PERGAMON

Phytochemistry 50 (1999) 209–214

PHYTOCHEMISTRY

Expression of barley ADP-glucose pyrophosphorylase in *Escherichia coli*: processing and regulatory considerations

Cheng Luo, Leszek A. Kleczkowski *

Department of Plant Physiology, Umeå University, 901 87 Umeå, Sweden

Received 16 June 1998

Abstract

Full length cDNAs for barley ADP-glucose pyrophosphorylase (AGPase) coding for the large subunits of the endosperm and leaf homologues of the enzyme (AGPase-S1 and -S2, respectively) and for the small subunit protein from endosperm (AGPase-B1), have been expressed in *Escherichia coli*. The cDNAs for AGPase-S1 and -S2 required different induction conditions for their maximal expression and they encoded immunologically distinct proteins. The AGPase-S1 that was produced by *E. coli* had the same M_r (58 kDa) as the corresponding protein in barley crude endosperm extracts, whereas the bacteria-produced AGPase-S2 (55 kDa) was larger than its counterpart from barley leaf preparations (53 kDa). An enzymatically active AGPase expressed in *E. coli* from a double construct containing cDNAs for AGPase-S1 and -B1 subunits was insensitive to the activation by 3-phosphoglycerate and to inhibition by inorganic phosphate, similarly to the enzyme in barley endosperm. Neither AGPase-S1 nor -B1 were active when expressed alone in the bacteria. The data are discussed with respect to possible mechanisms of intracellular targeting of immature AGPase-S proteins in barley tissues and regarding previous data on effector regulation of the barley enzyme. © 1998 Elsevier Science Ltd. All rights reserved.

Keywords: *Hordeum vulgare*; *Gramineae*; *Poaceae*; Barley; Starch synthesis; *Brittle-2*; *Shrunken-2*; Heterologous expression; Transit peptide

1. Introduction

ADP-glucose pyrophosphorylase (AGPase), a two gene encoded enzyme, is the key component of starch biosynthesis machinery in all plants (Tsai & Nelson, 1966; Kleczkowski, Villand, Lönneborg, Olsen, & Lüthi, 1991; ap Rees, 1995; Martin & Smith, 1995). The enzyme from many species is tightly regulated by the ratio of 3-phosphoglycerate (PGA) to inorganic phosphate (P_i), (Sanwall, Greenberg, Hardie, Cameron, & Preiss, 1968; Kleczkowski, Villand, Preiss, & Olsen, 1993) and its activity may be directly related to the quantity of starch in plant tissues (Müller-Röber, Sonnewald, & Willmitzer, 1992; Giroux et al., 1996). In barley, there are at least two distinct isoenzymes of AGPase that are localized in seed endosperm and leaves, respectively, and encoded by two sets of mRNAs, each corresponding to the small (AGPase-B) and large (AGPase-S) subunits (Villand et al., 1992a; Kleczkowski et al., 1993c; Kleczkowski, Villand, Lüthi, Olsen, & Preiss, 1993; Thorbjørnsen, Villand, Kleczkowski, & Olsen, 1996). The isoenzymes differ in their regulatory properties, with the endosperm form insensitive to the effector regulation (Kleczkowski et al., 1993a,c) and they are localized in different cell compartments. Whereas the leaf form in all plants is believed to be located exclusively in chloroplasts (Sanwall et al., 1968; ap Rees, 1995), the seed enzyme in barley and maize has been found largely confined to the cytosol, as determined by cell fractionation techniques (Thorbjørnsen, Villand, Denyer, Olsen, & Smith, 1996; Denyer, Dunlap, Thorbjørnsen, Keeling, & Smith, 1996). The cytosolic location of the major isoenzyme of AGPase in cereal seeds is still a controversial issue (reviewed in ap Rees (1995); Kleczkowski (1996)) and needs further confirmation.

Despite great interest in possibilities of manipulating starch levels through changes in regulatory properties of AGPase in transgenic plants (e.g. Giroux et al. (1996)), relatively little is known about the structure/

* Corresponding author.

