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Characterization of Recombinant Fungal Phytase (*phyA*) Expressed in Tobacco Leaves

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The *phyA* gene from *Aspergillus ficuum* coding for a 441-amino-acid full-length phytase was expressed in *Nicotiana tabacum* (tobacco) leaves. The expressed phytase was purified to homogeneity using ion-exchange column chromatography. The purified phytase was characterized biochemically and its kinetic parameters were determined. When the recombinant phytase was compared with its counterpart from *Aspergillus ficuum* for physical and enzymatic properties, it was found that catalytically the recombinant protein was indistinguishable from the native phytase. Except for a decrease in molecular mass, the overexpressed recombinant phytase was virtually the same as the native fungal phytase. While the temperature optima of the recombinant protein remain unchanged, the pH optima shifted from pH 5 to 4. The results are encouraging enough to open the possibility of overexpressing *phyA* gene from *Aspergillus ficuum* in other crop plants as an alternative means of commercial production of this important enzyme.

myo-Inositol hexakis phosphate, which is also known as phytic acid, is a storage form of phosphorus in soybeans, cottonseeds, and other legumes and cereals (1). When phytic acid-rich meals are fed to monogastric animals, they poorly digest phytic acid for the lack of active phytase in their digestive tract (2). Consequently, the manure rich in phytic acid may serve as a source of phosphate, which is then released to the groundwater by the action of microbial phytase. The abundance of phytic acid in animal feed also poses another risk to the animal. That is, they bind nutritionally important minerals and render the metal ions unavailable to monogastric animals (3, 4). In order to solve both the nutritional and environmental problems, an active phytase was sought by researchers over the last four decades (5). A phytase with higher activity was identified in *Aspergillus ficuum* (6). This enzyme, which is glycosylated, was purified, biochemically characterized, cloned, and overexpressed (7, 8, 9). In addition, the three-dimensional structure of this enzyme has also been deduced at 2.5 Å resolution (10). Because of all the available data, several commercial enzyme producers had targeted this enzyme for commercial exploitation. The fungal phytase gene had been overexpressed not only in commercial fungal strains, but also in *Nicotiana tabacum* (11). A thorough biochemical characterization of the expressed phytase in tobacco was not reported, however. In this communication, we report the purification and detailed biochemical characterization of the fungal phyA gene product, which is the most thoroughly studied phytase of all "histidine acid phosphatases." Except for minor variation in molecular mass of mature phytase, the overall kinetic properties of the enzyme have remained identical to the native fungal phytase. This bodes well for the prospect of 'biofarming' fungal phytase in other crop plants to produce this important enzyme in a cost-effective manner.

MATERIALS AND METHODS

Plasmid Development and Plant Transformation

The 5′ end of the *A. ficuum phyA* gene (Accession Number M94550) was modified by PCR using the primer VSPphy (5′-GCCAT-**GGCGCC**CTGGCAGTCCCCGCC-3′), resulting in a recombinant gene in which a *Nar* I site (bold) was incorporated immediately 5′ to the codon (underlined) specifying the first amino acid of the mature, processed phytase enzyme. The *Nar* I site allows for the fusion of phyA to a sequence specifying the soybean VSPβ secretory leader peptide (12). The 3′ end of the phyA gene was modified using the primer RIphy (5′-G**GAATTC**TAAGCAGAACACTCC-3′), thereby placing an *Eco* RI restriction site (bold) immediately 3′ to the stop codon (underlined). The effect of these changes is the removal of the fungal secretion signal peptide and associated intron and the addition of novel cloning sites to facilitate vector construction. The modified phyA gene was fused to VSPβ, which was in turn fused to the constitutive CaMV 35S promoter (13, 14). The addition of the man-

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nopine synthase terminator completed the expression cassette, which was subcloned into pCGN1578 to yield pTZ117 (15).

