RESEARCH PAPER

Functional interconnections of *Arabidopsis* exon junction complex proteins and genes at multiple steps of gene expression

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Abstract

The exon junction complex (EJC) is deposited on mRNA after splicing and participates in several aspects of RNA metabolism, from intracellular transport to translation. In this work, the functional and molecular interactions of *Arabidopsis* homologues of Mago, Y14, and PYM, three EJC components that participate in intron-mediated enhancement of gene expression in animals, have been analysed. AtMago, AtY14, and AtPYM are encoded by single genes that show similar expression patterns and contain common regulatory elements, known as site II, that are required for expression. AtPYM and AtY14 are phosphorylated by plant extracts and this modification regulates complex formation between both proteins. In addition, overexpression of AtMago and AtY14 in plants produces an increase in AtPYM protein levels, while overexpression of AtPYM results in increased formation of a complex that contains the three proteins. The effect of AtMago and AtY14 on AtPYM expression is most likely to be due to intron-mediated enhacement of AtPYM expression, since the *AtPYM* gene contains a leader intron that is required for expression. Indeed, transient transformation asssays indicated that the three proteins are able to increase expression from reporter constructs that contain leader introns required for the expression of different genes. The results indicate that the plant homologues of Mago, Y14, and PYM are closely interconnected, not only through their function as EJC components but also at different steps of their own gene expression mechanisms, probably reflecting the importance of their interaction for the correct expression of plant genes.

Introduction

The expression of eukaryotic genes requires multiple steps to achieve the synthesis of correct amounts of the encoded proteins. Introns, once regarded as non-functional pieces of nucleic acid that must be removed for correct gene expression, are now known to participate in almost every step from gene transcription to protein synthesis. In fact, there is abundant evidence that certain introns produce a significant increase in gene expression, an effect known as intron-mediated enhancement (IME) (Rose and Last, 1997; Rose and Beliakoff, 2000; Rose, 2002, 2004; Kim *et al.*, 2004; Hsu *et al.*, 2005). IME has been demonstrated in several eukaryotic organisms, including plants (Matsumoto *et al.*, 1998; Lu and Cullen, 2003; Nott *et al.*, 2003; Nott, 2004). Among the vast literature about introns that enhance gene expression in plants, examples are found of introns that participate at the transcriptional, post-transcriptional (i.e. transcript stability or nuclear export), and translational levels (Morello *et al.*, 2002; Belostotsky and Rose, 2005; Glisovic *et al.*, 2008; Lee *et al.*, 2009; Moore and Proudfoot, 2009). Even if it is not clearly known what properties of introns make them suitable to enhance gene expression, some clues were found in systematic studies of intron composition, length, and position within the transcription unit. Introns that participate in IME tend to be longer, to

be located near the transcription start site, and to have an increased number of repetitions of certain short nucleotide sequences (Gniadkowski *et al.*, 1996; Bourdon *et al.*, 2001; Nott *et al.*, 2003; Kim *et al.*, 2004). Based on these characteristics, Rose *et al.* (2008) have designed an algorithm (IMEter) that estimates the efficiency of an intron in IME.

Introns present in the 5'-non-coding region of *Arabidopsis COX5c* genes, encoding a subunit of mitochondrial cytochrome *c* oxidase, produce a significant increase in expression (Curi *et al.*, 2005). These introns have high IMEter scores (Rose *et al.*, 2008) and enhance expression of genes from different promoters, increasing both transcript levels and protein synthesis (Curi *et al.*, 2005; Cabello *et al.*, 2007). An effect of introns on translation is counterintuitive since introns are removed in the nucleus and translation takes place in the cytoplasm. This apparent paradox was solved when it was discovered that proteins deposited in the nucleus during splicing remain bound to the mRNA in the cytoplasm. These proteins form a complex that localizes near the junction of two exons (20–25 nucleotides upstream) and is thus known as the exon junction complex (EJC) (Lau *et al.*, 2003; Nott *et al.*, 2003; Wiegand *et al.*, 2003; Bono *et al.*, 2006; Lee *et al.*, 2009). It is through this complex that the effect of introns is transmitted to processes that occur after their removal. Many proteins have been described as participants in the EJC, but four of them form the core that is intimately associated with RNA and participates in most EJC functions; these are Mago, Y14, eIF4AIII, and MLN51 (Zhao *et al.*, 1998, 2000; Li *et al.*, 1999; Kim *et al.*, 2001; Tange *et al.*, 2005). Mago and Y14 form a dimer that joins the two other proteins and participates in mRNA localization, nonsense-mediated mRNA decay (NMD), and IME in animals (Le Hir, 2001; Dostie and Dreyfuss, 2002; Lau *et al.*, 2003; Shi and Xu, 2003). Another protein that has been shown to be involved in IME in animals is PYM, identified as a Partner of Y14 and Mago through its capacity to bind to the complex (Forler *et al.*, 2003). Rather than a *bona fide* component of the EJC, PYM has been described as a factor that links the EJC to the ribosome and participates in the dissociation and recycling of the complex (Diem *et al.*, 2007; Gehring *et al.*, 2009).

Homologues of Mago, Y14, and PYM have been described in plants (Swidzinski *et al.*, 2001; Park and Muench, 2007; Park *et al.*, 2009). Mago and Y14 from the tree *Taiwania cryptomerioides* were shown to interact and are expressed preferentially in root hairs (Chen *et al.*, 2007). Overexpression of TcMago in tobacco produced taller plants with increased root growth, suggesting that it is a regulator of plant growth. Interaction of Mago and Y14 homologues has also been described in *Physalis floridana* and *Arabidopsis* (He *et al.*, 2007; Park and Muench, 2007). Knock-out of *AtMago* is lethal in *Arabidopsis*, and its partial silencing leads to several defects in plant development, affecting root, shoot, and leaf growth and meristem, pollen, and seed development (Park *et al.*, 2009). Pollen from hemizygous knock-out plants shows defects in pollen tube growth, and a Mago homologue is essential for spermatogenesis in the fern *Marsilea* (Johnson *et al.*, 2004;

van der Weele *et al.*, 2007). Mago and Y14 function in one of two NMD mechanisms that exist in plants, according to studies in *Nicotiana benthamiana* (Kerényi *et al.*, 2008). PYM has been studied in *Arabidopsis*, where it interacts with AtMago and AtY14, both with the complex and with the isolated proteins, and shuttles between the nucleus and the cytosol (Park and Muench, 2007). Different portions of AtPYM are involved in the interaction with the complex or with the isolated proteins. These properties suggest that the interaction of PYM with EJC components is different in plants and animals.

