FEBS Letters 490 (2001) 44–48 FEBS 24585

Two isoforms of a nucleotide-sugar pyrophosphatase/phosphodiesterase from barley leaves (*Hordeum vulgare* L.) are distinct oligomers of HvGLP1, a germin-like protein

Milagros Rodríguez-López, Edurne Baroja-Fernández, Aitor Zandueta-Criado, Beatriz Moreno-Bruna, Francisco José Muñoz, Takashi Akazawa, Javier Pozueta-Romero*

Instituto de Agrobiotecnología y Recursos Naturales, Universidad Pública de Navarra/Consejo Superior de Investigaciones Científicas, Ctra. Mutilva s/n, 31192 Mutilva Baja, Navarra, Spain

Received 23 November 2000; revised 8 January 2001; accepted 8 January 2001

First published online 23 January 2001

Edited by Ulf-Ingo Flügge

Abstract Two isoforms of ADPglucose pyrophosphatase/phosphodiesterase (AGPPase) have been characterized using barley leaves (*Hordeum vulgare* L.). Whilst one of the isoforms, designated as soluble AGPPase1 (SAGPPase1), is soluble in low ionic strength buffers, the other, SAGPPase2, is extractable using cell wall hydrolytic enzymes or high salt concentration solutions, thus indicating that it is adventitiously bound to the cell wall. Both AGPPase isoforms are highly resistant to SDS, this characteristic being utilized to purify them to homogeneity after zymographic detection of AGPPase activity in SDS-containing gels. N-terminal and internal amino acid sequencing analyses revealed that both SAGPPase1 and SAGPPase2 are distinct oligomers of the previously designated HvGLP1, which is a member of the ubiquitously distributed group of proteins of unknown function designated as germin-like proteins (GLPs).

Key words: Cell wall; Cupin; Gluconeogenesis

1. Introduction

A widely distributed pyrophosphatase/phosphodiesterase activity has recently been found in plants that catalyzes the hydrolytic breakdown of several NDP-monosaccharides [1]. Among numerous substrates of physiological relevance tested, ADPG, the universal precursor for starch biosynthesis, was shown to act as the preferred substrate and therefore the enzyme was designated as AGPPase.

Experiments carried out to characterize different AGPPase isoforms have demonstrated the occurrence of both soluble and starch granule-bound, as well as intra- and extra-plastidial enzymes [1,2]. These observations have led us to the proposal that, essentially identical to the mechanisms proposed to control levels of nucleotide-sugars linked to glycoprotein and glycolipid biosynthesis in mammalian tissues [3–5], nucleotide-sugar hydrolytic enzymes may play a crucial role in controlling metabolic flow towards starch, cell wall polysaccharides, glycoproteins and glycolipids in plants [2]. According to this hypothesis, the extent to which these molecules accumulate in the plant cell will be inversely correlated to the activities of nucleotide-sugar hydrolases.

Among the different AGPPase isoforms found, a soluble isoform has been partially purified and characterized using barley leaves [1]. Subsequent investigations, which are reported in this communication, were conducted to further clarify the nature of this enzyme, and have shown that it belongs to a group of proteins of yet to be identified enzymatic properties, the GLPs.

2. Materials and methods

2.1. Plant material

Barley plants (*Hordeum vulgare* cv. Scarlett) were grown in greenhouse. For purification of AGPPase, 1.1 kg of 45 days old leaves were used.

2.2. Extraction and purification of AGPPase

Unless otherwise indicated, all steps were carried out at 4°C. For small-scale extractions, barley leaves were homogenized with 3-fold of extraction buffer (50 mM MES, pH 6.0/1 mM EDTA/2 mM DTT) in a Waring blender. When indicated, either 0.5 M NaCl or cell wall hydrolytic enzymes [6] were included in the homogenate. After 30 min at 25°C, the homogenate was filtered through four layers of Miracloth and centrifuged at $100\,000 \times g$ for 30 min. The supernatant was then used for subsequent analyses.