The binary vector pTZ117 was transformed directly into *A. tumefaciens* strain LBA4404 to yield the strain TZA6 (16), which was used in all subsequent transformation experiments. The transformation procedure for tobacco (*Nicotiana tabacum* W38) was essentially the explant method developed by Horsh *et al.* (17) with modifications as described previously (18). Transformation frequencies were comparable to those seen in previous studies. Rooted putative transformants of tobacco, potato, and alfalfa were screened in culture for phytase expression by activity assay. Plants shown to be expressing phytase were established under growth room conditions (16 h photoperiod of 280 μE m^{-2} s^{-1} and day and night temperatures of 21°C and 19°C, respectively) to allow for further evaluation of enzyme expression in mature plants.

Phytase Assay

Phytase assays were carried out in 1.0 ml volume at 58°C in 50 mM sodium acetate buffer at pH 5.0 similar to *Aspergillus ficuum* phytase assay (7). The liberated inorganic *ortho* phosphates were quantitated spectrophotometrically using a freshly prepared acetone-acid-molybdate (AAM) reagent consisting of acetone, 5.0 N sulfuric acid, and 10 mM ammonium molybdate (2:1:1, v/v). Adding 2.0 ml AAM solution per assay tube terminated phytase assay. After 30 seconds 0.1 ml of 1.0 M citric acid was added to each tube. Absorbance was read at 355 nm after blanking the spectrophotometer with appropriate control. A standard curve for inorganic ortho phosphate was made within the range of 10 to 500 nmoles.

Extraction of Phytase

Leaves were harvested from primary transformants after 3 weeks of growth in the field and stored in a −60°C freezer until fractionation. About 20 g of leaf tissue was homogenized in 25 mM sodium acetate buffer, pH 5.5 supplemented with 0.1 mM $CaCl_2$, 1.5 mM phenylmethylsulfonyl fluoride (PMSF), and 10% (v/v) glycerol using a chilled Waring Commercial Blender at low speed for 1 min. The leaf tissue extracts were spun at 12,000 rpm using SS-34 rotor in a refrigerated Sorval RC-5B centrifuge. After centrifugation, the pellet was resuspended in acetate buffer, homogenized for 1.0 min and centrifuged as before. The pellet was discarded and supernatants were collected and assayed for both protein concentration and phytase activity.

Purification of Phytase

Step 1: Macroprep High Q anion exchange chromatography. The supernatants from the leaf extract were dialyzed against 25 mM imidazole buffer, pH 7.0 supplemented with 5% (v/v) glycerol, by changing buffers three times. A Kontes Flex-Column (2.5 × 10 cm) was used to make a 10.0 ml column with Macro-Prep High Q (Bio-Rad) as anion-exchanger. An Econo System column chromatography workstation (Bio-Rad) was used to perform the chromatography. After loading the supernatant and washing with dialysis buffer, the column was developed running a linear salt gradient of 0 to 0.3 M NaCl in the buffer. A flow rate of 2.0 ml per minute was maintained for 50 min and 5.0 ml fractions were collected for measuring phytase activity and protein content. The column was then washed with 25 ml of 0.5 M and 1.0 M NaCl in the buffer, respectively.

Step 2: Macroprep S cation exchange chromatography. The active fractions from previous step were pooled and dialyzed against 25 mM Sodium Acetate, pH 3.75 supplemented with 5% (v/v) glycerol. A Kontes Flex-Column (1.5 × 5 cm) was used to make a 2.0 ml column with Macro-Prep High S cation-exchanger (Bio-Rad). After loading the dialyzed active fractions from the previous step the column was washed with acetate buffer and a linear salt gradient (0 to 0.3 M NaCl) was developed in the buffer at a flow rate 2.0 ml per min for 20 min. Finally, the column was washed with 0.5 M NaCl in the acetate buffer. Two ml fractions were collected and assayed for both activity and protein.

Protein Assay

The protein concentration of the column fractions and leaf extracts was determined by Coomassie Blue G-250 dye binding method (19).

Gel Electrophoresis and PAS Staining

Electrophoresis of the purified phytase was performed using Xcell II Mini-Cell and 4-12% NuPage Bis-Tris Gels (Novex, San Diego, CA). Successful separation was achieved by running at a constant 200 V for 70 min. After electrophoresis, the SDS slab gel was cut into identical halves. One half of the gel was stained with Coomassie Blue R-250 dye; the other half was fixed in a solution containing 50% methanol overnight for periodic acid-Schiff (PAS) staining. The PAS staining was done according to a procedure described elsewhere (20). Pre-stained, multicolored molecular weight markers (4-250 kDa) were used as standards.