0031-9422/98/$ - see front matter © 1998 Elsevier Science Ltd. All rights reserved.
PII: S0031-9422(98)00472-5

function properties of the enzyme. One of the key prerequisites to such studies is the establishment of a heterologous expression system where properties of AGPase can be manipulated via site-directed mutagenesis and other methods. Heterologous expression systems, using *Escherichia coli* as a host, have already been established for potato and maize AGPases (Giroux et al., 1996; Iglesias et al., 1993; Giroux & Hannah, 1994) and recently for barley AGPase-B proteins (Luo, Déjardin, Villand, Doan, & Kleczkowski, 1997). Barley AGPases appear to be especially suitable targets for such an investigation since their enzymatic/regulatory properties, as well as all relevant cDNA sequences, have been characterized (Villand et al., 1992a; Kleczkowski et al., 1993a,c; Thorbjørnsen et al., 1996b; Eimert et al., 1997) and their gene loci mapped in barley genome (Kilian et al., 1994). In addition, a barley endosperm isoenzyme of AGPase has been described which, unlike other AGPases, is remarkably insensitive to effectors and shows nearly full activity without activation by PGA (Kleczkowski et al., 1993a). cDNAs encoding subunits of this AGPase contain all the information conferring effector insensitivity and should be of value in designing recombinant AGPases, with their properties tailored to a given environment. The heterologous system may also help in addressing the question of the subcellular location of AGPase, by comparing the M_r's of heterologously expressed full length proteins with those present in plant extracts. In this way, any specific proteolytic processing of a given protein, which is a prerequisite for an effective targeting of a nuclei-encoded protein to the plastid compartment (Keegstra, Olsen, & Theg, 1989), can be directly verified.

In the present study, cDNAs corresponding to barley AGPase-S1, -B1 and -S2 were expressed in *E. coli*. Expression conditions, M_r's of the expressed proteins and their activities were studied. The results are discussed with respect to possible intracellular targeting and processing of AGPase isoenzymes in barley and considering previous data on effector sensitivity of the barley seed enzyme.

2. Results and discussion

2.1. Expression of the large subunit homologues of barley AGPases in E. coli

Constructs containing cDNAs encoding the large subunit of AGPase from barley seed endosperm and leaves have been expressed in *E. coli*, using the isopropylthio-β-D-galactoside (IPTG) inducible expression system (Fig. 1). The AGPase-S1 protein was detected with specific antibodies, which were raised against a synthetic peptide based on a 17 amino acid internal

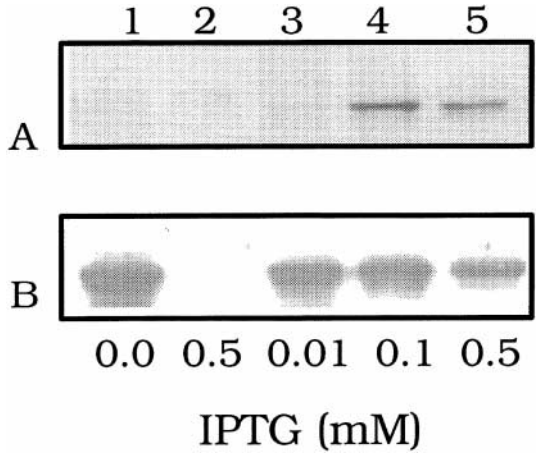

Fig. 1. Immunoblot detection of the IPTG-induced production of barley AGPase-S1 (A) and AGPase-S2 (B) proteins in *E. coli*. Bacterial cells were transformed with full length cDNAs corresponding to AGPase-S1 and AGPase-S2, respectively. The cells were treated with a given concentration of IPTG (lanes 1–5) for 2 h. Crude bacterial proteins were loaded onto 12% SDS-PAGE gel (ca. 5 mg protein per lane). Following the electrophoresis and transfer on an immunoblot membrane, AGPase-S1 and -S2 were detected using antibodies against AGPase-S1-based peptide (Kleczkowski et al., 1993a) and those against native tomato enzyme (Chen & Janes, 1997), respectively. Lanes 1 and 3-5, pET construct containing respective cDNA for AGPase-S; lane 2, pET construct without the insert.