Purification of AGPPase was performed as described elsewhere ([1]; cf. Table 2), except that 0.5 M NaCl was included in the elution buffer of the gel filtration step, and a further zymographic purification step was carried out. The partially purified enzyme preparation obtained after the gel filtration step was loaded without heat denaturation onto an SDS 4–12% NuPAGE Bis-Tris gel (Novex). After electrophoresis, the gel was incubated for 15 min in 5 mM bis-PNPP and 50 mM sodium acetate buffer, pH 5.0 until a yellow band appeared, which indicated the position where the AGPPase-mediated hydrolytic breakdown of bis-PNPP took place. The band was then cut and AGPPase eluted at 4°C in 10 mM Tris–HCl, pH 7.5, 10 mM β-mercaptoethanol and 0.2 M NaCl. The eluted protein was desalted by ultrafiltration on Centricon YM-10 (Amicon, Bedford, MA, USA) for subsequent analyses.

The native molecular mass of AGPPase was determined by gel filtration on a Superdex 200 column (Pharmacia LKB) using a Bio-Rad kit of protein standards. Protein content was measured by the Bradford method using the Bio-Rad prepared reagent.

*Corresponding author. Fax: (34)-948232191.
E-mail: javier.pozueta@unavarra.es

Abbreviations: ADPG, ADPglucose; AGPPase, ADPG pyrophosphatase/phosphodiesterase; GLP, germin-like protein; HvGLP1, *Hordeum vulgare* germin-like protein 1; PAGE, polyacrylamide gel electrophoresis; PNPP, *p*-nitrophenyl phosphate; SAGPPase, soluble ADPglucose pyrophosphatase/phosphodiesterase; UDPG, UDPglucose

PII: S0014-5793(01)02135-4

2.3. Sequencing of AGPPase

Purified SAGPPase1 and SAGPPase2 were electrophoretically separated in a 12% SDS–PAGE according to Laemmli [7] and blotted onto polyvinylidene difluoride membrane (Immobilon P; Millipore Corp.). The protein bands corresponding to both isoforms of AGPPase were excised and used for N-terminal and internal peptide sequences using an Applied Biosystems model 473A amino acid sequencer.

2.4. Enzyme assays

Measurements of AGPPase activities were performed as described by Rodríguez-López et al. [1]. Measurements of oxalate oxidase and superoxide dismutase activities were performed as described by Vallelian-Bindschedler [8] and Yamahara et al. [9], respectively. The unit is defined as the amount of enzyme which catalyzes the production of 1 μmol of product per min.

2.5. Reagents

Pectolyase Y-23 was purchased from Seishin Co. Cellulase Onozuka R-10 and Macerozyme R-10 were from Yakult Pharmaceutical Ind. Co. Ltd. Other enzymes and chemical reagents were purchased from Sigma. Glycoprotein staining was performed employing a periodic acid-Schiff reagent kit from Sigma.

3. Results and discussion

3.1. Presence of different soluble AGPPase isoforms in barley leaves

Experiments carried out to identify possible different AGPPase isoforms revealed that, essentially similar to the case of the suspension-cultured cells of sycamore (*Acer pseudoplatanus* L.) [2], there occur both soluble and particulate AGPPase activities in barley leaves (not shown). Although the majority of the AGPPase is shown to be tightly bound to the starch granules (Zandueta-Criado et al., manuscript in preparation) results presented in this work deal with the characterization of enzyme(s) responsible for the soluble activity.

Experiments conducted to optimize the conditions of extracting soluble AGPPase described previously [1] revealed that the efficiency was enhanced when 0.5 M NaCl was added to the extraction buffer. Preparations obtained using buffers with or without 0.5 M NaCl were separately subjected to gel filtration chromatography. As presented in Fig. 1, a single ca. 45–65 kDa AGPPase isoform (designated as SAGPPase1) was identified in samples obtained using low ionic strength extraction buffer, whereas two additional ca. 250 kDa and ca. 600 kDa isoforms (designated as SAGPPase2 and SAGPPase3, respectively) were obtained when 0.5 M NaCl was included in the extraction buffer. These results indicate that both SAGPPase2 and SAGPPase3 are adventitiously bound to particulate constituent(s) of the plant cell. Assuming that they are bound to the cell wall, solubilization was attempted using cell wall hydrolyzing enzymes. As shown in Fig. 1, only SAGPPase2 was found to be solubilized after treatment with Cellulase, Macerozyme and Pectolyase. The overall results thus strongly indicate that SAGPPase2 is ionically bound to the cell wall.