Phytase A Antibodies

For Western blotting two antibodies were used; one raised against native *A. ficuum* phytase and the other against a synthetic 16-mer peptide mimicking the residues from 151 through 170 of the fungal phytase (*phyA*). A 6.0 mg portion of the 16-mer *A. ficuum* phytase (*phyA*) specific peptide with sequence $-^{151}$IEEGFFQSTKLKD-PRAQPGQQSS^{170} was conjugated to antigenic protein Keyhole Limpet Hemocyanine (KLH) using carbodiimides as the coupling reagent. The conjugated protein was dialyzed extensively to remove the free peptide. Antibodies were raised in Female New Zealand rabbits. The IGGs were purified using Avid AL (BioProbe International, Tustin, CA) affinity column chromatography.

Western Blotting

After electrophoresis, the phytase was transferred onto a PVDF membrane using XCell II blot module with transfer buffer (12 mM Tris, 96 mM glycine, and 20% methanol). The transfer was achieved by running at 125 mM constant current for 90 min under cold conditions. The blots were treated with 5% bovine serum albumin (BSA) in Tris buffered saline (TBS) containing 0.1% Tween 20. After washing 3 times with the TBS buffer, incubated with the above antibodies for 1 hr. The peroxidase vecstatin ABC reagents (Vector Laboratories, Inc., Burlingame, CA) containing biotinylated anti-rabbit IgG, avidin and biotinylated horseradish peroxidase were used to identify the phytase.

Phytase Activity Inhibition by myo-Inositol Hexasulfate and Phenylglyoxal

For myo-inositol hexasulfate (MIHS) inhibition of phytase, the enzyme activity was performed at 58°C and pH 5.0 and 2.5. First, concentration dependent inhibition curves were established for MIHS at both the pHs. Then, K_m for phytate was determined in both the absence and presence of MIHS. MIHS sufficient to inhibit 50% of the phytase activity was included in each assay tube, which was 50 μM and 1 μM, at pH 5.0 and 2.5, respectively.

For phenylglyoxal inactivation of phytase, the enzyme was incubated at different concentrations of phenylglyoxal in 50 mM bicarbonate at pH 8.0. The inhibitor-enzyme mix was incubated at 37°C for 1 hr. A 50 μl aliquot was taken from this mix and added to 875 μl 50 mM acetate, pH 5.0. After 20 min incubation at room temperature the assay tubes were placed at 58°C for temperature equilibration and then phytase assay was carried out by adding 75 μl phytate.

TABLE 1

Comparison of Phytase Activity in the Leaves of Transgenic and Control Tobacco

Parameters	Control plants	Transgenic plants
Amount of leaves used to grind	2.55	3.6
Volume of leaf extract	10.2	14.5
Phytase activity per ml	70 nKat	371 nKat
Phytase activity per gm of leaves	280 nKat	1494 nKat
Phytase activity per mg of protein	41 nKat	203
Total protein in mg	17.45	26.52
Total activity	714 nKat	5380
Phytase* (in mg)	0.237	1.79

* Specific activity of 3000 nKat/mg is used for estimation.

RESULTS

Overexpression of Phytase in Tobacco Leaves

Phytase gene from *Aspergillus ficuum* was overexpressed in tobacco leaves. Table 1 summarizes the comparison of phytase activities in the leaves of control and transgenic tobacco plants. The activity data show that phytase activity had increased about 7.5-fold in the leaves produced by transgenic plant (Table 1). The same leaf extract showed about a 5-fold increase in specific activity for phytase.

Purification

The purification of overexpressed *phyA* protein in tobacco was achieved by using both anion and cation exchange columns. The results are shown in Table 2. Based on specific activity, a 3-fold purification was needed to purify the overexpressed phytase. The purified phytase after the last step of purification (Macro-Prep High S) gave a specific activity of 3280 nkat/mg of protein with an overall yield of about 46 percent.

