Purification, characterization, gene cloning and preliminary X-ray data of the *exo-inulinase* from *Aspergillus awamori*

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Extracellular *exo-inulinase* has been isolated from a solid-phase culture of the filamentous fungus *Aspergillus awamori* var. 2250. The apparent molecular mass of the monomer enzyme was 69 ± 1 kDa, with a pI of 4.4 and a pH optimum of 4.5. The enzyme hydrolysed the β -(2 $\rightarrow$ 1)-fructan (inulin) and β -(2 $\rightarrow$ 6)-fructan (levan) via *exo*-cleavage, releasing fructose. The values for the Michaelis constants K_m and V_{max} in the hydrolysis of inulin were 0.003 ± 0.0001 mM and 175 ± 5 μ mol $\cdot$ min⁻¹ $\cdot$ mg⁻¹. The same parameters in the hydrolysis of levan were 2.08 ± 0.04 mg/ml and 1.2 ± 0.02 μ mol/min per mg, respectively. The gene and cDNA encoding the *A. awamori* *exo-inulinase* were cloned and sequenced. The amino acid sequence indicated that

the protein belongs to glycoside hydrolase family 32. A surprisingly high similarity was found to fructosyltransferase from *Aspergillus foetidus* (90.7% on the level of the amino acid sequence), despite the fact that the latter enzyme is unable to hydrolyse inulin and levan. Crystals of the native *exo-inulinase* were obtained and found to belong to the orthorhombic space group $P2_12_12_1$ with cell parameters $a = 64.726$ Å (1 Å = 0.1 nm), $b = 82.041$ Å and $c = 136.075$ Å. Crystals diffracted beyond 1.54 Å, and useful X-ray data were collected to a resolution of 1.73 Å.

Key words: family 32, inulin degradation, levan.

INTRODUCTION

Inulin is a widespread polyfructan with linear chains of β -(2 $\rightarrow$ 1)-linked fructose residues attached to a terminal sucrose molecule [1]. Inulin serves as a storage polysaccharide in the Compositae and Gramineae, accumulating in the underground organs of several plants of the Asteraceae, including *Vernonia herbacea*, Jerusalem artichoke (*Helianthus tuberosus*) and chicory (*Cichorium endivia*) [2]. Inulinases are enzymes that degrade the β -(2 $\rightarrow$ 1)-linkages of β -fructan chains by two mechanisms. *Exo-inulinases* (β -D-fructopyranoside fructohydrolase, EC 3.2.1.80) sequentially split-off the terminal β -(2 $\rightarrow$ 1)-fructofuranosidic bonds, whereas *endo-action* enzymes (β -D-fructan fructanohydrolase, EC 3.2.1.7) hydrolyse the internal linkages in inulin to release a set of inulo-oligosaccharides as the main reaction products [3,4]. Inulin is of great interest because it is a relatively inexpensive and abundant substrate for the production of rich fructose syrups [2,5]. Due to bad solubility in water, it is hydrolysed in an acid medium at high temperature (80–100 °C), although under these conditions fructose can be degraded easily, resulting in undesirable side products, such as difructose anhydrides. Meanwhile, microbial and plant inulinases hydrolyse inulin into fructose or other oligosaccharides under mild reaction conditions, representing an alternative approach to production of targeted compounds. Successful biotechnological application of inulinases clearly explains the increased attention being drawn to these enzymes [6]. *Exo-inulinases* have been

isolated from *Aspergillus ficuum* [7] and some other fungal species [8,9]. Nucleotide and deduced amino acid sequences were reported for the *exo-inulinases* from chicory [10] and yeast [11]. Until now, however, there have been no proper data on the three-dimensional structures or the subsite architecture of the active centres of these enzymes. In this report we describe the purification, enzymic properties and sequencing of the *exo-inulinase* from *Aspergillus awamori* var. 2250, and preliminary X-ray data collected for the crystallized protein.

