

The Product of the Rice *myb7* Unspliced mRNA Dimerizes with the Maize Leucine Zipper Opaque2 and Stimulates Its Activity in a Transient Expression Assay*

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***myb7* mRNA is present in rice in spliced and unspliced forms, splicing being enhanced by anoxia. The protein (Mybleu) encoded by the unspliced mRNA is composed of an incomplete Myb domain followed by a leucine zipper; however, it lacks canonical sequences for DNA binding, transcriptional activation, and nuclear localization. We show here that in transiently transformed tobacco protoplasts, Mybleu is able to enhance the transcriptional activity of the maize leucine zipper Opaque2 on its target *b32* promoter. The Mybleu transactivation effect is strictly dependent on the presence of Opaque2 and is driven by Mybleu-Opaque2 heterodimers. Mybleu is located in the nucleus, both in rice and in transformed tobacco protoplasts. In rice, the protein is expressed in regions corresponding to undifferentiated cells of roots and coleoptiles. Therefore, *myb7* mRNA encodes, depending on its splicing, two transcription factors belonging to separate classes. One of them, Mybleu, has novel structural characteristics, suggesting the existence of new mechanisms acting in the activation of transcription.**

Mybs are a family of transcription factors widely represented in viruses, insects, mammals, and plants. The common feature of Myb factors is the presence of a conserved domain consisting of imperfect repeats of 50–53 amino acids, called “tryptophan clusters” because of the highly conserved tryptophan residues involved in stabilizing the structure of the DNA binding domain (1). In plants, *myb* genes are present as large families (6–100 members) involved in the control of a wide range of biochemical pathways (2–6), including responses to biotic and abiotic stresses (7–13).

Screening a cDNA library from anaerobically grown rice coleoptiles, we isolated *myb7*, a cDNA derived from a Myb-encoding unspliced mRNA (8). Some features of *myb7* sequence indicate that it may be post-transcriptionally regulated; in particular, two unspliced introns are present in positions that are conserved with respect to other plant *myb* genes. Both

spliced and unspliced *myb7* mRNAs are present *in vivo* (7).

In aerobically grown rice roots, we observed a higher proportion of unspliced *myb7* RNA, with respect to the spliced form. The spliced form is instead predominant in rice roots during anoxia, a stress situation that generally inhibits splicing (14). These data suggest that post-transcriptional regulation controls the ratio between *myb7* unspliced and spliced forms (7). This hypothesis is supported by the observation that the 5' region of the first intron can direct the synthesis of an in-frame leucine zipper, a functional domain present in several transcription factors (15–18).

The putative polypeptide encoded by the unspliced *myb7* mRNA consists of an incomplete Myb domain followed by the leucine zipper: we therefore named it Mybleu. Mybleu does not have the characteristics of a functional transcription factor. Indeed, it should be unable to bind DNA because of the presence of an incomplete Myb repeat. Moreover, neither a putative transcriptional activator region nor a consensus for nuclear targeting can be identified in Mybleu. The synthesis of the putative Mybleu polypeptide may, however, provide the cell with a mechanism able to regulate the dimerization and hence activity of other transcription factors.

Our main goal in this study was to determine whether Mybleu is able to interact with a leucine zipper protein and modulate its activity. To this purpose, we have transiently expressed Mybleu in tobacco protoplasts together with the maize leucine zipper Opaque2 (O2)¹.

Transient assays are used widely to provide functional information on transcription factors (19 and references therein). O2 is a very well characterized plant leucine zipper transcription factor that transactivates the *b32* promoter both in maize endosperm and in transient assay in tobacco protoplasts (18, 20, 21). Moreover, recent data suggest that a bZIP protein functionally similar to O2 may exist in rice (22).

We show that Mybleu does not inhibit, but instead enhances the transactivation on the *b32* promoter by O2. This effect seems to be driven by heterodimers, which are quantitatively formed between O2 and Mybleu. We also show that Mybleu is located in the nucleus both in rice cells and in transformed tobacco protoplasts.