sequence of AGPase-S1 (Kleczkowski et al., 1993a). The expression of AGPase-S1 depended strongly on the inducer concentration, with the highest expression recorded at 0.1 mM IPTG. In contrast, the expression of AGPase-S2, monitored on immunoblots with the antibodies raised against native AGPase from tomato fruits (Chen & Janes, 1997), did not require any IPTG-dependent induction (Fig. 1). In fact, the presence of IPTG appeared to reduce slightly the level of expression of AGPase-S2 when compared with the IPTG-less conditions. This is probably not surprising, since the *lacUV5* promotor controlling the T7 RNA polymerase gene in the AGPase-S2 pET expression system allows some degree of transcription in the uninduced state (Dubendorff & Studier, 1991). Apparently, in the case of AGPase-S2 expression, even a slight background activity of the promotor was sufficient for maximal expression.

In contrast to our earlier work on the expression of the AGPase-B proteins in *E. coli*, which were overexpressed up to 35% of total bacterial proteins (Luo et al., 1997), we have failed to produce large quantities of any of the AGPase-S homologues, regardless of the IPTG concentration. There were no new protein band(s) on Coomassie Blue stained SDS-PAGE gels for crude extracts of the recombinant *E. coli* when compared with those from wild type bacteria (data not shown). To our knowledge, the large subunit has not been produced in large quantities from any other plant

species (Giroux et al., 1996; Iglesias et al., 1993). The expression of the large subunit may have inhibitory or toxic effect on the growth of host *E. coli* cells (Villand, personal communication). Also, the newly synthesized AGPase-S may perhaps require the presence of the corresponding AGPase-B for stability, as recently proposed for the *Arabidopsis* enzyme in transgenic plants (Wang et al., 1997) (see below).

The bacteria-expressed AGPase-S1 (calculated M_r of 57.9 kDa (Villand, Olsen, Kilian, & Kleczkowski, 1992)) had the same electrophoretic mobility as the corresponding protein in barley endosperm extracts, as found by immunoblotting of the SDS-PAGE-resolved proteins [Fig. 2(A)]. In contrast, the M_r's of AGPase-S2 (54.8 kDa (Eimert et al., 1997)) was larger than the size of the corresponding partially purified leaf protein (ca. 53 kDa) [Fig. 2(B)]. This suggests that the AGPase-S2 undergoes proteolytic processing in leaves, consistent with targeting to the plastid compartment. Whereas the cytosol-based proteins do not require any proteolytic processing for intracellular targeting, such

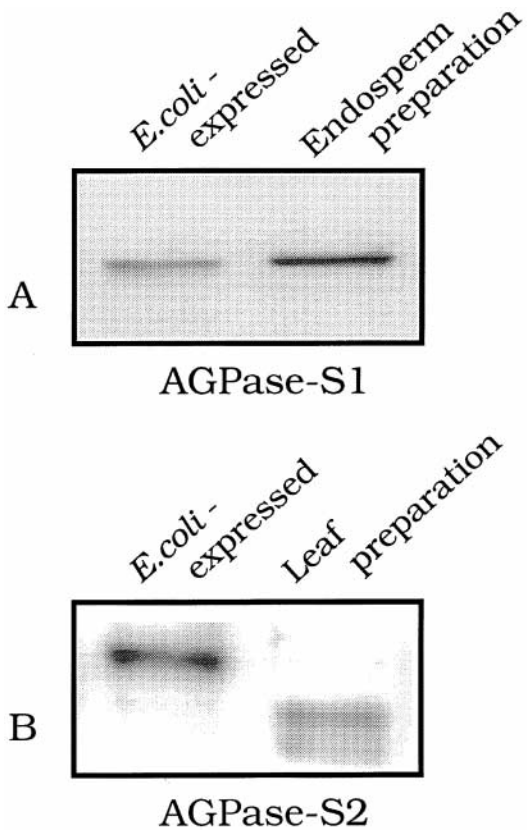

Fig. 2. Comparison of M_r's of barley AGPase-S proteins expressed in *E. coli* from full length cDNAs and those present in barley endosperm and leaf preparations. Proteins (ca. 5 mg per lane) were resolved by SDS-PAGE (10% gel) and AGPase-S bands were detected by immunoblotting. AGPase-S1 and -S2 proteins were identified using antibodies against AGPase-S1-based peptide (Kleczkowski et al., 1993a) and those against native tomato enzyme (Chen & Janes, 1997), respectively.

a processing is mandatory for all nuclei-encoded proteins that are targeted to the plastid compartment (Keegstra et al., 1989).