In this work, the functional interactions of *Arabidopsis* homologues of Mago, Y14, and PYM and of the respective genes have been investigated. It was observed that the three genes display similar expression patterns and depend on common elements for their expression, suggesting the existence of transcriptional co-regulation. In addition, AtPYM expression is enhanced by the two other proteins and is subjected to an autoregulatory mechanism through a process that depends on its leader intron, and the interaction of AtY14 and AtPYM is regulated at the post-translational level through phosphorylation of both proteins. The results indicate that the function of the three proteins is tightly regulated, probably reflecting the importance of their interaction for the correct expression of plant genes. In this sense, it was observed that the three proteins participate in IME in plants, as they do in animal cells.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana Heyhn. ecotype Columbia (Col-0) was purchased from Lehle Seeds (Tucson, AZ, USA). Plants were grown on soil in a growth chamber at 22–24 °C under long-day photoperiods (16 h of illumination by a mixture of cool-white and GroLux fluorescent lamps) at an intensity of ~90 µE m^{-2} s^{-1}. Alternatively, plants were grown in Petri dishes containing 0.5× Murashige and Skoog (MS) medium and 0.8% agar.

Reporter gene construct and plant transformation

A fragment spanning 2135 bp upstream of the ATG initiation codon of the *AtMago* gene (At1g02140) was obtained by PCR amplification of *Arabidopsis* genomic DNA using primers pMagoF and pMagoR (see Supplementary Table S1 available at *JXB* online). The resulting fragment, containing *Sal*I and *Bam*HI sites, was cloned in the same sites of pBI101.3. A similar strategy was used to clone a fragment spanning nucleotides –2177 to –1 and –2253 to –1 with respect to the ATG initiation codon of the *AtY14* (At1g51510) and *AtPYM* (At1g11400) genes, respectively. Complementary primers were used to introduce mutations or perform deletions using overlap extension mutagenesis by PCR (Silver *et al.* 1995). The sequences of the oligonucleotides used are shown in Supplementary Table S1. All constructs were checked by DNA sequencing.

The respective constructs were introduced into *Agrobacterium tumefaciens* strain LB4404, and transformed bacteria were used to obtain transgenic *Arabidopsis* plants by the floral dip procedure (Clough and Bent, 1998). Transformed plants were selected on the basis of kanamycin resistance and positive PCR carried out on genomic DNA with gene-specific primers and the *gus* (β-glucuronidase) primer 5′-TTGGGGTTTCTACAG-GAC-3′. Thirty independent lines for each construct were further reproduced, and homozygous T$_3$ and T$_4$ plants were

used to analyse *gus* expression. Plants transformed with pBI101.3 or pBI121 were obtained in a similar way.

β-Glucuronidase assays

The GUS activity of transgenic plants was analysed by histochemical staining using the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc) as described by Hull and Devic (1995). Whole plants or separated organs were immersed in a 1 mM X-gluc solution in 100 mM sodium phosphate, pH 7.0, and 0.1% Triton X-100, and, after applying a vacuum for 5 min, they were incubated at 37 °C until colour development. Tissues were cleared by immersing them in 70% ethanol. Specific GUS activity in protein extracts was measured using the fluorogenic substrate 4-methylumbelliferyl β-D-glucuronide (MUG) essentially as described by Jefferson *et al.* (1987). Total protein extracts were prepared by grinding the tissues in extraction buffer (50 mM sodium phosphate, pH 7.0, 10 mM EDTA, 10 mM β-mercaptoethanol) containing 0.1% (w/v) SDS and 1% Triton X-100, followed by centrifugation at 13 000 *g* for 10 min. GUS activity in supernatants was measured in extraction buffer containing 1 mM MUG and 20% methanol. Reactions were stopped with 0.2 M Na_2CO_3 and the amount of 4-methylumbelliferone was calculated by relating relative fluorescence units to those of a standard of known concentration. Controls were made to ensure that activity measurements were within the linear range as a function of time and protein concentration. The protein concentration of extracts was determined as described by Sedmak and Grossberg (1977).

Transient transformation of Arabidopsis seedling

Arabidopsis transient transformation was carried out following the protocol proposed by Li *et al.* (2009) with modifications. The day before co-cultivation, liquid cultures of *A. tumefaciens* were inoculated from frozen glycerol stocks. After growth at 28 °C in 2 ml of LB medium with appropriate antibiotics for 24 h, a saturated culture was diluted into 10 ml of fresh LB medium to OD_{600}=0.3 and grown until the OD_{600} reached 1.5. Bacterial cells were harvested by centrifugation at 6000 *g* for 5 min and washed once with 10 ml of washing solution containing 10 mM $MgCl_2$ and 100 μM acetosyringone. After centrifugation at 6000 *g* for another 5 min, the pellet was resuspended in 1 ml of washing solution. In a sterilized 12-well plate, 30–40 transgenic *Arabidopsis* seedlings of 5 d old were soaked with 20 ml of co-cultivation medium containing 0.25× MS, 1% sucrose, 100 μM acetosyringone, 0.005% (v/v) Silwet L-77, and *A. tumefaciens* cells at a final density of OD_{600}=0.5. Co-cultivation was carried out in darkness in a growth chamber for 36–40 h. Then, β-glucuronidase assays were performed.

RNA isolation and analysis

Total RNA was prepared with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For northern blot analysis, specific amounts of RNA were electrophoresed through 1.5% (w/v) agarose/6% formaldehyde gels. The integrity of the RNA and equality of RNA loading were verified by ethidium bromide staining. RNA was transferred to Hybond-N nylon membranes (GE Healthcare) and hybridized overnight at 68 °C to a ^{32}P-labelled cDNA probe, comprising the entire GUS coding region isolated from vector pBI101.3, in buffer containing 6× SSC, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) bovine serum albumin (BSA), 0.1% (w/v) Ficoll, 0.2% (w/v) SDS, and 10% (w/v) polyethylene glycol 8000. Filters were washed with 2× SSC plus 0.1% (w/v) SDS at 68 °C (four times, 15 min each), 0.1× SSC plus 0.1% (w/v) SDS at 37 °C for 15 min, dried, and exposed to Kodak BioMax MS films. To check the amount of total RNA loaded in each lane, filters were then re-probed with a 25S rDNA from *Vicia faba* under similar conditions to those described above, except that hybridization was performed at 62 °C and the wash

with 0.1× SSC was omitted. Quantitative analysis was performed with ImageQuant™ TL Software (GE Healthcare).