Therefore, a heterodimer consisting of two natural leucine zipper transcription factors, one complete and the other lacking

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¹ The abbreviations used are: O2, Opaque2; CAT, chloramphenicol acetyltransferase; GUS, β -glucuronidase; kb, kilobases; DAPI, 4,6-diamidino-2-phenylindole; PAGE, polyacrylamide gel electrophoresis; bZIP, basic leucine zipper.

5 B32—5'-GGACGAGTTGACGTTGAACC-3' corresponding to the B5 O2 binding site of the *b32* promoter (20).

Oligonucleotides were annealed and labeled as described by Ciceri *et al.* (38). 10 μ g of transformed protoplasts nuclear extract for each sample were incubated 10 min at room temperature in binding buffer (10 mM Hepes, pH 7.9, 1 mM EDTA, 2 mg/ml bovine serum albumin, 100 μ g/ml salmon sperm DNA, 10% glycerol) containing KCl at three different concentrations (40, 70, 100 mM). The probe was then added to the extracts and the reactions allowed for 20 min at room temperature. Samples were then loaded onto a 4% polyacrylamide gel in 23 mM Tris borate, 0.5 mM EDTA, pH 8. Gels were run at 4 $^{\circ}$ C, dried, and autoradiographed. The presence and the relative amount of O2 and Mybleu in the extracts were assayed by Western blot (data not shown).

Immunocytochemical Analysis

Immunocytochemical analysis on tobacco transformed protoplasts was performed as reported (43). Phosphatase activity was detected by 4-nitro blue tetrazolium chloride (Sigma) in a 5-min reaction (40). Nuclei were stained with the DNA-specific fluorochrome DAPI (2 μ g/ml). Nitrocellulose filters were examined and photographed under a Zeiss Axioplan fluorescence microscope, using an excitation filter LP 400 to detect DAPI fluorescence.

For immunocytochemical assays on rice tissues, roots and coleoptiles were spread on nitrocellulose filters by applying them with gentle pressure. Tissue prints were then processed for immunodetection as described above.

RESULTS

Mybleu Has a Synergistic Effect on O2-driven Transactivation—The presence of a leucine zipper domain and the absence of both DNA binding and transactivation domains suggest that Mybleu might act as a dominant repressor, according to the results obtained with other incomplete transcription factors (44–47). We therefore investigated the action of Mybleu on the activity of O2, using as a target the *b32* promoter. The *in vivo* assays were performed in transiently transformed tobacco protoplasts, using the plasmids shown in Fig. 1A. We first verified whether cotransformations of protoplasts with several plasmids carrying the same strong promoter region (CaMV 35S) influence the expression of the individual constructs. Increasing amounts (10–60 μ g/test) of pCaMVNeo did not affect the expression of 10 μ g of pCaMVCAT or pCaGUS (data not shown). Therefore, we compared the GUS expression driven by the *b32* promoter in the presence of O2, Mybleu, or both. Surprisingly, Mybleu did not inhibit O2 transactivation, but had a stimulatory effect (Fig. 1B).

The fold stimulation of Mybleu on O2 activity was always similar, within a wide range of amounts of plasmids expressed (0.1–10 μ g/test; Fig. 1B). The stimulation driven by 1 μ g of O2 seems higher at lower concentrations of the *b32* promoter because our unit of measure is the GUS activity driven by the *b32* promoter in the absence of O2. This is, of course, markedly higher at higher concentrations of the *b32* promoter; for this reason the transactivation efficiency (fold stimulation) of a given amount of O2 seems lower at higher concentration of *b32* promoter, unless the amount of the promoter is limiting.

A pCaMV-O2 dependent (0.1–10 μ g range) linear enhancement of the GUS expression was observed both when 1 μ g and 10 μ g of pB32GUSII were used (data not shown). These results suggest that the effect of Mybleu on O2 activity does not depend on its interaction with an unknown tobacco cellular factor that may sequester O2. If a tobacco factor able to interact with O2 and inhibit its activity existed, it would be saturable. In this case, the Mybleu effect would be higher at lower O2, and Mybleu concentrations and the O2-driven transactivation of the *b32* promoter would be less efficient at lower concentrations of O2. The possibility of a direct activity of Mybleu on the *b32* promoter was also ruled out because in the absence of O2, Mybleu did not stimulate the activity of the *b32* promoter (Fig. 1B).