The data are consistent with our recent evidence on the expression of barley AGPase-B (small subunit) homologues in *E. coli* (Luo et al., 1997). By comparing the M_r of proteins expressed from full length cDNAs with those present in barley extracts, we have found that the endosperm AGPase-B1 does not undergo any specific post-translational proteolytic processing. On the other hand, AGPase-B2 from leaf extracts was ca. 5 kDa shorter than when expressed in the bacterial system (Luo et al., 1997). Thus, both the small and large subunits of the barley endosperm-type AGPase remain intact following translation, whereas the corresponding subunits of the leaf-type of the enzyme are proteolytically processed, with the mature proteins being smaller than their precursors. These results confirm the current view (Villand & Kleczkowski, 1994; Thorbjørnsen et al., 1996a,b; Denyer et al., 1996) that the endosperm-type of barley AGPase is located in the cytosol, whereas the leaf-type is targeted to plastids (ap Rees, 1995; Sanwall et al., 1968). Based on gene expression studies, the former enzyme appears to be confined solely to the endosperm, whereas the latter occurs abundantly in leaves and, to some degree, in the endosperm (Villand et al., 1992a; Eimert et al., 1997). The presence of the cytosolic and plastidial AGPases in barley seeds has recently been demonstrated directly by subcellular fractionation methods (Thorbjørnsen et al., 1996a).

2.2. Activities and regulation of barley AGPase subunits expressed in E. coli

One of the most recognised enzymatic properties of AGPase from a range of plant species and tissues is a several-fold activation of the enzyme by PGA and inhibition by P_i (Sanwall et al., 1968; Müller-Röber et al., 1992; Kleczkowski et al., 1993c; Iglesias et al., 1993). In many tissues, the effector regulation of AGPase directly affects rates of starch synthesis, providing one of the most recognised models for an in vivo metabolic control of a major plant pathway (Kleczkowski, 1994). Barley endosperm AGPase is the notable exception to this rule, with PGA causing only ca. 25% activation (when assaying in ADP-glucose formation) and with Pi acting as a weak inhibitor (Kleczkowski et al., 1993a). In studies on the expression of a double construct containing cDNAs for both AGPase-B1 and -S1 in *E. coli*, we have found that the activity of the newly formed protein had characteristics that were analogous or identical to those found for AGPase from barley endosperm (Table 1). The enzyme was only slightly activated by PGA and was not affected by P_i. In comparison,

Table 1. Activities and effector regulation of barley AGPase subunits that were expressed in *E. coli* from single constructs containing either AGPase-B1 or -S1 and a double construct containing both AGPase-B1 and -S1

Construct	Activity (%)		
	no effectors	+ PGA	+ P_i
AGPase-B1	0	0	0
AGPase-S1	0	0	0
AGPase-B1 and -S1	100* (100)[b]	127 (125)[b]	101 (98)[b]

Assays were carried out in the ADP-glucose synthesis direction. Concentrations of PGA and P_i were 1 mM each.
[a]Corresponds to 0.091 units per mg protein.
[b]Numbers in parentheses correspond to relative activities of barley endosperm AGPase, purified as in Kleczkowski et al. (1993a) and assayed under the same assay conditions as for the bacteria-expressed enzyme.