MATERIALS AND METHODS

General methods

The molecular mass of the protein was estimated by SDS/PAGE on a 10% polyacrylamide gel according to the Laemmli method [12] using the molecular-mass calibration kit LMW (14400–94000 Da) from Pharmacia Biotech, Uppsala, Sweden. Determination of the apparent molecular mass of the protein in non-denaturing conditions was performed by analytical chromatography on a Superose 12 column (10 mm $\times$ 300 mm; Pharmacia Biotech) equilibrated with 20 mM sodium acetate, pH 4.2/100 mM NaCl. The MW-GF-200 kit for molecular masses of 12000–200000 Da from Sigma was used. The flow rate of the elution was 0.2 ml/min. Native electrophoresis was performed in accordance with [6] using the MW-ND-500 kit for molecular masses of 14000–500000 Da from Sigma.

Abbreviations used: Endo H, *endo*- β -N-acetylglucosaminidase H.

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The nucleotide sequence for *A. awamori* inulinase was submitted to the EMBL Nucleotide Sequence Database under the accession number AJ315793.

Isoelectric focusing was carried out with Servalyt Precoates plates 3-10 (Serva Feinbiochemica, Heidelberg, Germany). Protein was measured by following the Lowry procedure with BSA as a standard [13]. To obtain deglycosylated forms of the protein, the native exo-inulinase was treated with an endo- β -*N*-acetylglucosaminidase H (Endo H) from *Streptomyces griseus* [14]. The inulinase (100 μ g) was incubated with 0.3 units of Endo H in 20 mM sodium phosphate buffer, pH 6.0, at 37 °C overnight. Treatment of the inulinase with *Trichoderma reesei* α -galactosidase, *Saccharomyces cerevisiae* α -glucosidase and α -mannosidase from jack bean (*Canavalia ensiformis*) (Sigma) was performed as described in [15].

Micro-organism growth and inulinase purification

A. awamori var. 2250 used in this study was a wild-type strain from the culture collection of Voronezh Technological Academy, Voronezh, Russia. This micro-organism was preserved by monthly transfers to malt extract agar, and stored at 4 °C. Fresh spore suspensions were obtained from 5 day-old cultures on Petri dishes containing malt extract agar, by the addition of 5 ml of sterilized water. Conventional Erlenmeyer flasks (500 ml) with a 2 cm layer of wet wheat bran were inoculated with $\approx$ 10 ml of spore mixture of *A. awamori*. Solid-state fermentation was carried out at 32 ± 2 °C for 96 h. All purification procedures were carried out at 4 °C. Crude enzyme preparation was extracted from the fungal growth surface with 2.5 l of water and separated by centrifugation (3000 *g* for 30 min). The supernatant was concentrated 10-fold by use of hollow fibres (Kirishi, Kirishi, Russia) and loaded on to a Sephadex G-50 column (80 mm $\times$ 800 mm) equilibrated with 20 mM Tris/HCl buffer, pH 7.5 (buffer A). The column was then washed with the same buffer (total volume, 1.2 l) at a flow rate 10 ml/min.

The crude inulinase solution was loaded on to a DEAE-Sephadex A50 column (60 mm $\times$ 150 mm) equilibrated with the same buffer and eluted with 1 M NaCl in buffer A (total volume, 200 ml) at a flow rate of 3 ml/min. The fraction obtained was concentrated on an Amicon PM-30 membrane to 20 ml, desalted by dialysis against the same buffer, and loaded on to a column (21.5 mm $\times$ 150 mm) of DEAE-5PW (Pharmacia Biotech) equilibrated with buffer A. The inulinase fractions were eluted with a linear gradient (0–0.5 M) of NaCl in buffer A (total volume, 384 ml) at a flow rate of 2.4 ml/min, concentrated on the Amicon PM-30 membrane to 4 ml, and dialysed against 20 mM sodium acetate buffer, pH 4.2 (buffer B). Obtained protein solution was chromatographed on a Mono S HR (5/5) column (5 mm $\times$ 50 mm) from Pharmacia Biotech, with a linear gradient (0–0.5 M) of NaCl in buffer B (total volume, 24 ml). The elution flow rate of 0.6 ml/min was used. Finally, inulinase was concentrated to 4 ml by using Amicon PM-30 membrane, transferred to 1.7 M $(\text{NH}_4)_2\text{SO}_4$ in 20 mM sodium acetate buffer, pH 5.0, and applied on a phenyl-Superose column (5 mm $\times$ 50 mm; Pharmacia Biotech) equilibrated with the same buffer. Inulinase fractions were eluted with a linear gradient (0–100%) of 20 mM sodium acetate buffer, pH 5.0, at a flow rate of 0.6 ml/min (total volume, 24 ml), dialysed against the same buffer, then against deionized water, and lyophilized.