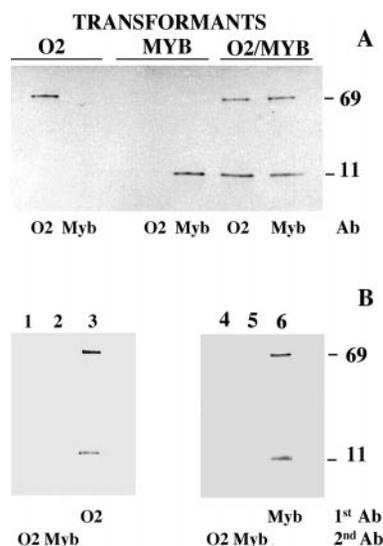


FIG. 2. **Coimmunoprecipitation of Mybleu and O2.** Panel A, nuclear proteins were extracted from tobacco protoplasts transformed with pCaMV-O2, pCaMVMybleu, or both (O2, MYB, and O2/MYB, as indicated at the top of the figure) and immunoprecipitated with anti-O2 (O2 lanes) or anti-Mybleu (Myb lanes). Panel B, nuclear proteins were extracted from tobacco protoplasts cotransformed with pCaMV-O2 and pCaMVMybleu and immunoprecipitated (1st Ab) with anti-O2 (O2) or anti-Mybleu (Myb) (lanes 3 and 6). Each supernatant was again immunoprecipitated (2nd Ab) with either the same antiserum (lanes 1 and 5) or with the other one (lanes 2 and 4). After SDS-PAGE, proteins were blotted on a nitrocellulose filter, which was then incubated with a mixture of anti-O2 and anti-Mybleu antisera. Molecular mass markers are indicated in kDa.

Finally, as shown in Fig. 1B, the GUS activities induced by 8 μ g of pCaMV-O2 or 4 μ g of pCaMV-O2 and 4 μ g of pCaMVMybleu are similar and significantly higher than the activity induced by 4 μ g of pCaMV-O2.

Mybleu Interacts Directly with O2—The possible direct physical interaction between Mybleu and O2 was tested by immunoprecipitation experiments. Nuclear extracts of protoplasts transformed with pCaMV-O2, pCaMVMybleu, or both plasmids (10 μ g each) were immunoprecipitated with anti-O2 or anti-Mybleu antiserum and analyzed by Western blot with a mixture of both antisera. As shown in Fig. 2A, both antisera (Ab O2 and MYB) immunoprecipitated specifically the correspondent antigen in single transformants (O2 and MYB). The specificity of immunoprecipitations was confirmed by the absence of immunoprecipitated proteins in pCaMV-O2 transformed protoplasts by Mybleu antiserum and *vice versa*. Therefore, the coimmunoprecipitation of both factors with any of the two antisera in the double transformants (O2/MYB) indicates a direct interaction. Immunoprecipitation appeared quantitative for both proteins, irrespectively of the antiserum used, suggesting a complete interaction between Mybleu and O2 (Fig. 2B, lanes 3 and 6). Consistently, using anti-Mybleu antiserum, we were unable to immunoprecipitate any protein from the supernatant after immunoprecipitation with anti-O2 antiserum and *vice versa* (Fig. 2B, lanes 2 and 4). We were finally unable to detect O2 or Mybleu analyzing by Western blot the supernatants of the immunoprecipitations, indicating that the two proteins were quantitatively available for immunoprecipitation (data not shown).

Mybleu and Opaque2 Form Heterodimers—To determine the assembly state of Mybleu and O2, nuclear extracts from tobacco protoplasts transformed with plasmids expressing O2, Mybleu, or both (10 μ g/each) were loaded onto preformed linear sucrose gradients (5–25% w/v) and subjected to sedimentation velocity centrifugation. A mixture of molecular mass markers