Molecular Weight, Purity, and Glycosylation

Judging from the high specific activity of purified phytase (step 3), we concluded that the protein was homogeneous. SDS-PAGE data of purified phytase had confirmed our conclusion (Figure 1). Like recombinant *Aspergillus niger* phytase, the overexpressed fungal phytase in tobacco leaves also showed diffused protein band indicating extensive glycosylation. The computed molecular weight of phytase expressed in tobacco gave a value of 73-88 kDa. Under the identical condition, the commercial phytase (Gist-brocade) gave a value of 80-114 kDa. Thus, the recombinant phytase gave a 17% lower molecular mass than the native phytase. When the duplicate gel was visualized for glycoprotein via PAS staining, it gave positive result. Thus, the overexpressed phytase in tobacco leaves also were glycosylated similar to fungal phytase (7).

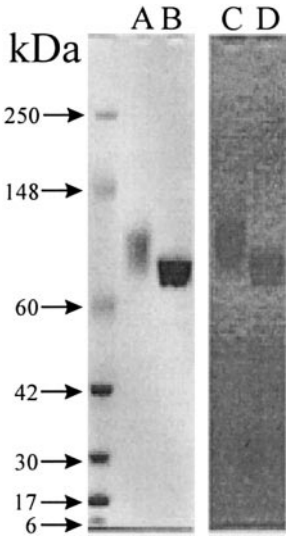

FIG. 1. SDS-PAGE profile of native *A. ficuum* phytase and recombinant phytase in tobacco leaves. The extreme left lane shows molecular weight standards. Lanes A and B show the banding pattern for native *A. ficuum* and overexpressed phytase, respectively. Lanes C and D are identical to Lanes A and B excepting that the proteins were stained with PAS for visualization of glycoproteins.

Kinetic Characterization

pH optima. Although pH optima of the recombinant phytase show the characteristic bi-hump peaks like the native fungal phytase, there is a noticeable downward shift in the optima. Unlike the native *A. ficuum* phytase whose activity is highest at pH 5.0 to 5.5 (7), the recombinant phytase expressed in tobacco leave gave highest activity at pH 4.0; while the second peak was discerned at pH 2.0 (Figure 2).

Temperature optima. The temperature optima profile of recombinant phytase paralleled the native fun-

TABLE 2

Purification of Recombinant Fungal Phytase from Tobacco Leaves

Steps	Volume (ml)	Activity (nKat/ml)	Total activity (nKat)	Total protein (mg)	Specific activity (nKat/mg)	Yield (%)
1. Leaf tissue extract	80	374	29,911	147.34	203	100
2. Macro-Prep High Q	40	420	16,800	11.57	1,452	56.1
3. Macro-Prep High S	5.4	2,582	13,943	4.25	3,280	46.6

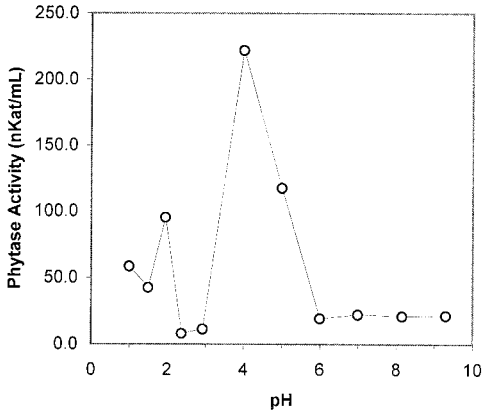

FIG. 2. The pH versus activity of overexpressed phytase in tobacco leaves. The buffer used for pH 1.0-2.5 was 50 mM glycine-HCl; 3.0-5.0 50 mM sodium acetate; 6.0-9.0 50 mM imidazole.

gal phytase (Figure 3). Like native phytase, its activity also peaked at 58°C. Similarly, the recombinant phytase also lost about 40% and 80% activity when assayed at 65°C and 70°C, respectively.