N-terminal amino acid and internal microsequence analyses

The purified protein was electroblotted on to PVDF membranes (Sequi-Blot PVDF membrane; Bio-Rad, Richmond, CA, U.S.A.) using 25 mM Tris/192 mM glycine/0.1% SDS as the cathode buffer and 25 mM Tris/192 mM glycine/20% methanol as the anode buffer. The bands on the PVDF membrane were excised and the protein N-terminal amino acid sequences were de-

termined subsequently by automated Edman degradation of 1 pmol of protein using a Beckman LF3400D protein-peptide microsequencer equipped with an online model 126 Gold system microgradient HPLC and a model 168 diode array detector (Beckman Instruments). For internal sequencing, the inulinase was treated with CNBr or trypsin, and peptides were purified by capillary reversed-phase HPLC [16].

Enzyme assays and kinetic analysis

Inulin was purchased from Merck (Darmstadt, Germany), levan from *Microbacterium levaniformans* was a gift from Dr A. Miasnikov, Danisco-Cultor, Oy, Finland. Stachyose and raffinose were from Sigma. Exo-inulinase activity was determined by the quantification of fructose released from inulin. Standard assays (0.2 ml) were carried out in 20 mM sodium acetate buffer, pH 4.5, and contained 0.5 mg of inulin and crude or purified enzyme extract corresponding to at least 0.2 μ g of pure exo-inulinase. After 10–30 min incubation at 37 °C the reaction was stopped by adding 1 ml of Somogyi copper reagent and boiling for 10 min. Following this, liberated fructose was measured according to the Somogyi–Nelson method [17]. One unit of enzymic activity was defined as the amount of inulinase required to produce 1 μ mol of fructose from inulin/min at pH 4.5 and 37 °C. Activities of the exo-inulinase towards raffinose, stachyose and levan were evaluated in the same manner.

The effect of pH and temperature on the hydrolysis of inulin was studied by the Somogyi–Nelson method. The effect of pH on activity was measured at 37 °C for 10 min over the pH range 3–9 in 100 mM sodium citrate buffer. The influence of pH on stability of the enzyme was studied by incubating samples of the enzyme at 20 °C for 16 h in 100 mM sodium phosphate/citrate buffers ranging from pH 3 to pH 9, followed by measuring the residual activity of the enzyme under standard conditions. The effect of temperature on activity was measured at pH 4.5 in 20 mM sodium acetate buffer over a temperature range of 30–80 °C using 0.02 units of the enzyme and 2 mM inulin. After 10 min of incubation at the appropriate temperature, reaction was stopped by addition of 1 ml of copper reagent followed by standard measurements [17]. To evaluate temperature stability of the exo-inulinase, the enzyme was incubated without substrate at various temperatures in 20 mM sodium acetate buffer, pH 4.5. Equal aliquots of the reaction mixture were taken after appropriate time intervals, and the residual activity of the enzyme was measured at pH 4.5 under standard conditions. The Michaelis constant was determined for each substrate from the Michaelis–Menten equation by non-linear regression analysis [18]. Rates were determined at between six and 15 different substrate concentrations ranging from $\approx 0.1 \times K_m$ to $4\text{--}8 \times K_m$. The specificity of the exo-inulinase was studied during inulin and levan hydrolysis. The reactions were stopped after different intervals of time by boiling for 2 min and the resulting products were analysed by HPLC on a Dextro-PakTM cartridge column (8 $\times$ 10 mm; WATO85650 from Waters, Milford, MA, U.S.A.), using isocratic elution in water (flow rate, 1 ml/min) with refractometric detection. Quantitative measurements were carried out by integrating peaks corresponding to fructose liberated during the enzymic reaction. Fructose and fructo-oligosaccharides with degrees of polymerization of 2–5 were used as standards. Glucose formed during inulin hydrolysis was measured according to the glucose oxidase method [19].

