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...
the transcriptional and binding domains, is able to activate transcription in plant cells. Myb7 presence in rice strongly suggests that such a mechanism is a natural one.

**EXPERIMENTAL PROCEDURES**

**Plant Material**

Maintenance of *in vitro* shoot cultures of *Nicotiana tabacum* (cultivar Petit-Havana, SRI) and preparation of apical segments from rice (cultivar Arborio) roots and coleoptiles were described previously (23, 24).

**Production of Anti-Myb7 Antisera**

The intronic *KpnI*-XbaI fragment, coding for the 33 COOH-terminal amino acids of Myb7, absent in the Myb protein encoded by the spliced mRNA (7), was inserted into the pMAL-p2 expression vector cut with EcoRI and XhoI (25). The maltose-binding protein-Myb7 fusion protein (molecular mass 44 kDa, as expected) was isolated following standard protocols (25). 4 mg was used to immunize New Zealand White rabbits. Periplasmic and cytoplasmic extracts (25) of untransformed and transformed *Escherichia coli*, before and after isopropyl-β-d-thiogalactopyranoside induction, were subjected to Western blot as positive and negative controls.

**Plasmid Constructs**

pCaMVCAT (4.2 kb), pCaMVNeo (4.4 kb), pCaGUS (5.3 kb), pCaMV-O2 (4.7 kb) expressing, respectively, CAT, neomycin phosphotransferase II, GUS, and O2 open reading frames, all under the constitutive CaMV 35S promoter, and pB32GUSII (4.1 kb), expressing the GUS open reading frame under the b32 promoter, have been described previously (20, 23, 26, 27). The NheI-ScaI fragment of unspliced myb7 cDNA (8), which encodes Myb7, was inserted between the SmaI and ScaI sites of pCaGUS, to yield pCaMVMyb7 (4.4 kb). Representation of the plasmids used in this work is in Fig. 1A.

**Transient Expression in Tobacco Protoplasts**

Mesophyll protoplasts from tobacco leaves were isolated according to Nagy and Maliga (28) and Potrykus and Shillito (29) and transformed according to Bilang et al. (30). Protoplast viability was checked by the fluorescein diacetate method (31). Protoplast extracts were used for GUS and CAT activity determinations. Protein concentration was determined by the method of Lowry as modified by Peterson (32). Results are the means of at least four transformation experiments.

**Determination of GUS and CAT Activities**

GUS activity was determined according to Jefferson (33). CAT activity was assayed as described by Sambrook et al. (34). Protoplast transformations with GUS-expressing constructs always included an internal reference marker (10 μg of pCaMVCAT plasmid), and the level of GUS expression was referred as a ratio between the coexpressed GUS and CAT activities. Transactivation of the GUS expression was referred as a ratio between the coexpressed GUS activity and negative controls.

**Isolation of Nuclear Fractions and Immunological Analysis**

Cytoplasmic and nuclei-enriched subcellular fractions from frozen root and coleoptile segments and from transformed protoplasts were isolated as described (24) and then loaded on discontinuous Percoll gradient to obtain a pure nuclear fraction (35). The purity of the nuclear preparations was assessed by UV fluorescence microscopy after DAPI (1 μg/ml) staining. Nuclear or cytoplasmic proteins from rice roots and coleoptiles and nuclear proteins from transformed protoplasts (800 μg/ml) were immunoprecipitated with anti-O2 or anti-Myb7 antisera, as described by D’Amico et al. (36). Total nuclear or cytoplasmic extracts and immunoprecipitated proteins were fractionated by SDS-PAGE (37) and blotted onto nitrocellulose membrane (PROTRAN BA 85, 0.20 μm, Schleicher & Schuell) (38). Immunodetection of total extracts was performed by Western blot according to Harlow and Lane (39). The detection of immunoprecipitated proteins was performed using only the primary rabbit antiserum (anti-Myb7 and anti-O2), coupled to alkaline phosphatase (40). Nuclear extracts from transformed tobacco protoplasts used to perform gel shift experiments were prepared as described by Schmidt et al. (41).