Kinetic parameters. Table 3 shows the comparison between native fungal phytase and over-expressed recombinant phytase in tobacco. Fungal phytase shows a slightly lower K_m value than the recombinant phytase. The turnover number is very similar for both the phytases. The kinetic perfection of the native fungal and recombinant phytase, as judged by the ratio of K_{cat} over K_m, is not very dissimilar. The native fungal phytase showed only slightly higher kinetic perfection over the recombinant phytase (Table 3). When the inhibition constant, K_i, was determined for *myo*-inositol hexasulfate, a potent inhibitor of phytase, the K_i values were 4.6 and 2.5 μM for the native and recombinant phytase, respectively.

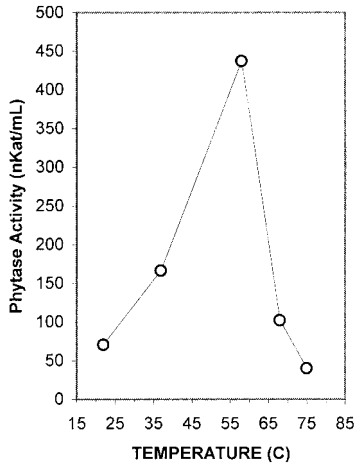

FIG. 3. Temperature versus activity of overexpressed phytase in tobacco leaves. The enzyme assay was performed at pH 4.0.

TABLE 3

Kinetic Parameters of Native Fungal Phytase and Recombinant Phytase in Tobacco

Kinetic parameters	Native fungal phytase	Recombinant and over expressed phytase
K_m (μM)	27	65
K_i for *myo*-inositol hexaphosphate (μM)	4.6	2.5
Turnover number per sec	348	360
K_{cat}/K_m (M^{-1} sec^{-1})	1.29 ± 10^7	5.53×10^6

Phenylglyoxal Inactivation

Phenylglyoxal, a modifier of arginine, is known to inhibit fungal phytase (21). We performed inactivation of both native fungal and overexpressed recombinant phytase in tobacco by phenylglyoxal. The results are shown in Figure 4. Both the native and recombinant phytases were severely inhibited by phenylglyoxal. The enzymes were inactivated completely by 0.9 μM modifier concentration. At 0.1 μM phenylglyoxal concentration 50% activity of both the phytases were inactivated. Thus, the sensitive arginine residues responsible for active site formation in both the native and recombinant phytases were modified with the same intensity. The easy accessibility of phenylglyoxal to the active site of recombinant phytase suggests that overall tertiary structure of the protein has not changed due to overexpression in tobacco leaf tissues. The specific activity and turnover number of the recombinant phytase support this conclusion.

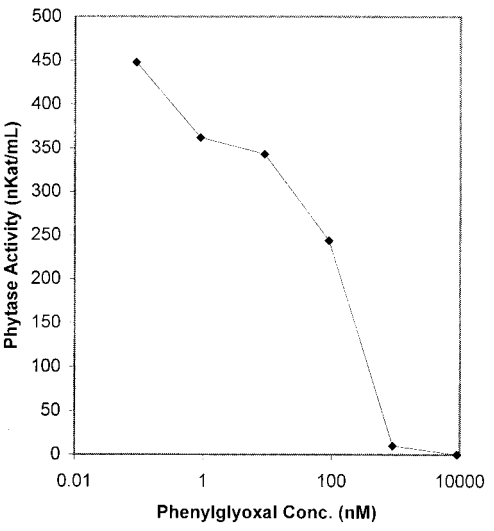

FIG. 4. Inhibition of recombinant phytase by phenylglyoxal. Enzyme activity measured as a function of increasing concentration of phenylglyoxal. The *X*-axis representing the inhibitor concentration is in log scale.

DISCUSSION

The phytase gene, an important member of "histidine acid phosphatase" from *Aspergillus ficuum,* was successfully introduced and expressed in tobacco leaves with no apparent loss of activity. We extracted the proteins from tobacco leaves and then purified phytase using a series of ion-exchange chromatographies. The purified phytase was then biochemically characterized and its kinetic properties compared with that of native fungal phytase.

