested whether down-regulation of the MIPS1 promoter in the mips1 mutants could involve increased DNA methylation. First, we used zebularine, a cytosine analog, which on incorporation into DNA leads to global DNA demethylation and transcriptional reactivation of epigenetically silent genes and transgenes in Arabidopsis (28). Zebularine was applied for 48 h to 10-day-old mips1-1 plantlets transformed with PMIPS1::MIPS1-HA, and an increase of the MIPS1 promoter activity was observed in treated lines compared with the non-treated.

Figure 3. MIPS1 is epigenetically regulated. (A) MIPS1-HA and ATXR6-GFP interact in vivo. PATXR6::ATXR6-GFP and PMIPS1::MIPS1-HA constructs were transfected in tobacco leaves via Agrobacterium-mediated infiltration. After protein extraction, ATXR6 or MIPS1 was immunoprecipitated using anti-GFP or anti-HA antibodies, respectively, or anti-IgG antibodies as negative controls, and immunoprecipitates were analyzed with anti-GFP and anti-HA antibodies. As positive controls, we used whole cell lysate. The black star indicates IgG. (B) Methylation of H3K27 is increased in the mips1-1 mutant. H3K27me1 state at the whole MIPS1 locus was analyzed by ChIP on chromatin extracted from Col0 and mips1-1 plants using an anti-H3K27me1 antibody. Graph represents quantity of ChIP DNA in % of input in Col0 (black bars) and mips1-1 (grey bars). Error bars represent SD values from at least three repetitions. Insert: because fragments 5 and 6 are situated immediately upstream of the TSS of the MIPS1 gene, we specifically analyzed the relative enrichment in H3K27me1 in the proximal MIPS1 promoter. Error bars represent SD values from at least three repetitions. Primers used are the same as in Figure 2C. (C) Methyl-DNA immunoprecipitation analysis of DNA methylation in Col0, mips1-1, Ws and mips1-2 (arrow indicates the band that is quantified on right; star indicates primer). Quantification after input normalization is showed on the right of the gel.
ones (Supplementary Figure S7). Second, the mips1-1 PMIPS1::uidA line was crossed with the ddm1-2 mutant deficient for the DDM1 gene encoding an SWI/SNF2 chromatin remodeling factor, showing a reduction in the DNA methylation level. Both GUS staining and transcript level analysis by qRT-PCR showed that the transcriptional activity of the MIPS1 promoter is increased by 50- to 100-fold compared with the wild type in the mips1-1/ddm1-2 mutant (Figure 1A and B). Third, we analyzed DNA methylation in the promoter of MIPS1 by methyl-DNA immunoprecipitation using a monoclonal antibody raised against 5-methylcytidine (5 mC) (Figure 3C). An increase in DNA methylation was observed in mips1-1 and to a lesser extent in mips1-2 compared with Col0 andWs, respectively, indicating that increased H3K27 methylation of the MIPS1 promoter is indeed associated with increased DNA methylation. Importantly, we found the same regulatory mechanism to operate in the wild-type background because the activity of the MIPS1 promoter was increased in the ddm1-2 mutant compared with the wild type (Supplementary Figure S8). These results suggest a direct role of DNA methylation in the control of MIPS1 promoter activity. As we previously show that expression of the PMIPS1::uidA construct is higher in the atxr5/6 mutant than in the wild type, the deposition of both H3K27me1 and 5mC co-operate in the mips1 and in the wild-type background to modulate MIPS1 transcription. We next sought to place this regulatory mechanism in a physiological context.

**Flagellin induces a MIPS1 down-expression through the MPK4 pathway**

Disruption of MIPS1 results in spontaneous cell death, and the transcriptomic profile of mips1-1 is similar to plants subjected to pathogen infection (2). Induction of programmed cell death during hypersensitive response is a well-known mechanism allowing plant resistance toward pathogens (12). Hence, we postulated that down-regulation of MIPS1 may be part of the pathogen response of plant cells. We therefore examined MIPS1 expression in flg22-treated plants. Col0 plantlets were elicited during 1 h with or without 1 μM flagellin, and accumulation of MIPS1 mRNA was monitored by qRT-PCR. Plants of the Ws ecotype, which lacks the receptor to flg22, were used as a negative control. Accumulation of MIPS1 transcripts was decreased in flg22-elicited Col0 compared with untreated plantlets or treated Ws plants (Figure 4A). This down-regulation could be due to a transcription inhibition and/or to a decrease of the transcript stability. Accordingly, PMIPS1::uidA plantlets treated with 1 μM flg22 for 1 h showed reduced promoter activity (Figure 4B). These results suggest that MIPS1 transcription is reduced after flg22 elicitation.