similar assays for AGPases from leaves resulted in over 10-fold activation of the enzyme by PGA and an almost complete inhibition when P_i was present in the assay (Sanwall et al., 1968; Kleczkowski et al., 1993b). In our studies, under assay conditions (i.e. 1 mM PGA), neither AGPase-B1 nor -S1, when expressed alone, showed any enzymatic activity regardless of the presence or absence of PGA (Table 1). Similar data were obtained for the individual subunits of AGPase from potato tubers when expressed in *E. coli* (Iglesias et al., 1993); in the case of the potato small subunit, the protein did show enzymatic activity but only after treatment with a very high nonphysiological concentration of PGA (Iglesias et al., 1993). Although previously we were able to produce large quantities of AGPase-B1 in *E. coli* (Luo et al., 1997), the double construct containing cDNAs for both AGPase-B1 and -S1 was only weakly expressed, similarly to the expression of the AGPase-S1 cDNA alone. Thus it appears that the presence of the large subunit may represent a limiting factor for the formation of an active small and large subunit complex of native AGPase. Also, the simultaneous expression of cDNAs for both subunit types of the enzyme may be an important prerequisite to stabilise the corresponding mRNAs and/or to prevent degradation of individual subunit proteins (e.g. Wang et al. (1997)).

The results (Table 1) support the notion (Kleczkowski et al., 1993a,b,c; Rudi, Doan, & Olsen, 1997) that barley endosperm AGPase, in contrast to AGPases from other species/tissues, is insensitive to effectors. This property was previously observed both for intact and proteolytically modified endosperm enzyme that lacked ca. 5 kDa of the *N*-terminus of the large subunit (Kleczkowski et al., 1993a), suggesting that proteolysis of the large subunit is not the cause of effector insensitivity. This was confirmed in the present study where we used full length cDNAs and where no indication of proteolytical processing in the bacterial system was observed. Analogous results were recently obtained for barley AGPase-S1 and -B1 cDNAs that were expressed together in insect cells via a baculovirus-based delivery system (Rudi et al., 1997). Since insect cells are eukaryotic, like barley, they are able to carry out protein modifications, such as protein glycosylation or phosphorylation (O'Reilly, Miller, & Luckow, 1992). Given that both insect cells- and *E. coli*-mediated expression of AGPase cDNAs brought about the enzyme with similar regulatory properties, it appears that post-translational modifications, if any, do not play a major role in the regulation of the barley endosperm enzyme. The (in)sensitivity to effectors may depend on hydrophobic interactions between subunits of AGPase and/or on conformational states of individual subunits (Kleczkowski et al., 1993b). Whether the insensitivity of barley endosperm AGPase to PGA/Pi regulation is functionally related to the cytosol location of the enzyme, as suggested (Villand & Kleczkowski, 1994), is still unknown at present.

3. Experimental

3.1. Plant material

Barley (*Hordeum vulgare*), cv. Bomi, plants were greenhouse-grown. Crude extracts of barley endosperm proteins were isolated in SDS-buffer (Kleczkowski et al., 1993a,c). Leaf AGPase was partially purified as in Luo et al. (1997).

3.2. Plasmid construction and heterologous expression

Standard DNA techniques, according to Sambrook, Fritsch, and Maniatis (1989), were used. The *E. coli* DH5a strain was used as a host for cloning the cDNAs. For all cloning steps, the identity of the constructed DNA was verified by sequencing.

Full open reading frames (ORFs) of *bepl10* (Villand et al., 1992b) and *blpl14* (Eimert et al., 1997), the cDNAs encoding AGPase-S1 and -S2, respectively, were amplified by polymerase chain reaction (PCR) techniques. For the amplification of *bepl10*, primers 5′-CCGGATCCATATGCAGTTCAGCAGCGTG-3′ (forward) and M13 (reverse) were used. The primers used for the amplification of *blpl14* were 5′-GGGCGGATCCATTGGTTGCGCCATGG-3′ and M13, respectively. Amplification products were cleaved with *Bam*HI and inserted to pBluescript (Stratagene). Following the insertion, the coding region of the *bepl10* construct was cleaved out by partial digestion with *Nde*I and complete digestion with *Bam*H1 and inserted to the corresponding sites of the expression vector pET-3a (Studier, Rosenberg, Dunn, &

Dubendorf, 1990). For the *blpl14* construct, its coding region was cleaved out by complete digestion with *Bam*HI and partial digestion with *Nco*I and inserted to the corresponding sites of the expression vector pET-3d (Novagen).

