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ORIGINAL RESEARCH PAPER

A novel tissue-specific plantain β-1,3-glucanase gene that is regulated in response to infection by *Fusarium oxysporum* fsp. *cubense*

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Abstract A new full-length β-1,3-glucanase cDNA, *MpGlu*, was isolated from a plantain (*Musa paradisica*) by the rapid amplification of cDNA ends (RACE) technique. Recombinant GST-MpGlu protein, expressed in *E. coli*, hydrolyzed (1→3),(1→6)-β-glucan of *Laminaria digitata* and inhibited the growth of *Fusarium oxysporum* fsp. *cubense* (race 4) suggesting that it is a β-1,3-glucanase. Southern blot analysis indicated that there is one copy of *MpGlu* in the plantain genome. *MpGlu* gene expression was detected in plantain leaves, peel, and pulp by RT-PCR. Northern blot analysis revealed that the expression of *MpGlu* was up-regulated by *Fusarium* infection. Subcellular localization analysis indicated that 28 residues at the *N*-terminal end are necessary for extracellular secretion, while 32 residues at the *C*-terminal end are necessary to target the protein into vacuoles.

Keywords β-1,3-glucanase · Hypersensitive reaction · *Musa paradisica* · Pathogenesis-related protein · Plantain · Suppression subtractive hybridization

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Introduction

β-1, 3-Glucanases are involved in pathogenesis in plants. They are classified as a family 17 hydrolase based on structural criteria (Henrissat and Davies 1997), and preferentially hydrolyze 1,3-β-D-glycosidic linkages in (1→3)-β-D- and (1→3),(1→6)-β-D-glucans in the cell walls of many pathogenic fungi. Apart from their role in plant defense, 1,3-β-glucanases are involved in diverse physiological and developmental processes such as endosperm formation (Wu and Bradford 2003), somatic embryogenesis in *Cichorium* (Helleboid et al. 1998; Helleboid et al. 2000), microsporogenesis (Worrall et al. 1992), pollen development (Hird et al. 1993), seed germination (Buchner et al. 2002; Leubner-Metzger 2005; Leubner-Metzger et al. 1995; Leubner-Metzger and Meins 2000), flower formation (Akiyama et al. 2004), and the response to wounding and abiotic stress (Obregon et al. 2001).

The subcellular localization of hydrolytic enzymes in plants can provide important information about the role of hydrolytic enzymes during defense against pathogens (Wubben et al. 1992). β-1,3-Glucanases vary in their spatial distribution; some accumulate in the cell walls and extracellular spaces of rhizomania-infected sugar beet roots (Burketová et al. 2003), while others localize in the cell walls of tissues such as the lemma, ovary, and rachis (Kang and Buchenauer 2002). β-1,3-Glucanases are secreted into both the vacuoles and the extracellular

space (Benhamou et al. 1989; Mauch and Staehelin 1989).

Previously, we isolated a cDNA from the plantain encoding a new 1,3-β-glucanase using the rapid amplification of cDNA ends (RACE) technique on an expressed sequence tag (EST), which was isolated from a suppression subtractive hybridization (SSH) library with cDNA of plantain leaves that had been inoculated with *Fusarium*. *MpGlu* was up-regulated after inoculation with *Fusarium*. In this work, we report the cloning, characterization, and recombinant expression of *MpGlu* in *E. coli*. The recombinant GST-MpGlu is antifungal against *Fusarium* in vitro. Removal of the *N/C*-terminal ends affected the subcellular localization of MpGlu.

Materials and methods

Plant and fungal material

The plantains used in this study were collect from Panyu and cultured in a greenhouse. *Fusarium* was collected and adjusted to an OD_{600} of 0.1. The plantain roots were cut and inoculated with the suspension at 25°C.

RNA extraction and isolation of a full-length *MpGlu* cDNA

Total RNA was extracted from plantain leaves using the Concert Plant RNA Reagent (Invitrogen). Specific 5′-RACE and 3′-RACE primers were designed based on the sequence of the EST isolated from the SSH library: 5′GSP1 (gene-specific primers)(5′-ATAAGGGTACACATTGACCAGGAGC-3)/NGSP2 (5′-GATGTTGGAGTTCCTGAGGGCTTGC-3′) and 3′GSP2 (AGCGAGGTGGTCAGTCTCTACAAA)/NGSP2 (5′-GCTGGCCTGCAAAACCAGATCAAGGT-3′). The 3′and 5′sequence of the β-1,3-glucanase cDNA were then obtained by RACE–PCR with a GeneRacer Kit (Invitrogen). The PCR product was cloned into the pMD18-T vector and sequenced.

DNA extraction and Southern blot analysis

High-molecular-weight genomic DNA was isolated from the green leaves of mature plantain plants. For Southern hybridization, genomic DNA was digested with *Xba*I overnight and 20 μg digested DNA was subjected to electrophoresis in 0.8% (w/v) agarose for 16 h at 4 V/cm. After depurination of DNA within the gel with 0.25 M HCl for 30 min at 25°C, the DNA was transferred to a Hybond-N^+ nylon membrane in 20 × SSC and hybridized with a ^{32}P-labeled gene-specific probe for 16 h at 65°C. The blots were then washed under high-stringency conditions and exposed to X-ray film.