Cloning, expression, and purification of recombinant proteins

The AtMago and AtY14 full-length coding sequences were amplified using oligonucleotides Mago-pGEXF and Mago-pGEXR, or Y14-pGEXF and Y14-pGEXR, respectively, and inserted in-frame into the *Bam*HI and *Eco*RI sites of the expression vector pGEX-3X (Smith and Johnson, 1988). For expression, *Escherichia coli* cells bearing the corresponding plasmids were grown and induced as described previously (Palena *et al.*, 1998). Purification of the fusion products was carried out essentially as described by Smith and Johnson (1988), with modifications described by Palena *et al.* (1998). The AtPYM coding sequence was cloned in-frame with the maltose-binding protein (MBP) in the *Eco*RI and *Pst*I sites of plasmid pMAL-c2 (New England Biolabs), using oligonucleotides Pym-pMALF and Pym-pMALR. Purification of the recombinant protein was performed as indicated by the manufacturers of the pMAL-c2 system.

Western blotting

Antibodies against AtMago, AtY14, and AtPYM were obtained by immunization of rabbits with the corresponding purified recombinant proteins using standard protocols. Controls using pre-immune sera in western blots of plant extracts showed the absence of immunoreactive bands before immunization. Antisera were pre-adsorbed with *E. coli* extracts expressing glutathione *S*-transferase (GST) or MBP, depending on the protein used to raise antibodies, before their use in western blots. Control experiments using recombinant AtMago, AtY14, and AtPYM showed no cross-reaction of antibodies with their non-target proteins (Supplementary Fig. S1 at *JXB* online). Plant protein extracts were prepared according to the protocol proposed by Milosevic and Slusarenko (1996). Fractions were solubilized in SDS sample buffer (100 mM TRIS-HCl, pH 8.0, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.002% Bromophenol blue), heated at 100 °C for 5 min, and analysed by SDS–PAGE using 12.5–15% polyacrylamide gels. Native PAGE was carried out using standard buffers without SDS or β-mercaptoethanol. The gels were blotted onto nitrocellulose membranes overnight. The membranes were blocked for 1 h at room temperature in 3% skim milk and probed with polyclonal antibodies against AtMago, AtY14, or AtPYM (dilutions 1:2500, 1:2000, and 1:5000, respectively) for 1 h at room temperature in 3% skim milk. The blots were washed and subsequently incubated at a 1:2500 dilution in the same buffer for 1 h at room temperature with anti-rabbit immunoglobin conjugated with horseradish peroxidase. After extensive washing, the blots were developed with the SuperSignal® West Pico Chemiluminescent Substrate (Pierce).

Pull-down assays

For pull-down assays, 3 μg of MBP–AtPym were incubated with either 3 μg of GST–AtMago or GST–AtY14 in binding buffer [20 mM HEPES pH 7.5, 50 mM KCl, 2 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithiothreitol (DTT), 0.5% (w/v) Triton X-100, 10% (v/v) glycerol, and 22 ng μl^{-1} BSA] at 4 °C for 30 min. Amylose beads (30 μl) were then added and the mixture was incubated at 4 °C for 3 h with gentle agitation. The beads were harvested and washed with buffer containing 20 mM TRIS-HCl (pH 7.4), 130 mM NaCl, 1 mM EDTA, and 0.07% (v/v) β-mercaptoethanol. Proteins were eluted from the beads by incubation in SDS sample buffer at 65 °C for 10 min and then were analysed by SDS–PAGE and western blotting.

In vitro phosphorylation assay

In vitro phosphorylation was carried out following a strategy suggested by Gong *et al.* (2002) with some modifications. Recombinant proteins (3 μg) were incubated in the presence of a kinase

buffer (20 mM TRIS-HCl pH 7.2, 5 mM MgCl$_2$, 0.5 mM CaCl$_2$, 2 mM DTT, 10 μM ATP, and 8 μCi of [γ-^{32}P]ATP) and a plant extract enriched in protein kinases (3 μg) obtained according to the protocol proposed by Toroser *et al.* (2000). Reactions were incubated for 30 min at 30 °C and stopped by boiling in 5× SDS sample buffer. Samples were analysed by SDS–PAGE and the gel was dried and exposed to Kodak BioMax MS films.

Dephosphorylation of protein extracts

Differents amounts of calf intestinal alkaline phosphatase (2–8 U) were incubated with 30 μg of protein extract in reaction buffer (50 mM TRIS-HCl pH 9.3, 10 mM MgCl$_2$, 1 mM ZnCl$_2$, 10 mM spermidine) at 37 °C for 30 min. Reactions were stopped with the addition of 5× SDS sample buffer and boiling, and analysed by SDS–PAGE and western blotting.

Results

Genes encoding AtMago, AtY14, and AtPYM share expression characteristics and mechanisms

Homologues of the EJC components Mago, Y14, and PYM are encoded by single genes in *Arabidopsis*. To analyse the

expression patterns conferred by the respective promoter regions of these genes, intergenic sequences located upstream of the translation start sites were cloned in the binary vector pBI101.3, that contains the *uidA* (*gus*) reporter gene, and used to transform *Arabidopsis* plants. Ten independent transformants for each construct were analysed by histochemical detection of GUS activity. The expression patterns originated by the three promoter regions were very similar (Fig. 1A–C, left panels). Preferential expression was observed in root and shoot meristems and vascular tissues of leaves, hypocotyls, and cotyledons. In mature plants, expression was higher in roots, and pollen and was also present in the receptacle of flowers and siliques.