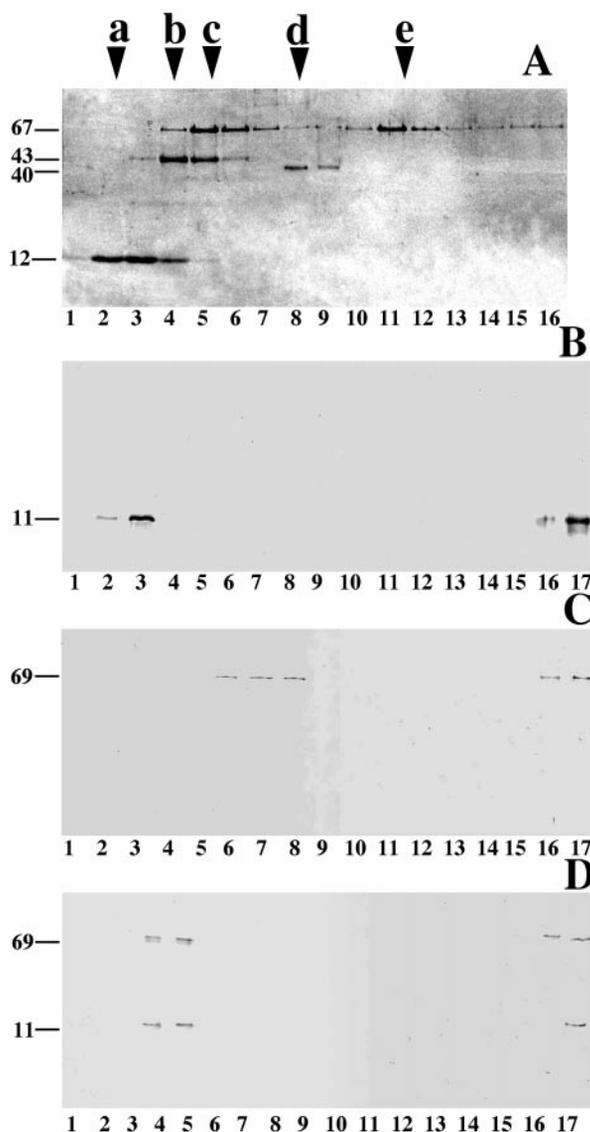


FIG. 3. Mybleu and O2 heterodimer formation. Molecular markers (*panel A*) and nuclear extracts from tobacco protoplasts transformed with plasmids expressing Mybleu (*panel B*), O2 (*panel C*), or both (*panel D*) were loaded onto a preformed sucrose sedimentation velocity gradient. Each fraction (*lanes 1–15*), after SDS-PAGE, was analyzed by Coomassie Brilliant Blue R-250 staining (*panel A*) or by Western blot using a mixture of anti-Mybleu and anti-O2 antisera as primary antiserum. *Lanes 16*, gradient pellet; *lanes 17*, nuclear extracts of transformed protoplasts before gradient sedimentation. Molecular mass markers are indicated in kDa. Markers: *a*, cytochrome *c* (12 kDa); *b*, ovalbumin (43 kDa); *c*, bovine serum albumin (67 kDa); *d*, aldolase (142-kDa trimer); *e*, catalase (232-kDa tetramer).

was also centrifuged. Each fraction of the marker gradient was analyzed by SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue R-250 (Fig. 3, *panel A*). Each fraction of the nuclear extract gradients was analyzed by Western blot using a mixture of anti-Mybleu and anti-O2 antisera as primary antiserum. In the single transformants, the position of both O2 and Mybleu along the gradient matched the one expected for the respective homodimers (Fig. 3 *panel B*, fractions 2 and 3, and *panel C*, fractions 6–8). In the double transformant, both proteins migrated in the position expected for heterodimers (Fig. 3, *panel D*, fractions 4 and 5). The identity of the two bands was confirmed assaying an aliquot of fraction 5 of the double transformant gradient by Western blot, using anti-Mybleu, anti-O2, or both primary antisera (data not shown). These