We hypothesized that MIPS1 could act as a positive transcriptional regulator of its own expression. To determine whether MIPS1 binding to chromatin correlates with a promoter transcriptional state, we investigated whether MIPS1 could bind to its promoter after flg22 treatment. A ChIP experiment followed by qRT-PCR demonstrated that MIPS1 was released from chromatin after flg22 treatment (Figure 4C). This was accompanied by both a decrease in the euchromatin mark H3K9ac and an increase in the heterochromatin mark H3K27me1 (Figure 4D and E and Supplementary Figure S9), especially in the proximal promoter and first exon of the gene, suggesting that MIPS1 can directly impact the chromatin state of its own promoter after flg22 treatment.

Two MAPK signaling pathways are activated by flg22: MKK4/MKK5–MPK3/MPK6 activates pathogen responses, whereas MEKK1–MPK4 acts negatively on the same responses. To specify the signaling pathway governing MIPS1 mRNA down-regulation after flg22 treatment, we analyzed the expression of MIPS1 in the mpk4 mutant line. qRT-PCR revealed that MIPS1 is down-regulated in the mutant context (Figure 4). Together, these results suggest that flagellin induces MIPS1 down-expression through the MPK4 pathway.

**DISCUSSION**

We previously showed that MIPS1 protects plant cells against cell death under high light intensity or long days (2). In plants, cell death is important for forming body plans and specific organ shapes (29). Therefore, control of MIPS1 expression would be crucial for plant development. Here we have identified a transcriptional regulation of MIPS1 expression.

Analysis of MIPS1 promoter activity in the mips1 mutant backgrounds or by addition of MI to the growth medium indicates that the MIPS1 protein itself is a positive regulator of MIPS1 transcription. This hypothesis is in agreement with our previous observation that MIPS1 can accumulate also in the nucleus and can interact with the HMTs ATXR5 and ATXR6 in the yeast two-hybrid system (2). Here we have both confirmed the nuclear localization of MIPS1 and showed that MIPS1 binds to ATXR6 both in vivo and in vitro. ChIP analysis of the MIPS1 promoter revealed that ATXR5 and ATXR6 are required for the deposition of H3K27me1, and that this inhibitory mechanism is enhanced in the mips1 background. This result together with the observation that MIPS1-GFP can bind to the MIPS1 promoter strongly suggests that MIPS1 itself inhibits ATXR5/6 activity at this locus. This hypothesis is indeed confirmed by the observation that MIPS1 can inhibit ATXR6 activity in vitro. MIPS1 recruitment on the MIPS1 promoter would hence be required to prevent ATXR5/6-dependent histone methylation and associated DNA methylation, thereby allowing normal MIPS1 transcription. Transcriptionally silent genes often show promoter methylation (30), and genome-wide analysis of chromatin modifications has revealed that DNA methylation and H3K27me1 are often associated in promoters (27). Accordingly, we found DNA methylation to be increased at the MIPS1 promoter in the mips1 background. The MIPS1 locus occupies a sub-telomeric position on chromosome IV, and a Transposable element (TE), present in the distal part of the MIPS1 promoter, is constitutively methylated.
at the DNA level and at the H3K27 level (27). Although we cannot rule out that the MIPS1 protein may have an indirect effect on histone methylation in the vicinity of the MIPS1 gene, its ability to bind to its own promoter and to inhibit ATXR6 in vitro strongly suggests that MIPS1 directly operates at the MIPS1 locus to prevent heterochromatin spreading from the nearby TE (Figure 5). Indeed, transposable elements have been shown to be involved in local heterochromatin spreading not only in several organisms, including Drosophila melanogaster and mouse (31), but also in plant species such as Brassica napus (32) or melon, in which a DNA transposon is responsible for the spreading of DNA methylation into the CmWIP1 promoter, leading to sexual determination (33). H3K27me1 was increased in the absence of mips1 or on flagellin treatment, especially in the proximal promoter and at the beginning of the coding sequence, suggesting that MIPS1, when associated with the MIPS1 promoter, functions as a barrier against the propagation of this repressive chromatin mark. The relatively large distance between the peak of MIPS1 fixation in regions 1 and 2, and the region where H3K27me1 is increased in the absence of MIPS1 (regions 6 and 7), may reflect the formation of loop at this locus. Indeed, histone abundance is low in regions 4 and 5, and histone depletion facilitates chromatin loops (Supplementary Figure S10) (34).

The requirement of the MIPS1 protein for MIPS1 expression is observed even when the MIPS1 promoter is at an ectopic position in the genome as evidenced by the behavior of the pMIPS1::GUS construct or even on a plasmid as observed in transient expression assays. At least two hypotheses can account for this. One possibility would be that ATXR5- and ATXR6-mediated H3K27 methylation is guided by sequence information. Alternatively, the MIPS1 protein may be required to recruit positive regulators of MIPS1 expression.