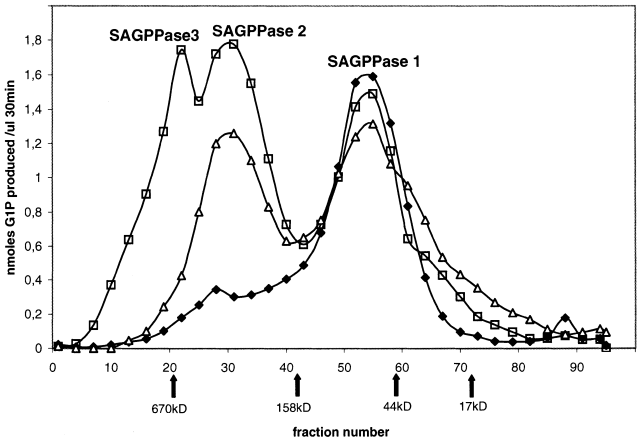

Fig. 1. Partial purification of different isoforms of AGPPase using gel filtration chromatography. Barley leaves were homogenized as described in Section 2, filtered through Miracloth and subjected to 100 000×*g* for 30 min prior to gel filtration (♦). In (□) and (△), 0.5 M NaCl and cell wall hydrolytic enzymes were supplemented to the homogenate, prior to 100 000×*g* centrifugation.

3.2. Purification of SAGPPase1 and SAGPPase2

Unlike SAGPPase3, both SAGPPase1 and SAGPPase2 are able to bind to concanavalin A-Sepharose beads (not shown) and are resistant to high temperature treatments, indicating that the latter AGPPase isoforms are glycosylated.

Since glycoproteins are known to bind poorly to SDS [10], we tested the ADPG and bis-PNPP hydrolytic activities of both SAGPPase1 and SAGPPase2 in the presence of 2% SDS. Remarkably, and essentially identical to other enzymes which were refractory to SDS [11,12], both SAGPPase1 and SAGPPase2 are highly resistant to SDS (see below). By taking advantage of this particular characteristic, partially purified SAGPPase1 and SAGPPase2 (gel filtration step, Table 1) were run on SDS–NuPAGE without prior heat denaturation. Gels were subsequently dipped into a bis-PNPP-containing solution, and AGPPase activities were revealed as yellow bands in the gel (Fig. 2a). The eluted bands were then subjected to a further step of SDS–NuPAGE separation in which

Table 1
Purification of SAGPPase1 and SAGPPase2 from Barley leaves

	Total volume (ml)	Total protein (mg)	Total activity (mU)	Specific activity (mU/mg protein)	Purification (fold)
Supernatant (100 000×*g*)	3 300	10 989	232 980	21.2	–
Ammonium sulfate 50%	3 300	2 646.6	232 385	87.8	4.1
pH 4.2/62°C	3 300	148.5	182 589	1 230	58
Ammonium sulfate 50%	30	86.16	182 250	2 115	100
Gel filtration:					
SAGPPase1	2.13	24.59	43 063	1 750	83
SAGPPase2	0.78	11.42	24 786	2 170	102
NuPAGE:					
SAGPPase1	0.63	0.19	43 000	226 647	10 690
SAGPPase2	0.23	0.07	24 500	354 085	16 700

ADPG hydrolytic activities were measured as described in [1].