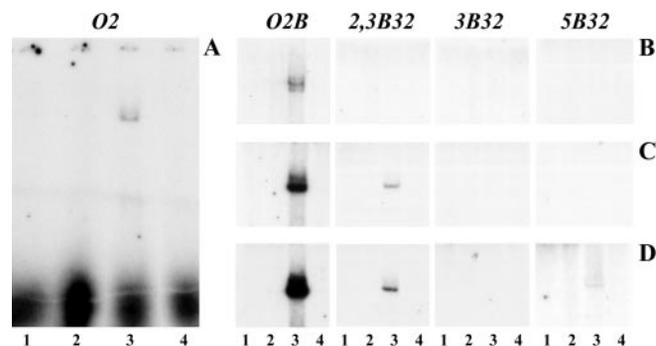


FIG. 4. Gel mobility shifts using nuclear extracts from transformed tobacco protoplasts. Extracts from protoplasts untransformed (*lanes 1*) and transformed with pCaMVMybleu (*lanes 2*), pCaMVO2 (*lanes 3*), and both (*lanes 4*) were tested in gel mobility shift assay. The oligonucleotides used are indicated at the top. *Panel A*, binding reaction with oligonucleotide O2 under 40 mM KCl. *Panels B, C, and D*, 40, 70 and 100 mM KCl, respectively.

results demonstrate that Mybleu and O2 form heterodimers and that heterodimer formation is dominant over O2-O2 homodimer assembly.

Gel Shift Assay with Transformed Tobacco Extracts—To analyze directly the DNA binding properties of O2 and Mybleu, we assayed the ability of homo- and heterodimers to bind the O2 binding sites by gel shift experiments (Fig. 4). We used 10 μ g of nuclear extract from protoplasts transformed with pCaMVMybleu (*lanes 2*), pCaMVO2 (*lanes 3*), or both constructs (*lanes 4*) and, as a control, the nuclear extract of mock transformed protoplasts (*lanes 1*). Under the salt conditions described for O2-DNA binding (41), we were able to detect O2-mediated probe retardation using the oligonucleotides corresponding to the O2 binding site present in the zein promoter (oligonucleotides O2 and O2B, containing respectively one and two O2 binding sites (Ref. 38); Fig. 4, *panels A and B, lanes 3*). The results obtained using several oligonucleotides under different salt conditions are shown in *panels B, C, and D* (40, 70, and 100 mM, respectively). Increasing the KCl concentration, we could also detect probe retardation using oligonucleotide 2,3 B32 (20) (*panels C and D, lanes 3*). Oligonucleotide 5 B32 is retarded only under very high salt concentration (*panel D, lane 3*). We were not able to detect a retarded complex using the 3 B32 oligonucleotide.

The fact that the extract from O2-expressing protoplasts, but not the one from protoplasts expressing both O2 and Mybleu, produced a retarded complex is consistent with the fact that O2-homodimers are not detectable in cotransformed cells (Figs. 2 and 3).

Mybleu Is a Nuclear Protein—In Mybleu there is no canonical nuclear localization sequence. To determine whether the transactivation activity of Mybleu in the presence of O2 results from O2-mediated nuclear localization of Mybleu, we performed immunolocalization experiments and Western blots on nuclear and cytoplasmic extracts. Fig. 5 shows protoplasts transformed with pCaMVMybleu (MYBLEU column), pCaMVO2 (O2 column), and both (O2/MYBLEU column) (10 μ g/each). Immunocytochemical localization (*odd rows*) was performed using antiserum against either O2 or Mybleu, as primary antibodies; as controls, antisera against the cytoplasmic protein actin and preimmune serum were used. Nuclei were evidenced by DAPI staining (*even rows*).

Immunocytochemical localization, performed on protoplasts transformed with individual constructs, indicated that Mybleu is able to enter the nucleus in the absence of O2. A similar result was obtained by Western blot analysis of nuclear and