Figure 4. Flagellin induces a MIPS1 down-expression through the MPK4 pathway. (A) Flg22 affects MIPS1 expression. Col0 plantlets were elicited during 1 h with or without 1 μM flagellin; total mRNA was extracted; and real-time PCR was performed. Plants from the Ws ecotype, which is not sensitive to flg22, were used as a negative control. (B) MIPS1 promoter activity is decreased after 1 h of 1 μM flagellin treatment. (C) MIPS1 is released on chromatin after flagellin treatment. ChIP assay from PMIPS1::MIPS1-GFP transgenic plants treated or not treated with flg22 was performed using an anti-GFP antibody. Immunoprecipitated DNA was analyzed by real-time PCR. Graph represents the enrichment of analyzed chromatin fragments relative to input chromatin. Error bars represent SD values from at least three repetitions. Position of primers pairs on the MIPS1 gene is indicated above (Figure 2C). (D) Methylation of H3K27 increases in the MIPS1 promoter after flg22 treatment. H3K27me1 state in the proximal MIPS1 promoter was analyzed by ChIP on chromatin extracted from Col0 treated or not treated with flg22 using an anti-H3K27me1 antibody. Graph represents relative enrichment compared with input. Error bars represent SD values from at least three repetitions. Primers used are the same as in Figure 3B. (E) Acetylation of H3K9 decreases in the MIPS1 promoter after flg22 treatment. H3K9ac state in the proximal MIPS1 promoter was analyzed by ChIP on chromatin extracted from Col0 treated or not treated with flg22 using an anti-H3K9ac antibody. Graph represents relative enrichment compared with input. Error bars represent SD values from at least three repetitions. Primers used are the same as in (D). (F) MIPS1 is down-regulated in mpk4 mutants. MIPS1 mRNA accumulation was quantified by qRT-PCR using Col0 as a control.
Although the dual function of MIPS1 in MI biosynthesis and transcriptional regulation may seem surprising, a few other examples of enzymes involved both in primary metabolism and chromatin metabolism have been documented. Recently in yeast, homocitrate synthase, which catalyzes the first step of lysine biosynthesis in the cytoplasm, has been shown to play a role in DNA damage repair independent of its catalytic activity (35). Similarly, we did not find any effect of MI in the control of MIPS1 expression: addition of MI in the growth medium did not restore the activity of the MIPS1 promoter in the mips1 mutant, suggesting that the MIPS1 protein itself, but not its catalytic activity, is required for this regulation.

Similarly, ARG5, which catalyzes the second step in ornithine biosynthesis, is also a nuclear and mitochondrial transcription factor (36). Yet another example is the kinase Gal1, which catalyzes a key step in catabolism of galactose and functions as a general activator of GAL gene transcription (37). However, here we showed that MIPS1 intervenes in the control of the epigenetic status of its own promoter.

Data reported here unveiled a complex control of MIPS1 expression at the transcriptional level, raising the question of the physiological relevance of such sophisticated mechanisms in planta. An essential component of Arabidopsis biotic stress responses resides in the plant capacity to reprogram its gene expression. Particularly, the stimulation of stress signaling pathway after pathogen attack is integrated into the nucleus through a set of transcription factor, which prioritizes defense over growth-related cellular functions (38). Importance of chromatin modifications and remodeling in the transcriptional regulation of defense-related genes is rapidly emerging (39). Salicylic acid (SA) plays an important role in establishing plant resistance against Pseudomonas syringae (40). The SA pathway is substantially controlled by histone modifications. Indeed, the Histone Deacetylase (HDAC) SRT2 suppresses the expression of the SA biosynthetic genes PAD4, EDS5 and SID2 (41), and loss of HDA19 activity results in enhanced basal expression of many downstream SA-responsive genes (e.g. PR1, PR4 and PR5) (42). All together, these reports suggest that chromatin remodeling plays a critical role in the control of Arabidopsis defense response. We previously showed that the cell death phenotype of mips1 is associated with improved resistance to pathogen attack. Thus, plant defense mechanisms may involve MIPS1 down-regulation to prevent pathogen proliferation. Our findings showed that flg22 treatment results in accumulation of heterochromatin marks on MIPS1 promoter in Arabidopsis and a release of MIPS1 from its own promoter, suggesting that on pathogen attack, MIPS1 is released from chromatin to allow ATXR5/ATXR6-dependent silencing of MIPS1. Analysis of mutants deficient for PAMP-induced signaling confirmed this hypothesis: the mpk4 mutant in which SA production and programmed cell death are constitutively activated (13,43) showed also a reduced MIPS1 expression. Further work will be required to elucidate how MAPK activation induces the release of MIPS1 from chromatin and subsequent reduction in MIPS1 promoter activity.

In summary, we have established an original picture of MIPS1 regulation directly involving MIPS1 in the control of its own transcription by inhibition of heterochromatin spreading. Thus, in plants, MIPS1 appears to have evolved as a bifunctional protein that connects cellular metabolism with chromatin functions and provide a new insight in the MIPS1 function.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online: Supplementary Table 1 and Supplementary Figures 1–10.

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