Protein crystallization and X-ray data collection

For crystallization trials, the lyophilized protein was dissolved in water at a concentration of 10 mg/ml, and 10 μ l drops of the

protein solution were mixed with 5 μ l of 15% poly(ethylene glycol) 3350 (Sigma) in 100 mM sodium acetate buffer, pH 4.0–5.0. Hanging drops were equilibrated against 1 ml of the same poly(ethylene glycol) solution at 17 °C. Needle-like crystals started to grow in 1 week and after 3 weeks reached a maximum size of 0.1 mm $\times$ 0.1 mm $\times$ 1.5 mm. For data collection, a single crystal was frozen quickly in a gaseous nitrogen flow at $\approx$ 100 K (Oxford Cryosystems). X-ray data were collected by the oscillation method on a 345 MAR Research image-plate detector at the Laboratório Nacional de Luz Síncrotron (LNLS) protein crystallography beam line [20]. The X-ray wavelength was set to 1.54 Å (1 Å = 0.1 nm) to maximize the signal-to-noise ratio and to optimize the speed of data collection [21]. The crystal-detector distance was set to 200 mm and the oscillation range was equal to 1°.

Isolation of nucleic acids and PCR cloning of the inulinase gene and cDNA

DNA isolation was performed by the method described in [22]. RNA isolation was carried out essentially as described by Chomczynski and Sacchi [23], starting from solid-phase-grown mycelium 4 days after inoculation with spores. *A. awamori* cDNA was obtained by reverse transcription of mRNA using the Ready-To-Go first-strand synthesis kit (Amersham Bioscience). PCR using either cDNA or genomic DNA as the template was carried out with turbo-*Pfu* (Stratagene) as the thermostable DNA polymerase under the conditions recommended by the supplier. Inverse PCR was performed essentially as described earlier [24]. The *Pfu*-generated fragments were cloned into the vector pCR4-TOPO blunt using the respective kit (Invitrogen). DNA sequencing was performed on an automated sequencer using fluorescent dyes as chain terminators. Two independent clones of each PCR product were sequenced in both directions to obtain a reliable sequence. In cases of conflict, a third independent fragment was amplified in order to decide on the correct sequence. DNA sequence analysis was performed on a Macintosh computer using the LaserGene software package (DNASar).

RESULTS AND DISCUSSION

The isolation procedures resulted in a 38-fold purification of the inulinase activity with a 10% yield (Table 1). Endo- and exo-inulinase activities were separated at the fourth purification step. Purification levels and percentage yields of the first three steps were calculated with the assumption that the endo- and exo-inulinase yields were equal. CNBr digestion of the protein produced the N-terminus of the purified exo-inulinase and three peptides. These were analysed, yielding the following sequences: N-terminal sequence, FNYDQPYRGQYHFSPQKNWMND-PNGL; peptide 1, NDPNGLLYHNGTYHLFFQYN; peptide 2, YTSYYPVAQTLP (the third peptide showed partial similarity with the N-terminal sequence, and was not analysed further). N-

Figure 1 Time course of inulin hydrolysis catalysed by the *A. awamori* exo-inulinase

Lower curve (●), formation of fructose during the reaction; upper curve (■), formation of glucose during the reaction. Inulin (3 mg) was incubated with the exo-inulinase (2 units) in 20 mM sodium acetate buffer, pH 4.5, at 37 °C. The products were separated on a Dextro-Pak™ cartridge column.

terminal sequence data confirmed the homogeneity of the purified protein. The molecular mass corresponding to a single protein band on SDS/PAGE was estimated to be 72 ± 1 kDa. Analytical gel filtration on a Superose 12 column showed a single symmetrical exo-inulinase peak, corresponding to 70 ± 5 kDa. Experimental data obtained from the native PAGE showed that the enzyme had a molecular mass of 70 ± 5 kDa. Taken together, all these results suggest that the enzyme consists of a single subunit. The isoelectric point determined by electrofocusing was 4.4. Maximal activity of the exo-inulinase occurred at pH 4.5 and 60 °C but inactivation started to occur at temperatures above 45 °C. More than 80% of the maximum activity was retained in the pH interval from 3.0 to 5.0. Significantly lower activity were observed at pH values below 3.5 and above 6.0. The remaining activity of the enzyme after 24 h of incubation at 50 °C was $\approx$ 90%, whereas at 60–70 °C the enzyme retained its activity for $\approx$ 1–2 h.