**Analysis of Myb7 and O2 Assembly by Sedimentation Velocity**

Nuclear extracts (500 μg) of protoplasts transformed with plasmids expressing O2, Myb7, or both were loaded on a continuous 5–25% (w/v) linear sucrose gradient (42). A mixture (50 μg each) of cytochrome c (12 kDa), ovalbumin (43 kDa), bovine serum albumin (67 kDa), aldolase (a 142-kDa trimer) and catalase (a 232-kDa tetramer) was used as a marker for molecular mass separation along the gradient. Samples were centrifuged at 39,000 rpm in a SW40 Ti rotor (Beckman, Fullerton, CA) for 40 h at 4 °C. After centrifugation, gradients were collected in 700-μl fractions. Density was measured with a refractometer. An aliquot (350–700 μl) of each fraction was trichloroacetic acid precipitated and analyzed by Western blot after 15% acrylamide SDS-PAGE (37–39).

**Electrophoretic Mobility Shift Assays**

This assay was performed according to a described procedure (41) with minor modifications. The following double strand oligonucleotides were used (the O2 binding sites are indicated in bold).

**Streptomycin Resistance (Streptomycin)**

**Myb7 Encodes a New Transcription Factor**

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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 μg/test) of pCaMVNeo did not affect the expression of 10 μg of pCaMV-CAT 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 μg/test; Fig. 1B). The stimulation driven by 1 μg 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 μg range) linear enhancement of the GUS expression was observed both when 1 μg and 10 μg 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).

myb7 Encodes a New Transcription Factor

Finally, as shown in Fig. 1B, the GUS activities induced by 8 μg of pCaMV-O2 or 4 μg of pCaMV-O2 and 4 μg of pCaMVMybleu are similar and significantly higher than the activity induced by 4 μg 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 (O2, MYB, and O2/MYB, as indicated at the top of the figure) and immunoprecipitated with antisera 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 antisera anti-O2 (O2) or anti-Mybleu (Myb) (lanes 3 and 6). Each supernatant was again immunoprecipitated (2nd Ab) with either the same antisera (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.

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

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 μg/ml). Nitrocellulose filters were examined and photographed under a Zeiss Axioplan fluorescence microscope, using an excitation filter LP 400 to detect DAPI fluorescence.

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bleu, anti-O2, or both primary antisera (data not shown). These double transformant gradient by Western blot, using anti-Mybleu, using a mixture of anti-Mybleu and anti-O2 antisera as primary antiserum. In the single transformants, the position of both O2 marker bands was confirmed assaying an aliquot of fraction 5 of the gradient by DAPI staining (even rows). The 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 μg 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 KCl, respectively). Increasing the KCl concentration, we could also detect probe retardation using oligonucleotide 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 pCaVMVMybleu (MYBLEU column), pCaMVO2 (O2 column), and both (O2/MYBLEU column) (10 μg each). Immunocytochemical localization (odd rows) was performed using antiserum against O2 or Mybleu, as primary antibodies; as controls, antiserum 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 cytoplasmic extracts. Each fraction of the nuclear extract was analyzed by SDS-PAGE, and the gel was stained with Coomassie Brilliant Blue R-250 (Fig. 3, 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).
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 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 effecter 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: heterodimers 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, Molkentin 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 effecter 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 unspecified factor, present also in tobacco protoplasts.

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**REFERENCES**

1. Ogata, K., Hojo, H., Aimoto, S., Nakai, T., Nakamura, H., Sarai, A., Ishii, S., and Nishimura, Y. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 6428–6432
2. Avila, J., Nieto, C., Canas, L., Benito, M. J., and Paz-Ares, J. (1993) *Plant J.* 3, 533–562
3. Jackson, D., Culezian-Macia, F., Prescott, A. G., Roberts, K., and Martin, C. (1991) *Plant Cell* 3, 115–125
4. Kranz, H. D., Deneckamp, M., Greco, R., Jin, H., Levy, A., Meissner, R. C., Petretti, K., Urzaizqui, A., Bevan, M., Martin, C., Smeekens, S., Tonell, C., Paz-Ares, J., Weisshaar, B. (1998) *Plant J.* 16, 263–276
5. Bender, J., and Fink, G. R. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 5655–5660
6. Marocco, A., Wissenbach, M., Becker, D., Paz-Ares, J., Saeldier, H., Salamini, F., and Rohde, W. (1989) *Mod. Gen. Genet.* 216, 183–187
7. Magaraggia, F., Solinas, G., Valle, G., Giovannazzo, G., and Coraggio, I. (1997) *Plant Mol. Biol.* 35, 1083–1089
8. Menguzzi, E., Valle, G., and Coraggio, I. (1995) *Plant Physiol.* 109, 1498 (PGR95-104)
9. Pandolli, D., Solinas, G., Valle, G., and Coraggio, I. (1997) *Plant Physiol.* 114, 747 (PGR97-079)