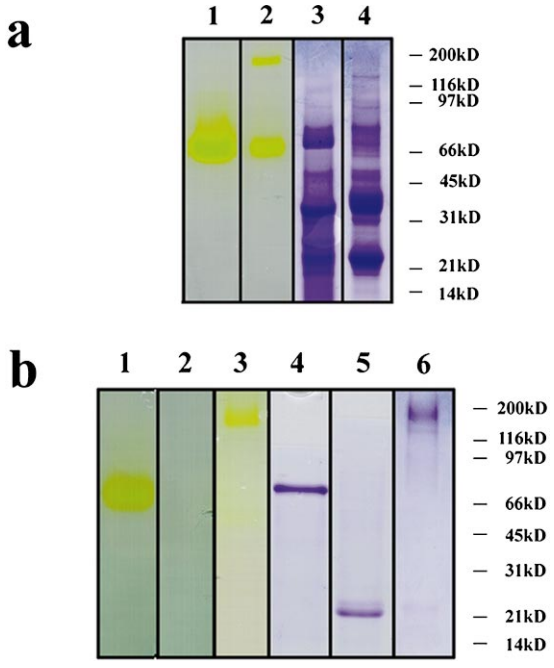

Fig. 2. Zymographic detection and Coomassie blue staining of SAGPPase1 and SAGPPase2. a: Partially purified SAGPPase1 (lanes 1 and 3) and SAGPPase2 (lanes 2 and 4) obtained after the gel filtration step (see Table 1) were subjected to SDS–NuPAGE separation. Each electrophoresed gel was subjected to zymographic detection of AGPPase (lanes 1 and 2) and Coomassie staining (lanes 3 and 4). b: The proteins eluted from the yellow bands running in a at the levels of ca. 65 kDa (SAGPPase1) and ca. 200 kDa (SAGPPase2) were subjected to a further step of SDS–NuPAGE. After electrophoresis, the gel was applied to zymographic detection of AGPPase (lanes 1–3) and Coomassie staining (lanes 4–6). In lanes 1 and 4 (SAGPPase1) and lanes 3 and 6 (SAGPPase2), proteins were loaded onto the gel without a previous heat denaturation step. In lanes 2 and 5, SAGPPase1 was heat denatured prior to electrophoretic separation.

bands of apparent ca. 65 kDa and ca. 200 kDa were detected for the case of SAGPPase1 and SAGPPase2, respectively (Fig. 2b). As presented in Table 1, we were able to purify SAGPPase1 and SAGPPase2 10 700- and 16 700-fold, the specific activities being 225 and 355 units/mg protein, respectively.

Similar to the case of other plant proteins described in the literature [11–13], when both SAGPPase1 and SAGPPase2 were heat denatured and subjected to SDS–NuPAGE separation, doublets of ca. 22 kDa and ca. 23 kDa polypeptides were revealed (lane 5 in Figs. 2b, 3a), the overall results thus indicating that, as will be discussed later, SAGPPase1 and SAGPPase2 are oligomers made up of the same polypeptide (see below).

3.3. Both SAGPPase1 and SAGPPase2 are distinct homooligomers of HvGLP1, a germin-like protein

The ca. 22 kDa and ca. 23 kDa polypeptide bands of the doublets observed after electrophoresis of the heat denatured SAGPPase1 and SAGPPase2 were subjected to peptide sequencing. N-terminal sequence comparisons between both polypeptides revealed that they share 100% homology over the 20 amino acids compared, the overall results thus strongly indicating that SAGPPase1 and SAGPPase2 are distinct homooligomers constituted by the same polypeptide. Glycoprotein staining analyses employing the periodic acid-Schiff

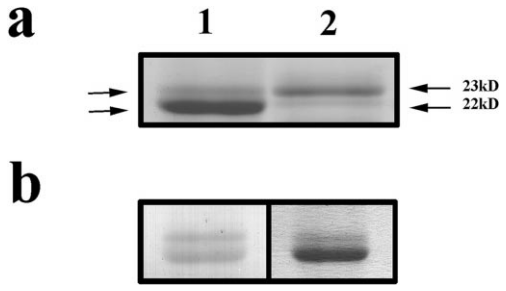

Fig. 3. SDS–PAGE of heat denatured SAGPPase1 and SAGPPase2. a: Coomassie blue staining of the doublets corresponding to SAGPPase1 (lane 1) and SAGPPase2 (lane 2). b: Schiff (lane 1) and Coomassie blue (lane 2) staining of SAGPPase1.