The resulting plasmids pET-3a-*bepl10* and pET-3d-*blpl14* were transformed into *E. coli* strains BL21(DE3)lysS and BL21(DE3), respectively (Novagen). These DE3 host cells contain the T7 RNA polymerase gene which is under the control of the *lacUV5* promotor (Dubendorff & Studier, 1991). All cells were grown at 37°C to $A_{0.5}$ at 600 nm (LKB Novaspec). The expression of AGPases-S was induced by addition of different conc. of IPTG. 2 h later the cells were collected by centrifugation at 6000 rpm for 10 min and the pellet was resuspended in one tenth of the original vol. with 10 mM Tris (pH 8.0) and 2 mM $MgCl_2$. The resulting extracts were immediately mixed with SDS-buffer for SDS-PAGE.

For studies on the activity of the heterologously expressed barley endosperm AGPase subunits, the ORF of small subunit cDNA (*BepsF1*) was amplified by using primers BS5P (GGATCCATGGATGTACCTTTGGCATCT) and BS3P (CGGGATCCTTATTTATTTATATGACTGTTCCACTAG) and inserted to the *Nco*I–*Bam*HI site of pET3d to create pTBSF. The AGPase-S1 (*Bepl10*) ORF was amplified by using the primers BL5P (GGATCCATATGCAGTTCAGCAGCGTGCTG) and BL3P (CGGGATCCGCACAGGTTGTCGCAGAAC) and inserted to the *Nde*I–*Bam*HI site of pET9a to create pT9BLF. To create the pT9BLSF double construct, the small subunit ORF and well as upstream Shine–Dalgarno sequences in pTBSF were amplified by using the primers 3DSD (CGGGATCCTTTAAGAAGGAGATATAC) and BS3P and inserted in the *Bam*HI site of pT9BLF. pT9BLSF was transferred into *E. coli* strain BL21[DE3] and induction of expression in liquid cultures was performed according to the manufacturers' (Novagen) instructions. Cells were harvested 2 h after induction and proteins were extracted for enzyme assays.

3.3. AGPase assay

AGPase was assayed in the direction of ADP-glucose synthesis, using Mg-ATP and ^{14}C-labelled glucose-1-P as substrates (Kleczkowski et al., 1993a,b). Reactions were initiated with the enzyme and were carried out for 10 min. For assays of AGPase expressed in *E. coli*, controls were run containing protein extracts from wild-type bacteria to subtract any endogenous activity of bacteria-own AGPase. Under assay conditions, we found no activity for *E. coli*-own AGPase. One unit of AGPase activity was defined as 1 μmol of ADP-glucose produced per 10 min.

3.4. Electrophoresis and immunodetection

Conditions of the electrophoresis were as in Luo et al. (1997). The SDS-PAGE-resolved proteins were transferred onto nitrocellulose membrane (Bio-Rad) using a Bio-Rad protein transfer system. Immunodetection of AGPase-S proteins was carried out using rabbit antibodies raised against a synthetic peptide that was based on a 17 amino acid internal sequence of AGPase-S1 (Kleczkowski et al., 1993a) and rabbit antibodies raised against native AGPase from tomato fruits (Chen & Janes, 1997). Binding of these primary antibodies to AGPase-S protein(s) was detected with donkey anti-rabbit secondary antibodies linked to horseradish peroxidase (Amersham); the peroxidase activity was visualised using ECL fluorescence reagents (Amersham). Protein content was determined using the Bio-Rad Assay, with bovine serum albumin as standard.

Acknowledgements

This research was supported, in part, by grants from the Swedish Foundation for Strategic Research and Swedish Natural Science Research Council.

References

ap Rees, T. (1995). In H. G. Pontis et al. (Eds.), *Sucrose metabolism, biochemistry, physiology and molecular biology* (p. 143). Rockville, MD: American Society of Plant Physiologist Series.

Chen, B.-Y., & Janes, H. W. (1997). *Plant Physiology, 113,* 235.

Denyer, K., Dunlap, F., Thorbjørnsen, T., Keeling, P., & Smith, A. M. (1996). *Plant Physiology, 112,* 779.

Dubendorff, J . W., & Studier, F. W. (1991). *Journal of Molecular Biology, 219,* 45.