Based on phytase activity, we estimated that about five-fold increase in the enzyme activity resulted from the recombinant phytase in the leaves of transgenic tobacco plant (Table 1). An earlier report on accumulation of *A. niger* phytase in transgenic tobacco leaves gave a much higher value (a 90-fold increase), but the value was solely based on the amounts of phytase determined by an immunolocalization technique (11). In this communication, we report the level of phytase solely based on enzyme activity. When *Aspergillus ficuum* phytase was expressed in *E. coli,* the enzyme was shown to be stored in inclusion bodies; no activity, however, could be observed (22). The folding pathway for fungal phytase could be different from that of prokaryote phytase.

Fungal phytase when overexpressed in tobacco leaves was found to be 17% less glycosylated than native phytase produced by *Aspergillus niger* (Figure 1). Despite this low glycosylation, the recombinant enzyme was fully functional and had the same kinetic parameters as the native fungal phytase (Table 3). It was reported recently that the fungal phytase when expressed in yeast, *Pichia pastoris,* under a glycosylating environment was able to secret functional enzyme into the media (23). However, when glycosylation was prohibited by the addition of tunicamycin, phytase activity was reduced significantly both inside and outside the yeast cells (23). Thus, glycosylation seems to affect both secretion and stability of fungal phytase.

Although the kinetic parameters of overexpressed fungal phyA gene product is near identical to the native phytase, the pH optima had shifted one pH unit; the recombinant protein in tobacco leaves showed a characteristic bi-hump pH optima peaking at pH 4.0 and 2.0 as opposed to pH 5 and 2.5 for the native fungal phytase (Figure 2). The only structural difference we can discern is the extent of glycosylation in these two species of phytase; the recombinant phytase produced in the tobacco leaves was shown to be have 17% less mass than the native phytase (Figure 1). It is now evident that glycosylation plays an important role in folding fungal phytase. When phyA gene from *Aspergillus niger* was expressed in *E. coli* the proteins not only accumulated in inclusion bodies but were inactive (22). Although, an insignificant amount of phytase activity was revived, the kinetic properties of the "revived" phytase remain unchanged as compared to the native phytase. The accumulation of inactive phytase in inclusion bodies of the bacteria had clearly indicated the role glycosylation plays in folding. The differences in pH optima of native and tobacco phytase may strengthen the view that post-translational modification of phytase such as glycosylation may play a leading role in structure-function relationship of this industrially important biocatalyst.

The temperature optima profile of overexpressed phytase in tobacco leaves showed remarkable similarities with that of native fungal phytase (7). Apparently, a somewhat diminished glycosylation of phytase in tobacco had not affected its temperature optima profile. Fungal phytase's inherent resistance to higher temperature and its ability to cleave phosphomonoester bonds at 58°C could be attributable to the presence of five disulfide bridges (24). The integrity and fidelity of disulfide bridges in recombinant phytase should have to be maintained to allow the right folding of the phytase molecule.

The K_m for phytate was about 2-fold lower in native fungal phytase as compared to the recombinant phytase, however, the K_i for *myo*-inositol hexasulfate were found to be very similar in both the phytases (Table 3). This indicates that the active site geometry in these two phytases may be near identical. Even the K_{cat}, a broad measure of overall efficiency in catalysis, remained virtually the same. The kinetic perfection as measured by the ratio of K_{cat} over K_m in native fungal phytase was only two-fold higher than it was in recombinant phytase. This is also indicative of very similar active site geometry in both the native and recombinant phytase.

To verify that the active site pocket of the recombinant phytase had not changed due to overexpression in tobacco leaves, we chemically probed both the native and overexpressed phytase with the arginine modifier, phenyl glyoxal. It was previously shown that phenyl glyoxal can modify sensitive arginyl residues in phytase and completely inactivate the enzyme (21). The inhibition profile of phenyl glyoxal in recombinant phytase was same as the native phytase (Figure 4). A 50% inhibition of activity in both native and overexpressed phytases had occurred at a concentration as low as 0.1 μM phenyl glyoxal. Whereas, the enzymes from both the sources were completely inactivated by phenyl glyoxal at 90 μM.

Judging from the catalytic characterization of cloned and overexpressed fungal phyA gene in tobacco leaves, we conclude that molecular biofarming of stable fungal enzymes has come of age. Biochemically speaking, there is not much difference in physico-chemical and catalytic properties between the fungally produced phytase and the one overproduced in tobacco leaves. Therefore, production of more stable hydrolytic enzymes from fungal sources in plants could open a new avenue for commercial exploits.