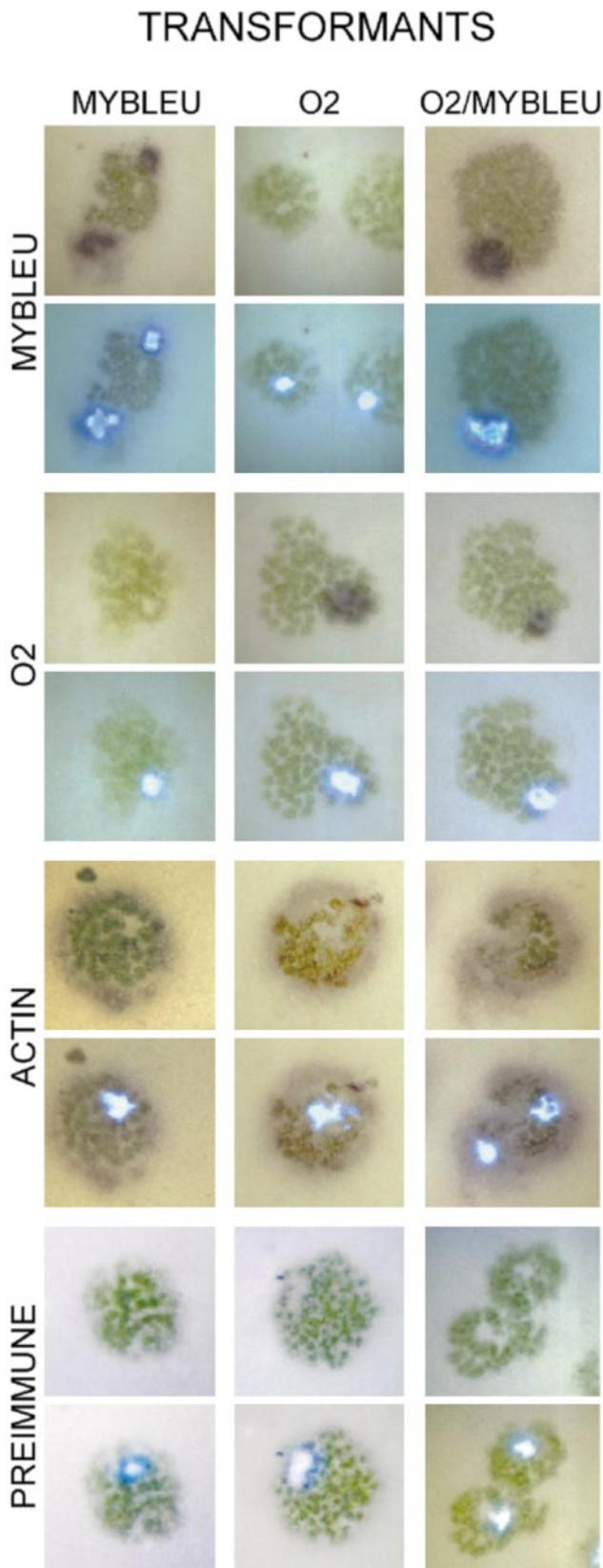


FIG. 5. Immunocytochemical localization of Mybleu and O2. Tobacco protoplasts were transformed with pCaMVMybleu (MYBLEU), pCaMV-O2 (O2) or both (O2/MYBLEU) and treated with different antisera, as described in "Immunocytochemical Analysis" under "Experimental Procedures." Primary antisera used were anti-Mybleu (MYBLEU), anti-O2 (O2), anti-actin (ACTIN), and preimmune serum. *Odd rows* show immunodetections; *even rows* show the same protoplasts stained with DAPI, to locate nuclei.