Analysis of the three promoter regions for the presence of common regulatory elements revealed that they all contain motifs known as site II (TGGGCC/T) that have been previously related to the expression of genes encoding ribosomal proteins and mitochondrial components (Tremousayge *et al.*, 2003; Welchen and Gonzalez, 2005, 2006). *AtMago* contains three site II elements, two of them in close proximity (around –110 from the translation start
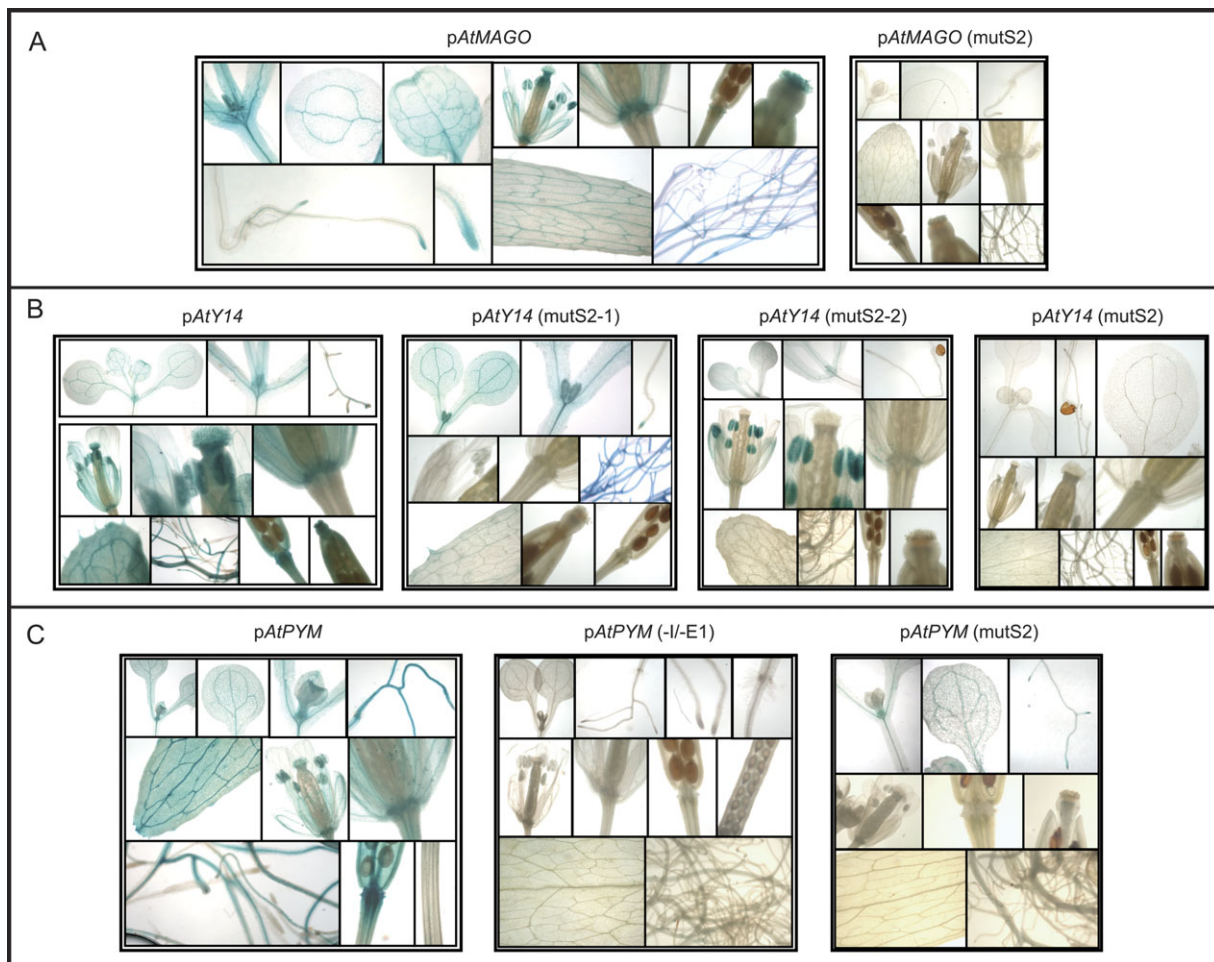
Fig. 1. Expression patterns conferred by the *AtMago* (A), *AtY14* (B), and *AtPYM* (C) upstream regions. Histochemical detection of GUS activity in plants transformed with the respective upstream regions (left panels), or with the same regions in which site II elements were mutagenized (mutS2; right panels). For the *AtY14* upstream region, the effect of mutations on two individual pairs of site II elements is shown (middle panels in B). The middle panel in C shows plants transformed with the *AtPYM* upstream region without the leader intron. See Supplementary Fig. S1 at *JXB* online for details about the location of elements.

site) and the third one at –148 (Supplementary Fig. S2 at *JXB* online). Mutation of the two proximal elements produced a complete loss of activity of the promoter (Fig. 1A, right panel). The same result was observed when the four site II elements present in the *AtY14* promoter were mutated (Fig. 1B, right panel). These elements occur in pairs, embedded in a 24 bp repeated sequence, in the *AtY14* promoter (Supplementary Fig. S2). Interestingly, mutation of the elements located in the proximal repeat (at –164) mainly affected expression in reproductive tissues, while mutation of the distal (–265) elements produced a decrease in expression in vegetative tissues (Fig. 1B, middle panels). Both groups of site II elements thus seem to participate in expression in different organs. Mutation of both *AtPYM* site II elements produced plants with reduced expression of the reporter gene, still detectable in seedlings but not in mature plants (Fig. 1C, right panel). Individual mutation of site II elements showed that they have an additive effect on expression in all tissues. The results obtained by histochemistry were confirmed using fluorometric measurements of GUS activity in extracts prepared from seedlings and organs from mature plants (Supplementary Fig. S3 at *JXB* online). A general conclusion of these experiments is that genes for the three EJC components analysed share expression patterns and characteristics, suggesting that they are implicated in similar processes during plant development.

The interaction of AtPYM and AtY14 is regulated by phosphorylation

Mago and Y14 are able to interact in the absence of other EJC components in several organisms, including plants (Mohr *et al.*, 2001; Fribourg, 2003; Kawano *et al.*, 2004; Chen *et al.*, 2007; Park and Muench, 2007; Chu *et al.*, 2009). The interaction of PYM with these proteins, however, seems to vary in different organisms. While PYM interacts only with the Mago–Y14 complex but not with the individual proteins in animals (Bono *et al.*, 2004; Gehring *et al.*, 2009), studies in *Arabidopsis* indicated that AtPYM is able to form separate complexes with AtMago and AtY14 (Park and Muench, 2007). Using recombinant forms of the proteins in pull-down assays, it was possible to confirm these observations that raise a question on the function of AtPYM in plants. In animals, it has been proposed that PYM produces the disassembly of the EJC through its interaction with the Mago–Y14 complex as the mRNA is read by the ribosome, thus allowing the recycling of EJC components (Gehring *et al.*, 2009). This function of PYM requires a dual behaviour (i.e. interaction with the complex but not with the isolated components) to be effective. It was therefore investigated if a dual behaviour producing cycles of interaction and disassembly may be achieved in plants through protein modification by phosphorylation. For this purpose, recombinant forms of AtMago, AtY14, and AtPYM were incubated with a plant extract that is rich in active protein kinases in the presence of radiolabelled ATP. Figure 2A shows that, apart from proteins from the extract, the recombinant forms of AtY14 and AtPYM were

phosphorylated, but AtMago was not. For AtY14 and AtPYM, two bands of close mobility were detected in plant extracts with specific antibodies, a behaviour that may be observed when proteins are modified by phosphorylation. However, experiments using alkaline phosphatase, that should remove accessible phosphates from the proteins, were not conclusive about the phosphorylation state of the proteins within the plant (not shown).