Eimert, K., Luo, C., Déjardin, A., Villand, P., Thorbjørnsen, T., & Kleczkowski, L. A. (1997). *Gene, 189,* 79.

Giroux, M. J., & Hannah, L. C. (1994). *Molecular and General Genetics, 243,* 400.

Giroux, M. J., Shaw, J., Barry, G., Cobb, B. G., Greene, T., Okita, T., & Hannah, L. C. (1996). *Proceedings of the National Academy of Sciences USA, 93,* 5824.

Iglesias, A. A., Barry, G. F., Meyer, C., Bloksberg, L., Nakata, P. A., Greene, T., Laughlin, M., Okita, T. W., Kishore, G. M., & Preiss, J. (1993). *Journal of Biological Chemistry, 268,* 1081.

Keegstra, K., Olsen, L. J., & Theg, S. M. (1989). *Annual Review of Plant Physiology and Plant Molecular Biology, 40,* 471.

Kilian, A., Kleinhofs, A., Villand, P., Thorbjørnsen, T., Olsen, O.-A., & Kleczkowski, L. A. (1994). *Theoretical and Applied Genetics, 87,* 869.

Kleczkowski, L. A. (1994). *Annual Review of Plant Physiology and Plant Molecular Biology, 45,* 339.

Kleczkowski, L. A. (1996). *Trends in Plant Science, 1,* 363.

Kleczkowski, L. A., Villand, P., Lönneborg, A., Olsen, O.-A., & Lüthi, E. (1991). *Zeitschrift für Naturforschung, 46c,* 605.

Kleczkowski, L. A., Villand, P., Lüthi, E., Olsen, O.-A., & Preiss, J. (1993a). *Plant Physiology, 101,* 179.

Kleczkowski, L. A., Villand, P., & Olsen, O.-A. (1993b). *Zeitschrift für Naturforschung, 48c,* 457.

Kleczkowski, L. A., Villand, P., Preiss, J., & Olsen, O.-A. (1993c). *Journal of Biological Chemistry, 268,* 6228.

Luo, C., Déjardin, A., Villand, P., Doan, D. N. P., & Kleczkowski, L . A. (1997). *Zeitschrift für Naturforschung, 52c,* 807.

Martin, C., & Smith, A. M. (1995). *Plant Cell, 7,* 971.

Müller-Röber, B., Sonnewald, U., & Willmitzer, L. (1992). *EMBO Journal, 11,* 1229.

O'Reilly, D. R., Miller, L. K., & Luckow, V. A. (1992). *Baculovirus expression vectors: a laboratory manual*. New York: W. H. Freeman and Co.

Rudi, H., Doan, D. N. P., & Olsen, O.-A. (1997). *FEBS Letters, 419,* 124.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular cloning: a laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sanwall, G. G., Greenberg, E., Hardie, J., Cameron, E. C., & Preiss, J. (1968). *Plant Physiology, 43,* 417.

Studier, F. W., Rosenberg, A. H., Dunn, J. J., & Dubendorf, J. W. (1990). *Methods in Enzymology, 185,* 60.

Thorbjørnsen, T., Villand, P., Denyer, L., Olsen, O. A., & Smith, A. M. (1996a). *Plant Journal, 10,* 243.

Thorbjørnsen, T., Villand, P., Kleczkowski, L. A., & Olsen, O. A. (1996b). *Biochemical Journal, 313,* 149.

Tsai, C. Y., & Nelson, O. E. (1966). *Science, 151,* 341.

Villand, P., & Kleczkowski, L. A. (1994). *Zeitschrift für Naturforschung, 49c,* 215.

Villand, P., Aalen, R., Olsen, O.-A., Lüthi, E., Lönneborg, A., & Kleczkowski, L. A. (1992a). *Plant Molecular Biology, 19,* 381.

Villand, P., Olsen, O.-A., Kilian, A., & Kleczkowski, L. A. (1992b). *Plant Physiology, 100,* 1617.

Wang, S.-M., Chu, B., Wue, W.-L., Yu, T.-S., Eimert, K., & Chen, J. (1997). *Plant Journal, 11,* 1121–1126.