reagent indicated that the doublets are ascribable to a higher degree of glycosylation of the ca. 23 kDa polypeptide over the ca. 22 kDa polypeptide (Fig. 3b). As presented in Fig. 3a, the patterns of band intensities of SAGPPase1 and SAGPPase2 were clearly distinguishable, suggesting that SAGPPase2 has a higher degree of glycosylation than SAGPPase1.

Most importantly, comparisons of both N-terminal and internal sequences of SAGPPase1 against sequences existing in the EMBL data bank (Fig. 4) showed that it corresponds to HvGLP1 [8], a member of a previously characterized group of plant proteins designated as GLPs, which in turn belongs to a superfamily of functionally diverse proteins existing in both prokaryotes and eukaryotes which are now called 'cupins' [14,15].

GLPs are known to be water-soluble glycoproteins with a monomer molecular mass of about 25 kDa. They share high sequence homology with cereal germins [11] and occur in the cell wall, cytoplasm, Golgi apparatus, vacuole and the plasma membrane [16–18]. Both germins and GLPs form oligomers that are highly resistant to both SDS and heat treatment, and migrate as doublets in denaturing gels after heat treatment [8,12,13,17,19–21]. Therefore, the overall characteristics of both SAGPPase1 and SAGPPase2 coinciding with those described for GLPs in the literature, confirm that they are homooligomers of HvGLP1, a GLP.

3.4. Both SAGPPase1 and SAGPPase2 are nucleotide-sugar pyrophosphatases/phosphodiesterases but not oxalate oxidases or superoxide dismutases

Unlike cereal germins which bear oxalate oxidase activities, no enzyme activity has yet been assigned to any GLPs, with the single exception of BuGLP, a moss GLP which is a manganese superoxide dismutase [9]. Both the oxalate oxidase and superoxide dismutase reactions supply H_2O_2 which is supposedly required for cross-linking reactions of cell wall polysac-

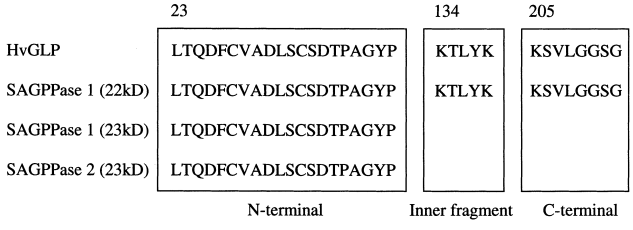

Fig. 4. Alignment of the amino acid sequences of the ca. 22 kDa and ca. 23 kDa polypeptides of SAGPPase1, SAGPPase2 and HvGLP1 (EMBL accession number Y15962, [8]).

Table 2
Substrate specificity of SAGPPase2

Substrates	K_m (mM)	V_{max} (% with respect to ADPG)
ADPG	0.8	100
CDPglucose	1.4	88
UDPG	1.9	106
ADPribose	0.5	80
Bis-PNPP	1.2	68

charides or for the induction of defense responses of plants against both biotic and abiotic stresses [19,22–25]. We therefore tested whether both SAGPPase1 and SAGPPase2 could catalyze oxalate oxidase and/or superoxide dismutase reactions. In conformity with the results obtained by Vallelian-Bindschedler et al. on HvGLP1 [8], we were repeatedly unable to detect such activities in SAGPPase1 and SAGPPase2 (not shown).

In our previous report we showed that SAGPPase1 is a pyrophosphatase/phosphodiesterase which hydrolyzes some low molecular weight phosphodiester bond-containing compounds such as nucleotide-sugars, whereas it does not hydrolyze PPi and nucleoside phosphomono-, di- or triesters [1]. Since both SAGPPase1 and SAGPPase2 are now found to be homooligomers of the same polypeptide, we examined the substrate specificity and kinetic parameters of SAGPPase2. As shown in Table 2, the overall characteristics of SAGPPase2 are identical to those described previously for SAGPPase1.