The mode of action of the enzyme was investigated by HPLC using inulin as a substrate. Data on the time course of inulin hydrolysis showed that fructose and glucose were released during the reaction (Figure 1). Glucose was produced from the non-reducing termini of inulin. Formation of fructosaccharides was not observed here. We therefore concluded that our enzyme is an exo-inulinase.

The exo-inulinase from *A. awamori* liberates fructose from fructosyl-containing polysaccharides and oligosaccharides. We found that the enzyme hydrolysed stachyose and raffinose, releasing fructose. Kinetic parameters of the hydrolysis are given

Table 1 Summary of the exo-inulinase purification

Purification step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Extract after ultrafiltration	200	2410	1952	2	1	100
Sephadex G-50 chromatography	300	1605	1659	2.6	1.3	85
DEAE-Sephadex chromatography	40	450	976	4.3	2.1	50
DEAE-5PW chromatography	8	36	585	16.1	8	30
Mono S chromatography	4	8	390	50	25	20
Phenyl-Superose chromatography	6	2.7	193	75	38	10

Table 2 Kinetic parameters of the reactions catalysed by exo-inulinase from *A. awamori* var. 2250

Substrate	K_m	V_{max} ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)
Inulin	0.003 ± 0.0001 mM	175 ± 5
Levan	2.08 ± 0.04 mg/ml	1.2 ± 0.02
Stachyose	10.1 ± 0.1 mM	80 ± 0.8
Raffinose	8 ± 0.2 mM	120 ± 4

in Table 2. Values for V_{max} for these natural substrates are comparable with those for inulin in contrast to the K_m values, which are significantly higher. Hydrolysis of levan by the exo-inulinase was investigated and kinetic parameters K_m and V_{max} were calculated by the method of initial rates (Table 2). It appears that the enzyme is capable of specifically degrading β -(2 $\rightarrow$ 1)- as well as β -(2 $\rightarrow$ 6)-linkages of fructo-oligosaccharides. Significant activity of the enzyme towards levan is somewhat surprising, since the highly homologous inulinase from *Aspergillus niger* was reported to have no detectable levan-hydrolysing ability [2]. HPLC analysis of the products formed during the hydrolysis of levan showed that, similarly to the hydrolysis of inulin, only fructose was accumulated.

Reverse transcriptase PCR of *A. awamori* mRNA using the oligonucleotides 5'-CCCCAGAAGAACTGGATGAA-3' and 5'-TCACCTTGGCCTCCGAATACCTCGAC-3' resulted in the amplification of a 1.4 kb cDNA fragment. These primers were deduced from the N-terminal amino acid sequence of the purified protein and the internal consensus amino acid sequence VEV-FGGQGE, which is shared between *Aspergillus foetidus* fructosyltransferase [25] and inulinases A and B from *A. niger* [26], and is close to the C-terminus of these enzymes. A fragment of slightly lower electrophoretic mobility in the agarose-gel electrophoresis was obtained by PCR from genomic DNA using the same set of primers, indicating the presence of an intron in the gene.

The flanking sequences harbouring the missing parts of the coding region of the *A. awamori* inulinase were obtained by inverse PCR after restriction of the genomic DNA with *Mbo*I and subsequent circularization, as described earlier [24]. The primer pairs 5'-GTGGCATCGAGTGGGAACA-3'/5'-GG-AAGAACAGATGGTAGGT-3' and 5'-CGCCAACCTCACC-GAGCAGAC-3'/5'-TCCGGTTGTGGTTGTGTTGGTG-3' were used for the amplification of the 5'- and 3'-flanking regions, respectively. The two resulting fragments of 480 bp (5' fragment) and 650 bp (3' fragment) both contained the expected *Mbo*I restriction site, indicating the the sequence joining the 5' and 3' ends of the genomic DNA fragments on circularization. The resulting DNA sequence featured an open reading frame of 1611 bp, flanked by a TATA-box motif 75 bp upstream of the deduced translation start site and a potential polyadenylation site 112 bp downstream of the stop codon. It was interrupted by one intron, identified by comparison of the sequences derived from genomic DNA and cDNA amplification at the position corresponding to amino acid 113 of the mature, processed inulinase. The precursor protein encoded by the cDNA had a size of 537 amino acids and was processed during maturation to a 518 amino acid glycosylated protein, as indicated by our analyses. The peptide part of the mature protein had a predicted molecular mass of 57149 Da and a predicted pI value of 4.89, which is compatible with the biochemical characteristics that we obtained for the glycosylated protein. Amino acid sequence identity with the *A. foetidus* fructosyltransferase was 90.7%. Together

Table 3 Data-collection statistics

$R_{sym} = \sum (I - \langle I \rangle) / \sum I$, where I is intensity and σ is the mean root square error. X-ray diffraction data were processed using the DENZO and SCALEPACK programs [34].