The effect of phosphorylation on the interaction of AtPYM with AtMago and AtY14 was then tested using pull-down assays with proteins that have been previously treated with the phosphorylating extract in either the presence or the absence (mock treatment) of ATP. As shown in Fig. 2B, phosphorylation of either AtY14 or AtPYM diminished the amount of recombinant AtY14 that was bound to resin that contained bound AtPYM, suggesting that formation of the complex requires that both proteins are dephosphorylated. In addition, once the complex is formed, it can be disrupted by phosphorylation, as suggested by an experiment in which the treatment with the phosphorylating extract was performed after the two proteins were allowed to interact (Fig. 2C). Binding of AtPYM to AtMago, on the other hand, was not affected by phosphorylation (Fig. 2D).

The formation of complexes containing AtMago, AtY14, and AtPYM in plant extracts was analysed using nondenaturing gels followed by immunodetection with specific antibodies. Two bands of similar mobility were detected with the three antibodies, indicative of co-migration of the three proteins, most probably due to their integration into two different complexes (Fig. 3A). These complexes most probably have different compositions and contain, in addition to AtMago, AtY14, and AtPYM, other EJC components that are known to associate with and dissociate from core components dynamically (Tange *et al.*, 2005). Interestingly, the intensity of the bands representing the complexes, for the three antibodies, was increased when extracts of plants overexpressing AtPYM were used. This would indicate that AtPYM overexpression produces an increase in complex formation, perhaps due to its interaction with AtMago and AtY14. Treatment of extracts with alkaline phosphatase produced an increase in the formation of the complex with higher mobility (Fig. 3B). It can be speculated that in *Arabidopsis* AtPYM would have a dual function in complex formation and dissociation according to its phosphorylation state, in agreement with the results obtained with the recombinant proteins.

Overexpression of AtMago or AtY14 in plants produces an increase in AtPYM levels

In the experiments described above, it was not possible to observe high mobility bands corresponding to the proteins dissociated from the complex. Probably the dissociated forms of the proteins migrated at the front of the non-denaturing gel or did not focus into a defined band. When the same antibodies and plant extracts were used in western blots from SDS–PAGE to analyse the expression levels of
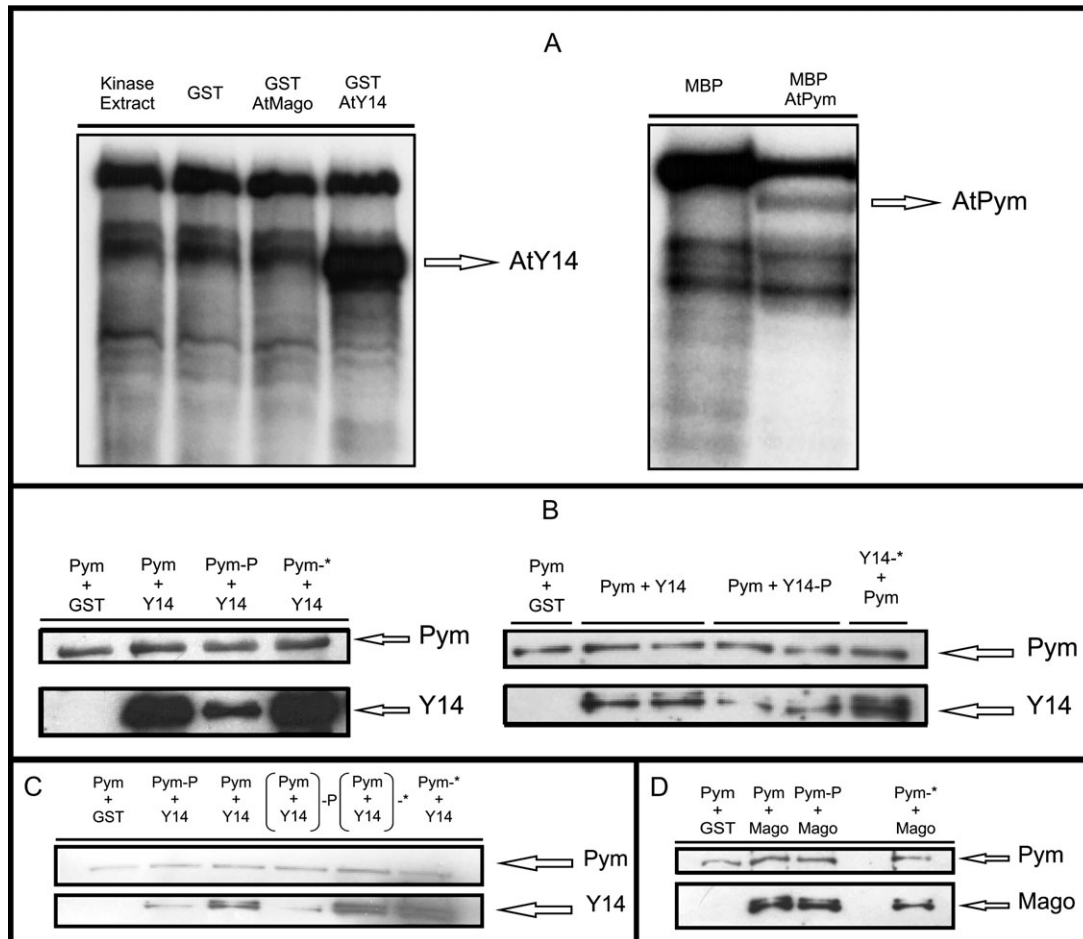
Fig. 2. The interaction of AtY14 with AtPYM is regulated by phosphorylation. (A) Phosphorylation assay of recombinant AtMago, AtY14, and AtPYM in the presence of a plant extract enriched in protein kinases. The phosphorylation assay was performed in the presence of labelled ATP and plant extract with or without the addition of GST, MBP, GST–AtY14, GST–AtMago, or MBP–AtPYM. Reaction mixtures were analysed by SDS–PAGE followed by autoradiography. Arrows indicate the migration of AtY14 and AtPYM. (B–D) Pull-down assays using GST–AtY14 or GST–AtMago and MBP–AtPYM binding to amylose resin. Where indicated, AtPYM or AtY14 were previously incubated with the phosphorylating extract either in the presence (P) or in the absence (*) of ATP. In C (lanes 4 and 5), treatment with the extract was performed after complex formation between AtY14 and AtPYM. The proteins were detected by western blots using specific antibodies.