Altogether these results demonstrate that, irrespective of their different subcellular localization, relative oligosaccharide content, native size and polymerization degrees, both SAGPPase1 and SAGPPase2 catalyze the hydrolytic breakdown of nucleotide-sugars and they are neither oxalate oxidases nor superoxide dismutases.

3.5. Additional remarks

Although one of the physiological roles attributable to AGPPase activities is to control the levels of ADPG linked to starch biosynthesis [1], a possibility has been discussed that they may also govern the intracellular levels of nucleotide-sugars necessary for the biosynthesis of cell wall polysaccharides, glycoproteins and glycolipids [2]. Remarkably, Vallelian-Bindschedler et al. [8] and Schweizer et al. [24] have shown that *HvGLP1* is a constitutive gene whose expression declined upon fungal infection, causing the disappearance of HvGLP1 in the infected areas concomitantly with a cell wall reinforcement. Therefore, based on the results presented in the present study and in the referred literature, it can be suggested that SAGPPase1 may entail a role in controlling the extent to which NDP-sugars can be channelled toward the synthesis of cell wall polysaccharides taking place in the Golgi apparatus and in the plasma membranes [26–28].

Some nucleotide-sugar hydrolases from both bacteria and animals, whose active sites face the external medium, play a paramount role in the nucleotide metabolism of the cell [29–33]. The suggested role of these 'ectoenzymes' is to act as a first step in the scavenging of extracellular nucleotide-sugars [34]. Furthermore, some extracellular nucleotide pyrophosphatases/phosphodiesterases have been reported in plants which suggested physiological role is the rescuing of nucleotides and Pi [35–37]. Therefore, it can be presumed that SAGPPase2, which is located outside the plant cell, may be involved in the scavenging of extracellular nucleotide-sugars.

Acknowledgements: We thank Maria José Villafranca (I.A.N.R., Spain) for expertised technical support. T.A. wishes to record his sincere appreciation for the support from the Spanish Ministry of Culture and Education. We are indebted to Pepa González-Nicolás (Universidad Autónoma de Madrid, Spain) for helpful and valuable contributions in microsequencing of AGPPase.