Parameter	Experimental results	
	All data	Last shell
Number of frames	88	
Space group	P2 ₁ 2 ₁ 2 ₁ (19)	
Cell dimensions at 85 K (Å)	$a = 64.726$ $b = 82.041$ $c = 136.075$	
Resolution range (Å)	30.0–1.73	1.77–1.73
Total number of reflections	196 637	6187
Number of unique reflections	68 047	3656
Redundancy	2.9	1.7
R_{sym} (%)	7.6	32.0
Completeness (%)	88.7	72.0
$\langle I/\sigma(I) \rangle$	8.3	2.9

with the earlier findings that no second related enzyme is present in *A. foetidus*, this indicates that the inulinase described here is the orthologue of the *A. foetidus* fructosyltransferase in *A. awamori*. This is particularly interesting since the two enzymes, despite their high sequence similarities, display rather distinct catalytic properties. Moreover, the subtle differences in the amino acid sequence between the two proteins do not offer an obvious clue to explain the different substrate specificity. Both are identical at positions 31–44, which has the sequence HFSPQKNWMND-PNG. This is a perfect match for the prosite pattern HX₂PX₄[L/I/V/M]NDPNG, which is indicative of the membership of the two enzymes in glycoside hydrolase family 32 [27]. All residues that resemble those in related enzymes (on the basis of structural predictions [28]) that have earlier been shown to be involved directly in the catalytic process [29–32] are conserved in both *Aspergillus* enzymes. These are, in particular, Asp-41, Asp-189 and Glu-241. Furthermore, both enzymes are identical in sequence in the six regions conserved among invertases and fructosyltransferases [25,33]. Therefore, an explanation of the differences in the catalytic properties of the two enzymes has to await the elucidation of their three-dimensional structures. Treatment of the native exo-inulinase with Endo H decreased the molecular mass of the protein to 63 ± 1 kDa (determined by SDS/PAGE), caused by cleavage of N-linked oligosaccharides. Based on the amino acid sequence data, the exo-inulinase has five potential sites of N-glycosylation. Additional treatment of the enzyme with glycosidases, as described in Materials and methods section, resulted in liberation of O-sugars and therefore a decrease of the mass of exo-inulinase to 61 ± 1 kDa. These results suggest that *Aspergillus* exo-inulinase is a glycoprotein with mixed-type glycosylation [15].

Crystals of the exo-inulinase obtained in the present study belonged to the orthorhombic space group P2₁2₁2₁, with cell parameters of $a = 64.726$ Å, $b = 82.041$ Å and $c = 136.075$ Å, and diffracted beyond 1.54 Å. Useful X-ray data (intensity, $I/2\sigma$) were collected to a resolution of 1.73 Å. X-ray diffraction data were processed using the programs DENZO and SCALEPACK [34]. The data-collection statistics are presented in Table 3. Freezing the crystals improved their stability in the X-ray beam significantly, allowing us to improve the data-collection statistics. Since no suitable model for molecular replacement has been found despite exhaustive sequence-comparison searches, we will

have to use the heavy-atom replacement method for the structure solution. Screening for heavy-atom derivatives is under way.

The present work was supported in part by a grant (no. 98-04-49719) from the Russian Foundation for Basic Research (RFFI), by a grant of 6th Expertise Competition from the Presidium of Russian Academy of Science. A.M.G., J.R.B.N. and I.P. gratefully acknowledge the financial support of FAPESP and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil. We thank Dr Arndt Heyer (Max Planck Institute of Molecular Plant Physiology, Golm, Germany) for his kind supply of cDNA constructs. M.A. thanks Dr Hieke Dürk for skilful technical assistance.

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Received 16 July 2001/29 October 2001; accepted 21 November 2001