the proteins under study in the different overexpressing lines, it was evident that AtPYM levels were higher than those of wild-type plants in lines that overexpress AtMago and AtY14 (Fig. 3C). These higher levels may originate from increased synthesis or stability of AtPYM caused by overexpression of the other proteins. Increased stability may originate from complex formation with AtMago or AtY14. However, increased complex formation was not observed in plants that overexpress these proteins as was evident in plants that overexpress AtPYM (Fig. 3A). This is noteworthy, since these plants also have higher levels of AtPYM. The reasons for this may be several, among them the fact that AtPYM levels were not as high in plants that overexpress AtMago or AtY14 in comparison with plants that overexpress AtPYM, the occurrence of post-translational modifications (i.e. phosphorylation) that influence complex formation, and the fact that the relative AtMago and AtY14 levels are different in plants that overexpress one of these proteins and plants that overexpress AtPYM.

Increased synthesis of AtPYM may be related to an effect of AtMago and AtY14 in IME of AtPYM expression. The *AtPYM* gene contains a leader intron in its 5'-non-coding region that was included in the constructs used to analyse expression of the gene. The influence of this intron on expression was evaluated in plants transformed with a construct in which the intron was removed. These plants showed no GUS staining in any tissue or organ analysed, and the GUS-specific activity was similar to that of plants transformed with a promoterless *gus* gene, suggesting that the leader intron is required for expression (Fig. 1C, middle panel; Supplementary Fig. S3 at *JXB* online). The same result was obtained when expression from a construct that retained the intron but contained a deletion of all except the three last nucleotides of exon 1 was analysed. This type of effect has been ascribed to a role for the EJC in IME, assuming that exon sequences located immediately upstream of the intron are necessary for the interaction of
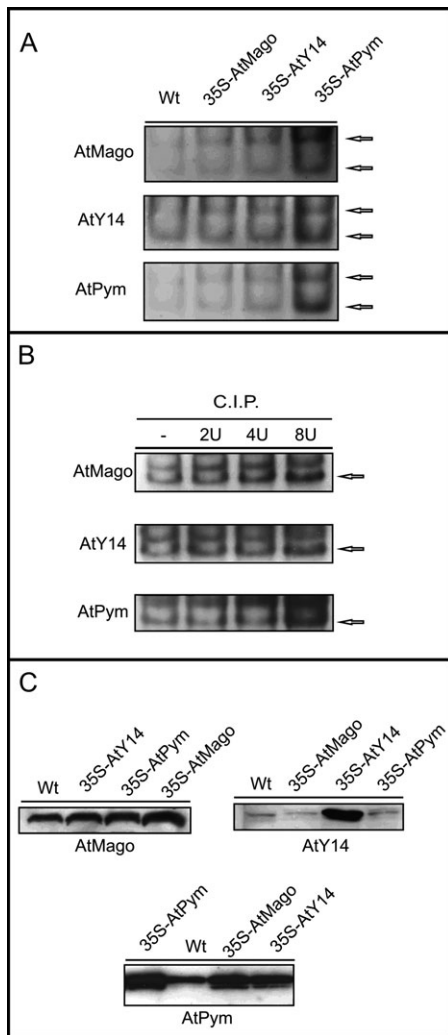
Fig. 3. Detection of AtMago, AtY14, and AtPYM in wild-type and overexpressing plants. Extracts from wild-type plants and from plants transformed with constructs that express AtMago, AtY14, or AtPYM under the control of the 35SCaMV promoter were analysed by non-denaturing (A, B) or denaturing (C) PAGE followed by western blots using specific antibodies (indicated beside each panel). In B, the extract was treated with different amounts of alkaline phosphatase before the analysis.

Fig. 4. The *AtPYM* leader intron increases GUS expression at different levels. GUS activity (A) and *gus* transcript levels (B) in plants transformed with different forms of the *AtPYM* promoter fused to *gus* were compared with those of plants transformed with pBI121, that contains the *gus* gene under the control of the 35SCaMV promoter. Two or three independent lines were analysed for each construct. GUS activity levels are expressed as the mean ($\pm$SD) of three independent measurements. Transcript levels were undetectable in plants transformed with modified forms of the *AtPYM* promoter (–I, without the leader intron; –E1, with the upstream portion of exon 1 deleted; mutS2, with mutagenized site II elements).

the EJC with the processed mRNA (Wiegand *et al.*, 2003; Nott *et al.*, 2004).

Previous studies with *AtCOX5c-2* showed that the presence of the leader intron influences expression of this gene at both the transcript and the protein levels (Curi *et al.*, 2005). A similar analysis for the *AtPYM* gene was performed by comparing GUS transcript and activity levels in plants transformed with fusions of the reporter gene to the *AtPYM* or the 35S cauliflower mosaic virus (CaMV) promoter. As shown in Fig. 4A, GUS activity levels produced by the *AtPYM* promoter (with the leader intron) were about one-half of those observed with 35SCaMV. Transcript levels, in turn, represented on average only 2% of those produced by 35SCaMV (Fig. 4B; Supplementary Fig. S4 at *JXB* online). It is concluded that relatively more
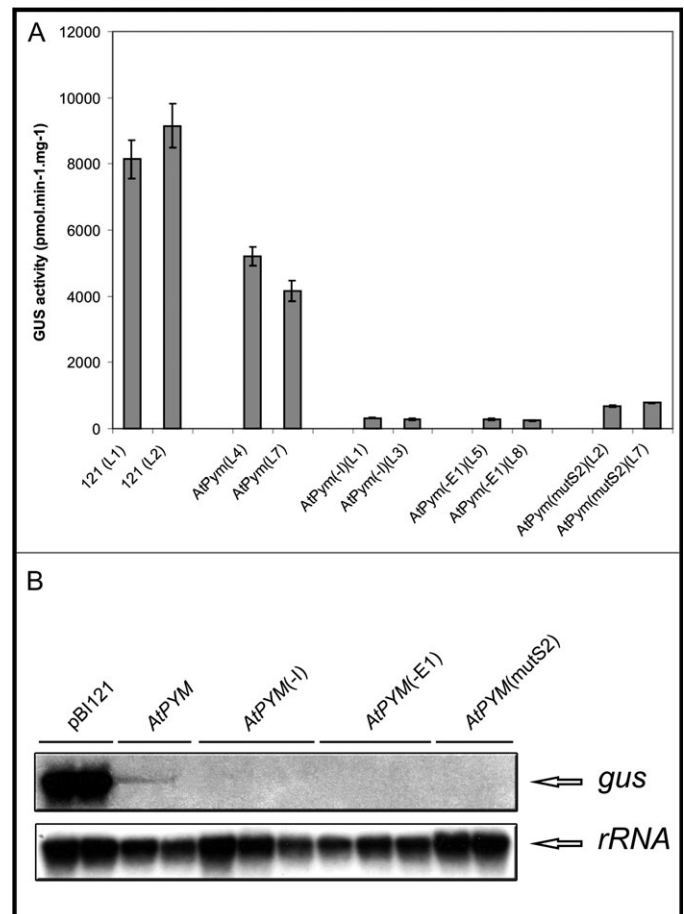
protein is produced from the transcript that originates from the *AtPYM* promoter, suggesting the existence of an effect of the intron on translation efficiency. Removal of the intron led to undetectable levels of transcript and activity (a total of 15 lines were analysed), showing that it is also involved at a transcriptional or post-transcriptional step (i.e. transcript processing, nuclear export, or stability) related to IME of gene expression.