REFERENCES

1. Reddy, N. R., Sathe, S. K., and Salunkhe, D. K. (1982) *Adv. Food Res.* **28,** 1–92.
2. Nelson, T. S., Shieh, T. R., Wodzinski, R. J., and Ware, J. H. (1968) *Poult. Sci.* **47,** 1985–1989.
3. Erdman, J. W., Jr. (1979) *J. Am. Oil Chem. Soc.* **56,** 736–741.
4. Maga, J. A. (1982) *J. Agric. Food Chem.* **30,** 1–9.
5. Dvorakova, J. (1998) *Folia Microbiol.* **43,** 323–338.
6. Shieh, T. R., and Ware, J. H. (1968) *Appl. Microbiol.* **16,** 1348–1351.
7. Ullah, A. H. J., and Gibson, D. M. (1987) *Prep. Biochem.* **17,** 63–91.
8. Mullaney, E. J., Gibson, D. M., and Ullah, A. H. J. (1991) *Appl. Microbiol. Biotechnol.* **35,** 611–614.
9. Van Hartingsveldt, W., Van Zeijl, C. M. J., Harteveld, G. M., Gouka, R. J., Suykerbuyk, M. E. G., Luiten, R. G. M., Van Paridon, P. A., Selten, G. C. M., Veenstra, A. E., Van Gorcom, R. F. M., and Van den Hondel, C. A. M. J. J. (1993) *Gene* **127,** 87–94.
10. Kostrewa, D. F., Gruninger-Leitch, F., D'Arcy, A., Broger, C., Mitchell, D., and van Loon, A. P. G. M. (1997) *Nat. Struct. Biol.* **4,** 185–190.
11. Verwoerd, T. C., van Paridon, P. A., van Ooyen, A. J. J., van Lent, J. W. M., Hoekma, A., and Pen. J. (1995) *Plant Physiol.* **109,** 1199–1205.
12. Mason, H. S., Guerrero, F. D., Boyer, J. S., and Mullet, J. E. (1988) *Plant Mol. Biol.* **14,** 845–856.
13. Harpster, M. H., Townsend, J. A., Jones, J. D. G., Bedbrook, J., and Dunsmuir, P. (1988) *Mol. Gen. Genet.* **212,** 182–190.
14. Odell, J. T., Knowlton, S., Lin, W., and Mauvais, C. J. (1988) *Plant Mol. Biol.* **10,** 263–272.
15. McBride, K. E., and Summerfeldt, D. R. (1990) *Plant Mol. Biol.* **14,** 269–276.
16. An, G., Ebert, P. R., Mitra, A., and Ha, S. B. (1988) *in* Plant Molecular Biology Manual (Gelvin, S. B., and Schilperoort, R. A., Eds.), Kluwer Academic Publishers, Dordrecht, Belgium.
17. Horsch, R. B., Fry, J. E., Hoffman, N. L., Eicholtz, D., Rogers, S. G., and Farley, R. T. (1985) *Science* **227,** 1229–1231.
18. Ziegelhoffer, T., Will, J., and Austin-Phillips, S. (1999) *Mol. Breed.*, in press.
19. Sedmak, J. J., and Grossberg, S. E. (1977) *Anal. Biochem.* **79,** 544–552.
20. Jay, G. D., Culp, D. J., and Jahnke, M. R. (1990) *Anal. Biochem.* **185,** 324–330.
21. Ullah, A. H. J., and Sethumadhavan, K. (1998) *Biochem. Biophys. Res. Commun.* **243,** 458–462.
22. Phillippy, B. Q., and Mullaney, E. J. (1997) *J. Agric. Food Chem.* **45,** 3337–3342.
23. Han, Y., and Lei, X. G. (1999) *Arch. Biochem. Biophys.* **364,** 83–90.
24. Ullah, A. H. J., and Mullaney, E. J. (1996) *Biochem. Biophys. Res. Commun.* **227,** 311–317.