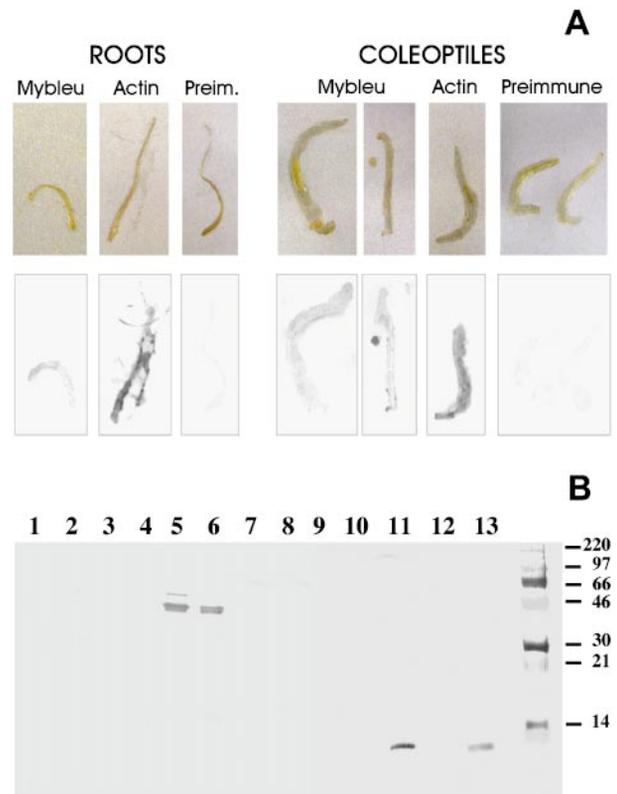


FIG. 6. Mybleu is present in rice. *Panel A*, tissue print immunolocalization. Rice roots and coleoptiles were spread on nitrocellulose filters and assayed with Mybleu, actin, and preimmune antisera. *Upper row*, rice tissues; *lower row*, immunodetection. *Panel B*, Western blot analysis using anti-Mybleu antiserum. Periplasmic extracts of *E. coli* (lanes 1–3), periplasmic (lanes 4–6), and cytoplasmic (lanes 7–9) extracts of *E. coli* expressing the maltose-binding protein-Mybleu fusion protein are shown. Lanes 1, 4, and 7, uninduced cells; lanes 2, 5, and 8, 4 h of isopropyl- β -D-thiogalactopyranoside induction; lanes 3, 6, and 9, 6 h of isopropyl- β -D-thiogalactopyranoside induction. Coleoptile subcellular fractions immunoprecipitated with anti-Mybleu antiserum are shown in lane 10 (cytoplasmic) and lane 11 (nuclear). Root subcellular fractions immunoprecipitated with anti-Mybleu antiserum are indicated in lane 12 (cytoplasmic) and lane 13 (nuclear). Molecular mass markers are indicated.

cytoplasmic extracts of protoplasts transformed with plasmids expressing O2, Mybleu, or both (data not shown).

Mybleu Is Present in Rice—We aimed to verify whether unspliced *myb7* mRNA, encoding Mybleu, was translated in rice. In fact, the existence of Mybleu in rice would strongly suggest a similar role in modulating activity of a natural rice transcription factor.

Rice coleoptiles grow under both aerobic and anaerobic conditions, whereas roots are unable to grow under anoxia. Because the level of the unspliced *myb7* mRNA (putatively encoding Mybleu) is decreased in anaerobically treated roots (7), we hypothesized that Mybleu could be involved in the process of cellular division. Immunoassay experiments on rice root and coleoptile prints (Fig. 6A) showed signals in regions corresponding to the dividing or undifferentiated cells, but not in the completely expanded cells of the same organs, supporting the above mentioned hypothesis. To confirm the presence of Mybleu and its nuclear localization, we therefore immunoprecipitated nuclear and cytoplasmic proteins extracted from meristem-enriched segments of both roots and coleoptiles. Western blot analysis of the nuclear proteins immunoprecipitated using Mybleu antiserum shows the presence of a single band with a molecular mass of 11 kDa, corresponding to the one expected for Mybleu (Fig. 6B, lanes 11 and 13). In cytoplasmic extracts,

the antiserum did not react with any protein (*lanes 10 and 12*). Western blot analysis of periplasmic extracts from *E. coli* expressing or not the Maltose binding protein-Mybleu fusion protein (used to produce the anti-Mybleu antiserum) was performed (*lanes 1–6*). The presence of a single band of expected molecular mass (44 kDa) in *lanes 5 and 6* demonstrated the specificity of the anti-Mybleu antiserum. We did not detect any protein reacting with preimmune serum (data not shown). We conclude that Mybleu is synthesized in apexes of rice roots and coleoptiles.

Finally, we were also able to detect Mybleu in nuclei of established suspension cultures of rice (data not shown).

DISCUSSION

The high amount of unspliced *myb7* mRNA with respect to the spliced form, the increase of the spliced form in roots during anoxia, and the presence of a leucine zipper in the polypeptide encoded by the unspliced mRNA suggest a regulatory role for *myb7* splicing (7).

We showed here that Mybleu is expressed *in vivo* in both root and coleoptile meristematic regions and in established cell cultures. The results of transient expression in tobacco protoplasts indicate that Mybleu enhances the activity of the bZIP O2. These results support the hypothesis mentioned above and suggest an *in vivo* function as transcriptional activator.