AtMago, AtY14, and AtPYM participate in intron-mediated enhancement of gene expression

The role of AtMago, AtY14, and AtPYM itself in enhancement of *AtPYM* gene expression was analysed using transient transformation assays of seedlings that

contain the *AtPYM* promoter (with or without the intron) fused to *gus* with constructs that express the respective cDNAs under the control of the 35SCaMV promoter. As shown in Fig. 5, transformation with each of the cDNAs produced a significant increase in GUS activity and this effect was dependent on the presence of the *AtPYM* intron. Transformation with other constructs (i.e. the pBI101.3

vector, that contains a promoterless *gus* gene, or a construct that expresses the Hahb-4 HD-Zip transcription factor; Dezar *et al.*, 2005), as controls, did not produce significant changes in GUS expression. It is concluded that the increase in AtPYM levels observed in plants that overexpress AtMago and AtY14 is at least in part related to a role for these proteins in IME. Indeed, results similar to those
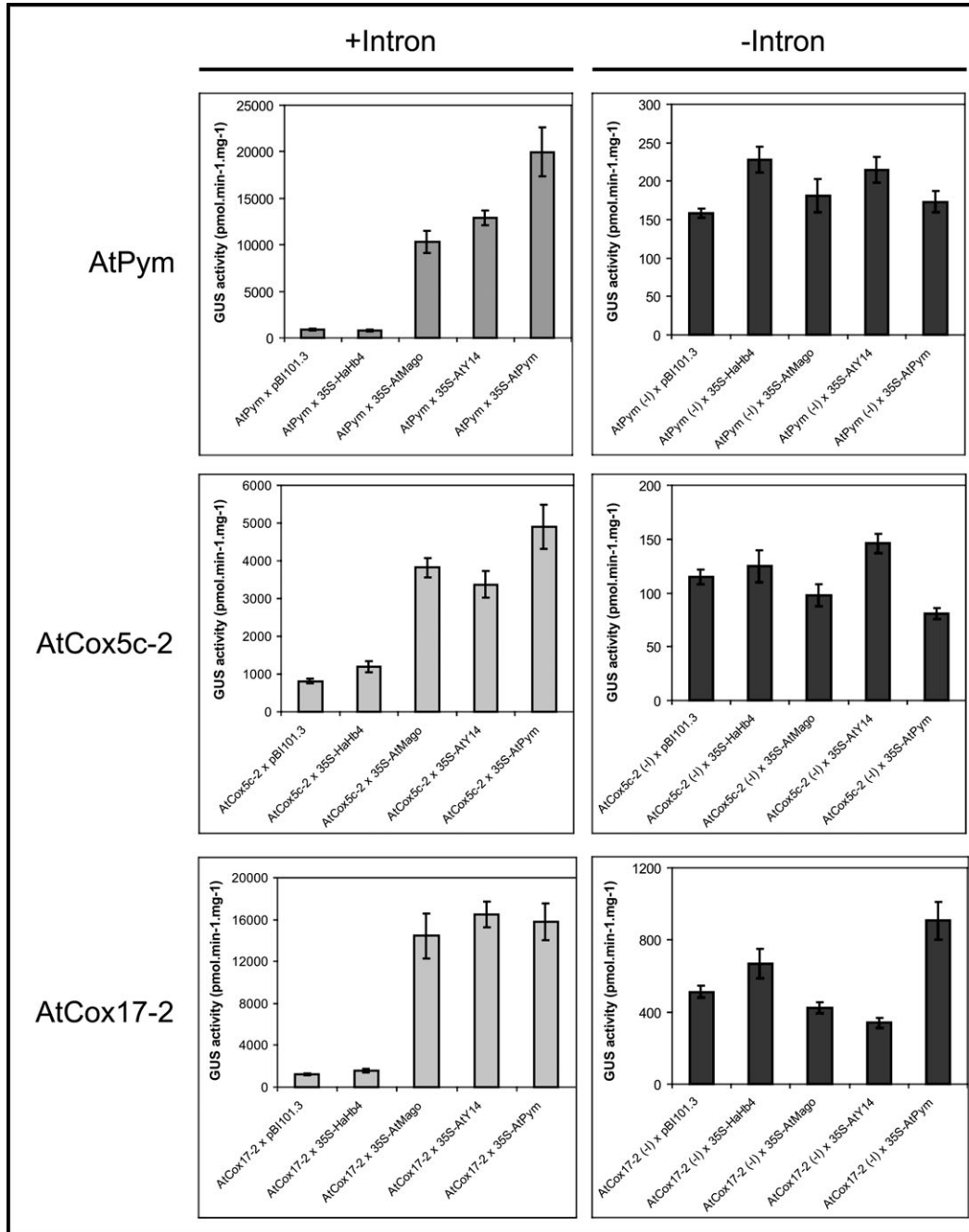

Fig. 5. Expression of AtMago, AtY14, and AtPYM produces an increase in intron-mediated gene expression. Stable transgenic lines expressing *gus* fusions of the upstream regions of *AtPYM*, *AtCOX5c-2*, or *AtCOX17-2* (with or without the leader introns; left and right panels, respectively) were transformed transiently with constructs that express AtMago, AtY14, or AtPYM under the control of the 35SCaMV promoter. Thirty-six hours after transformation, GUS activity levels were determined in extracts of transformed plants. As controls, plants were also transformed with the same *Agrobacterium* strain containing pBI101.3 (promoterless *gus* gene) or a construct expressing the HD-Zip transcription factor Hahb-4 under the control of the 35SCaMV promoter. The results are means (±SD) of three independent measurements.

observed for the *AtPYM* promoter were obtained with two other genes that depend on a leader intron for expression, namely *AtCOX5c-2* and *AtCOX17-2* (Fig. 5).