References

[1] Rodríguez-López, M., Baroja-Fernández, E., Zandueta-Criado, A. and Pozueta-Romero, J. (2000) Proc. Natl. Acad. Sci. USA 97, 8705–8710.
[2] Baroja-Fernández, E., Zandueta-Criado, A., Rodríguez-López, M., Akazawa, T. and Pozueta-Romero, J. (2000) FEBS Lett. 480, 277–282.
[3] Nelson, J.D., Jato-Rodriguez, J.J., Labrie, F. and Mookerjea, S. (1977) J. Endocrinol. 73, 53–58.
[4] van Dijk, W., Lasthuis, A.-M., Trippelvitz, L.A.W. and Muilerman, H.G. (1983) Biochem. J. 214, 1003–1006.
[5] Hickman, S., Wong-Yip, Y.P., Rebbe, N.F. and Greco, J.M. (1985) J. Biol. Chem. 260, 6098–6106.
[6] Pozueta-Romero, J., Frehner, M., Viale, A.M. and Akazawa, T. (1991) Proc. Natl. Acad. Sci. USA 88, 5769–5773.
[7] Laemmli, U.K. (1970) Nature 227, 680–685.
[8] Vallelian-Bindschedler, L., Mösinger, E., Métraux, J.-P. and Schweizer, P. (1998) Plant Mol. Biol. 37, 297–308.
[9] Yamahara, T., Shiono, T., Suzuki, T., Tanaka, K., Takio, S., Sato, K., Yamazaki, S. and Satoh, T. (1999) J. Biol. Chem. 274, 33274–33278.
[10] Lamport, D.T.A. (1980) in: The Biochemistry of Plants (Stumpf, P.K. and Conn, E.E., Eds.), Vol. 3, pp. 501–541, Academic Press, New York.
[11] Lane, B.G., Dunwell, J.M., Ray, J.A., Schmitt, M.R. and Cuming, A.C. (1993) J. Biol. Chem. 268, 12239–12242.
[12] Dumas, B., Freyssinet, G. and Pallett, K.E. (1995) Plant Physiol. 107, 1091–1096.
[13] Carter, C., Graham, R.A. and Thornburg, R.W. (1999) Plant Mol. Biol. 41, 207–216.
[14] Dunwell, J.M. (1998) Biotechnol. Genet. Eng. Rev. 15, 1–32.
[15] Dunwell, J.M., Khuri, S. and Gane, P.J. (2000) Microb. Mol. Biol. Rev. 64, 153–179.
[16] Carter, C., Graham, R.A. and Thornburg, R.W. (1998) Plant Mol. Biol. 38, 929–943.
[17] Lane, B.G., Grzelczak, Z., Kennedy, T., Kajioka, R., Orr, J., D'Agostino, S. and Jaikaran, A. (1986) Biochem. Cell Biol. 64, 1025–1037.
[18] Hurkman, W.J., Tao, H.P. and Tanaka, C.K. (1991) Plant Physiol. 97, 366–374.
[19] Berna, A. and Bernier, F. (1999) Plant Mol. Biol. 39, 539–549.
[20] Jaikaran, A.S.I., Kennedy, T.D., Dratweka-Kos, E. and Lane, B.G. (1990) J. Biol. Chem. 265, 12503–12512.
[21] Membré, N., Bernier, F., Staiger, D. and Berna, A. (2000) Planta 211, 345–354.
[22] Hurkman, W.J. and Tanaka, C.K. (1996) Plant Physiol. 111, 735–739.
[23] Caliskan, M. and Cuming, A.C. (1998) Plant J. 15, 165–171.
[24] Schweizer, P., Christoffel, A. and Dudler, R. (1999) Plant J. 20, 541–552.
[25] Zhou, F., Zhang, Z., Gregersen, P.L., Mikkelsen, J.D., Neergaard, E., Collinge, D.B. and Thordal-Christensen, H. (1998) Plant Physiol. 117, 33–41.
[26] Amor, Y., Haigler, C.H., Johnson, S., Wainscott, M. and Delmer, D.P. (1995) Proc. Natl. Acad. Sci. USA 92, 9353–9357.
[27] Delmer, D.P. (1999) Annu. Rev. Plant Physiol. Plant Mol. Biol. 50, 245–276.
[28] Kanshal, G.P., Szumilo, T. and Elbein, A.D. (1988) in: The Biochemistry of Plants (Preiss, J., Ed.), Vol. 14, pp. 421–463, Academic Press, New York.
[29] Mauck, J. and Glaser, L. (1970) Biochemistry 9, 1140–1147.
[30] Burns, D.M. and Beachman, I.R. (1986) Nucleic Acids Res. 14, 4325–4342.

[31] Evans, W.H. (1974) Science 250, 391–394.
[32] Abney, E.R., Evans, W.H. and Parkhouse, M.E. (1976) Biochem. J. 159, 293–299.
[33] Funakoshi, I., Kato, H., Horie, K., Yano, T., Hori, Y., Kobayashi, H., Inoue, T., Suzuki, H., Fukui, S., Tsukahara, M., Kajii, T. and Yamashina, I. (1992) Arch. Biochem. Biophys. 295, 180–187.
[34] Elovson, J. (1980) J. Biol. Chem. 255, 5807–5815.
[35] Salvucci, M.E. and Crafts-Brandner, J. (1995) Plant Physiol. 108, 1269–1276.
[36] Olczak, M., Kobialka, M. and Watorek, W. (2000) Biochim. Biophys. Acta 1478, 239–247.
[37] Abel, S., Nürnberger, T., Ahnert, V., Krauss, G.-J. and Glund, K. (2000) Plant Physiol. 122, 543–552.