Mybleu activity is synergistic rather than additive with respect to that of O2; in fact, Mybleu alone has no effect on *b32* promoter transactivation. Equal amounts of O2- or of a mixture of O2- and Mybleu- encoding plasmids have similar activities (in activating the *b32* promoter). When coexpressed, the two proteins form mainly, if not exclusively, heterodimers. This was demonstrated by the absence of O2 remaining in the supernatant after immunoprecipitation with anti-Mybleu antiserum and *vice versa* and by the sedimentation velocity experiment on the extract from cotransformed tobacco protoplasts. In fact, when coexpressed, both proteins are present in fractions expected to contain heterodimers and are no longer detectable in the fractions corresponding to the respective homodimers. These results demonstrate that the heterodimer is as active as the O2 homodimer in activating transcription. The disappearance of the O2 homodimers in the presence of Mybleu is also consistent with the results of the gel shift experiments; the presence of Mybleu abolishes the specific shift driven by O2. Our failure to detect a shift driven by the heterodimer may be due to a different affinity of the heterodimer with respect to the homodimer. Further factors and/or conditions acting *in vivo* might influence the binding affinity and stability and therefore the transcriptional activity.

Intron splicing is in general an accurate and efficient process, and relevant amounts of unspliced transcripts are detected only in cells subjected to severe stress (14, 48–50). However, some mRNAs encoding transcription factors are subjected to regulated splicing. This can produce mRNAs encoding different transcription factors with distinct functions (51–55). Natural and artificially incomplete transcription factors (lacking the activation domain) are able to interact with complete factors and act as negative dominants (44–47, 56).

However, Mybleu is not a repressor, but is a synergistic effector on O2 activity. An example of the *in vivo* presence of incomplete transcription factors, able to activate transcription only by interacting with the proper partner, is the Maf family of mammals (57). The Maf protein contains a basic DNA binding domain followed by an extended leucine zipper, but it lacks a recognizable activator domain. Both Maf homo- and heterodimers (with two members of the NFE2/CNC-bZIP family) are able to bind the NFE2 site present in the promoter region of γ -globin. Nevertheless, they exert opposite effects: het-

erodimers stimulate whereas homodimers inhibit the expression driven by the γ -globin promoter (57). Mybleu has no repressor activity as a homodimer, probably because it lacks a DNA binding domain. Our results recall the cooperative activation of muscle gene expression, driven by Mef2 and myogenic bHLH proteins (58). Mef2 and its HLH partner are produced *in vivo* as complete transcription factors and may activate promoters that contain either one, the other, or both of the regions recognized by each of them. Using deletion mutations, Molkenin *et al.* (58) demonstrated that this cooperation requires direct interactions and that only one factor containing a transactivation and a DNA binding domain is needed in the heterodimers.

We have shown here that Mybleu is a natural factor that has a positive transcriptional effect, although it seems to lack both a DNA binding and a transactivation domain. It might be a general synergistic effector of several leucine zipper factors, by changing their conformation and/or allowing the interaction with other proteins. Although the effect of Mybleu is not dramatic, it is clear that the heterodimer is able to activate transcription. We may hypothesize that Mybleu *in vivo* is able to dimerize with nuclear factor(s) that are unable to form homodimers or that the DNA binding properties of the heterodimers are different in some way from that of the homodimers, as suggested by gel shift experiments and as already demonstrated for other transcriptional factors (59–61). If this is the case, the differential comparative effect on different promoters of the heterodimers with respect to homodimers could have a relevant biological significance. Further work is in progress to clarify if such a mechanism is present. It should also be noted that the same RNA encodes two active transcription factors, belonging to different classes depending on the splicing. Finally, Mybleu represents a new class of transcription factors, characterized by the presence of a leucine zipper following an incomplete Myb domain. Further investigations are necessary to identify its natural partner in rice.

Although we were unable to identify any nuclear localization sequence along the Mybleu amino acid sequence, immunocytochemical and Western blot assays demonstrated its nuclear location both in rice and in transformed tobacco protoplasts. Several nuclear proteins, and among them some Myb factors, do not show any canonical consensus for nuclear localization (62–64). Some of these enter the nuclei only upon interaction with their partner, like APETALA and PISTILLATA (65), and Dp and E2F (66). If Mybleu enters the nucleus upon interaction with a partner, this should be a widespread or an unspecific factor, present also in tobacco protoplasts.

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