Discussion

Mago and Y14 are two interacting partners of the EJC core in animals (Kataoka *et al.*, 2000; Le Hir *et al.*, 2000, 2001). A third protein, PYM, has been identified as a partner of Y14 and Mago (Forler *et al.*, 2003). Genes encoding putative homologues of these proteins are present in *Arabidopsis* and other plant species (Swidzinski *et al.*, 2001; Park and Muench, 2007; Park *et al.*, 2009). In the present work, the functional links between the corresponding *Arabidopsis* genes and proteins have been investigated in order to gain insight into their role in plant gene expression. First, it was observed that the promoters of AtMago, AtY14, and AtPYM share expression characteristics, with preferential expression in meristems, roots, vascular tissues, and pollen. According to the role of the EJC in mRNA metabolism, it can be proposed that expression of its components may be linked to processes of cell growth or proliferation that require active protein synthesis. In this sense, it has been shown that the three genes depend on site II elements for their expression. These elements, first described in *PCNA* genes (Kosugi *et al.*, 1995), are present in a majority of genes encoding ribosomal proteins and mitochondrial components and were postulated as regulators of gene expression in proliferating tissues (Tremousaygue *et al.*, 2003; Welchen and Gonzalez, 2005, 2006). The expression of EJC components thus seems to be coordinated with other biogenetic processes within the cell. It is noteworthy that *Arabidopsis* genes encoding other putative EJC components often contain site II elements in their promoters (Supplementary Table S2 at *JXB* online). The observed expression patterns are in agreement with previous studies indicating that a decrease in AtMago levels produces alterations in meristem and pollen development and affects root and leaf growth (Park *et al.*, 2009).

The common expression patterns observed for AtMago, AtY14, and AtPYM suggest that the main functions of the three proteins are closely linked and that the physical interactions that were observed among them have a functional role in plant cells. As shown previously (Park and Muench, 2007), these interactions show conserved and varying features when plant and animal systems are compared. Among the latter, it is noteworthy that AtPYM is able to interact not only with the Mago–Y14 complex, as in animals, but also with the separated proteins. Recent studies on animal PYM indicated that it is bound to the ribosome where it functions as a linker of the EJC to the translation machinery and as a disassembly factor to allow recycling of EJC components (Diem *et al.*, 2007; Gehring *et al.*, 2009). This function of PYM requires the existence of a tight regulation of its interaction with the EJC to avoid complex dissociation before the mRNA reaches the ribosome. In animal cells, this would be achieved by

maintaining most of the protein tightly associated with the ribosome. In plants, it has been reported that AtPYM shuttles between the nucleus and the cytoplasm, and the existence in its sequence of putative nuclear localization and nuclear export signals has been described (Park and Muench, 2007). This double location would require mechanisms to regulate the interaction of PYM with EJC components. It was demonstrated here that the interaction of AtPYM with AtY14 (and probably the complex) can be modulated by phosphorylation of the two proteins. Phosphorylation of Y14 has been reported in animals, and this modification disrupts its interaction with other EJC components, but not with Mago (the effect on the interaction with PYM has not been tested; Hsu *et al.*, 2005). The Y14 sequence that is phosphorylated in animals is also present in AtY14, suggesting that the modification may occur in the same part of the protein. Phosphorylation of PYM has not been reported previously and may be specific to plants. This would provide an additional mechanism to avoid the dissociation of the EJC before it reaches the ribosome, by modulating the phosphorylation state of the interacting partners. The formation of complexes that contain the three proteins under study is suggested by the fact that they co-migrate in non-denaturing gels. In wild-type plants, these complexes seem to be present in low amounts, as suggested by the intensity of the signals obtained in western blots and in agreement with previous observations made by Park and Muench (2007). Notably, complex formation is increased in plants that overexpress AtPYM but not in plants that overexpress AtMago or AtY14, even if these plants also contain higher amounts of AtPYM. This is a further indication that the interaction of the three proteins is tightly regulated.

Increased AtPYM levels in plants that overexpress AtMago and AtY14 is most probably due to an effect of these proteins in IME of expression. Indeed, transient expression of these proteins produced a significant increase in expression from reporter constructs that contain leader introns of three different genes, including the AtPYM leader intron. Interestingly, AtPYM also increased the expression from its own promoter, suggesting that it may participate in an autoregulatory loop. At least part of the increase in expression promoted by the AtPYM intron seems to occur at the translational level. A role for PYM, Y14, and Mago in increasing translation efficiency has been previously reported in animals (Wiegand *et al.*, 2003; Nott *et al.*, 2004; Diem *et al.*, 2007; Lee *et al.*, 2009), and the present results show that similar mechanisms operate in plants. It is noteworthy that individual expression of the three proteins was effective in increasing IME. This suggests that the three proteins are limiting factors for the efficiency of the process. This notion has been put forward in animals considering that the amount of Mago and Y14 is much lower than the amount of exon junctions in total cellular RNA, indicating the importance of efficient recycling of EJC components (Gehring *et al.*, 2009).

In conclusion, the experiments described here show that the plant homologues of Mago, Y14, and PYM are closely

interconnected, not only through their function as EJC components but also at different steps of their own gene expression mechanisms. Co-regulated transcription through site II elements would help to keep a balance in the synthesis of the three proteins and link their expression to processes of cell growth or proliferation that require active protein synthesis. IME of AtPYM expression through the action of EJC components would be an additional factor to maintain this balance. Finally, post-translational regulatory mechanisms would fine-tune the interactions of the three proteins to optimize their functioning in gene expression within the cell. Overexpression of AtMago, AtY14, and/or AtPYM could also be useful to increase the expression levels of proteins of interest in plants through the use of constructs with suitable introns.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Analysis of cross-reactivity of antibodies raised against AtMago, AtY14, and AtPYM with the different recombinant proteins.

Figure S2. Scheme of the regions of AtMago, AtY14, and AtPYM fused to *gus* to analyse expression levels.

Figure S3. GUS activity levels in plants transformed with different constructs.

Figure S4. GUS activity and transcript levels in different lines of plants transformed with fusions to the *AtPYM* or the 35SCaMV promoters.

Table S1. List of primers used.

Table S2. Site II elements in the promoters of *Arabidopsis* EJC component genes.

Acknowledgements

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