Construction of *E. coli* expression vector

Primers 5-GCGGAATTCATGGCAACAAAAGCTTCTCTCT-3 and 5-ATACTCGAGCTAGAAGCTTATTTGGTAGACG-3 were used to clone the mature MpGlu gene. Purified PCR products were digested with *Xho*I and *Eco*RI and cloned into the corresponding sites of the *E. coli* vector pGEX-4T-1 (Amersham Pharmacia Biotech).

Expression and purification of recombinant protein in *E. coli*

In vitro expression was performed following the manufacturer's instructions (Amersham Pharmacia Biotech). The recombinant protein was purified using a glutathione–Sepharose 4B column and subjected to SDS-PAGE to validate the expression of the inserted fragment.

MpGlu activity assay

The activity of the GST-MpGlu fusion protein was determined in a standard assay mixture containing 0.25% (w/v) laminarin (*Laminaria digitata*), 50 mM sodium acetate (pH 6.0), and 3 μg recombinant protein in a total volume of 50 μl incubated at 37°C. The reaction was stopped by the addition of p-hydroxybenzoic acid hydrazide reagent, and the increase in reducing sugars was measured colorimetrically.

Assay of antifungal activity

The antifungal activity of recombinant GST-MpGlu was assayed using the hyphal extension inhibition assay. *Fusarium* was inoculated at the center of a potato/dextrose/agar plate. After incubation at 28°C for 36 h, GST-MpGlu was added to wells 2 cm away

from the center of the plate. The plate was further incubated at 28°C for 48 h and the inhibition of hyphal extension around the wells was observed.

Semi-quantitative RT-PCR

RT-PCR was performed using total RNA from mature leaf, pulp, mature roots, corm, and peel. The primers LF (5′-ATGGCAACAAAAGCTTC TCTCTCC-3′) and LR (5′-ATAAGGGTACACATT-GACCAGGAG C-3′) were designed to analyze the tissue expression pattern. The PCR conditions were 95°C for 5 min, followed by 25 cycles of amplification (94°C for 30 s, 58°C for 30 s, 72°C for 30 s), then 72°C for 10 min. The *actin* was amplified using the primers ActinF (5′-TGTAGGTGATGAGGCCC AAT-3′) and ActinR (5′-ATACCTGTGGTACG TCCGCT-3′) as a control. PCR products were analyzed on 1% (w/v) agarose gels.

Northern hybridization

Total RNA (20 μg per lane) was subjected to electrophoresis on a 1.2% (w/v) agarose gel and transferred to a Hybond-N^+ nylon membrane in 20 × SSC. A gene-specific probe was made by PCR from the conserved region of the *MpGlu* cDNA. The blots were hybridized with a ^{32}P-labeled gene-specific probe for 16 h at 65°C. The blots were then washed under high-stringency conditions and exposed to X-ray film. To ensure equal loading of RNA and the intactness of the ribosomal RNA, the gels were stained with ethidium bromide.

Subcellular localization of MpGlu-GFP in onion epidermal cells

To study the subcellular localization of MpGlu, various truncations of the MpGlu gene were fused in frame to the green fluorescent protein (GFP) gene and inserted into the vector pCAMBIAC1301 driven by the CaMV35S promoter. This agrobacterium with constructed vector was then transformed into onion (*Allium cepa* L.) epidermic cells. Sixteen hours after transformation, GFP fluorescence in onion epidermal cells was visualized with a confocal laser scanning microscope (Leica TCS-SP2).

Results and discussion

Cloning and sequence analysis of *MpGlu*

The complete cDNA sequence was nominated as *MpGlu*. It has a length of 1,144 bp with an open reading frame of 1,023 bp encoding a putative 340 amino acid protein. It has a 22 bp 5′untranslated region, a 99 bp 3′ non-coding region, and two tailing signals. The putative gene product of *MpGlu* has a calculated molecular weight of 36378.24 Da and an isoelectric point of 8.82. The deduced amino acid sequence contains an *N*-terminal secretory signal peptide of approximately 28 amino acids, as predicted by the SignalP Server (http://genome.cbs.dtu.dk/services/SignalP-2.0/), and a *C*-terminal glycosylation site.

The deduced amino acid sequence of the MpGlu had 99.1%, 66.4%, 56.3%, 54.4%, and 54.1% identity with those of other monocotyledon β-1,3-glucanases from *Musa acuminata*, *Lilium hybrid*, *Oryza sativa*, *Triticum aestivum*, and *Hordeum vulgare*, respectively (Fig. 1). It has two domains that are conserved with Family 17 glycohydrolases and an incomplete exo-beta-1,3-glucanase with a number of highly conserved blocks with considerable homology. Sequence similarity analysis revealed that the deduced amino acid of MpGlu had 99.1% identity with the endo-β-1,3-glucanase from banana fruit (*Musa acuminata*), and both have a negatively-charged catalytic central cleft harboring the two glutamate residues (Glu94 and Glu236) acting as a hydrogen donor and nucleophilic residue, respectively (Receveur-Bréchot et al. 2006) (Fig. 1). The homology of candidate regions containing the catalytic and substrate-binding sites of the enzymes have been found in other plant β-glucan endohydrolases.

Southern blot analysis of *MpGlu*

Southern blot analysis of *MpGlu* genomic DNA digested with *Xba*I, which does not cut within the *MpGlu* cDNA, was performed to estimate the *MpGlu* copy number in the plantain genome. After a high-stringency wash, a 482 bp probe containing the *MpGlu* conserved domain hybridized with single band, demonstrating that there was single *MpGlu* copy in the plantain genome (Fig. 2).

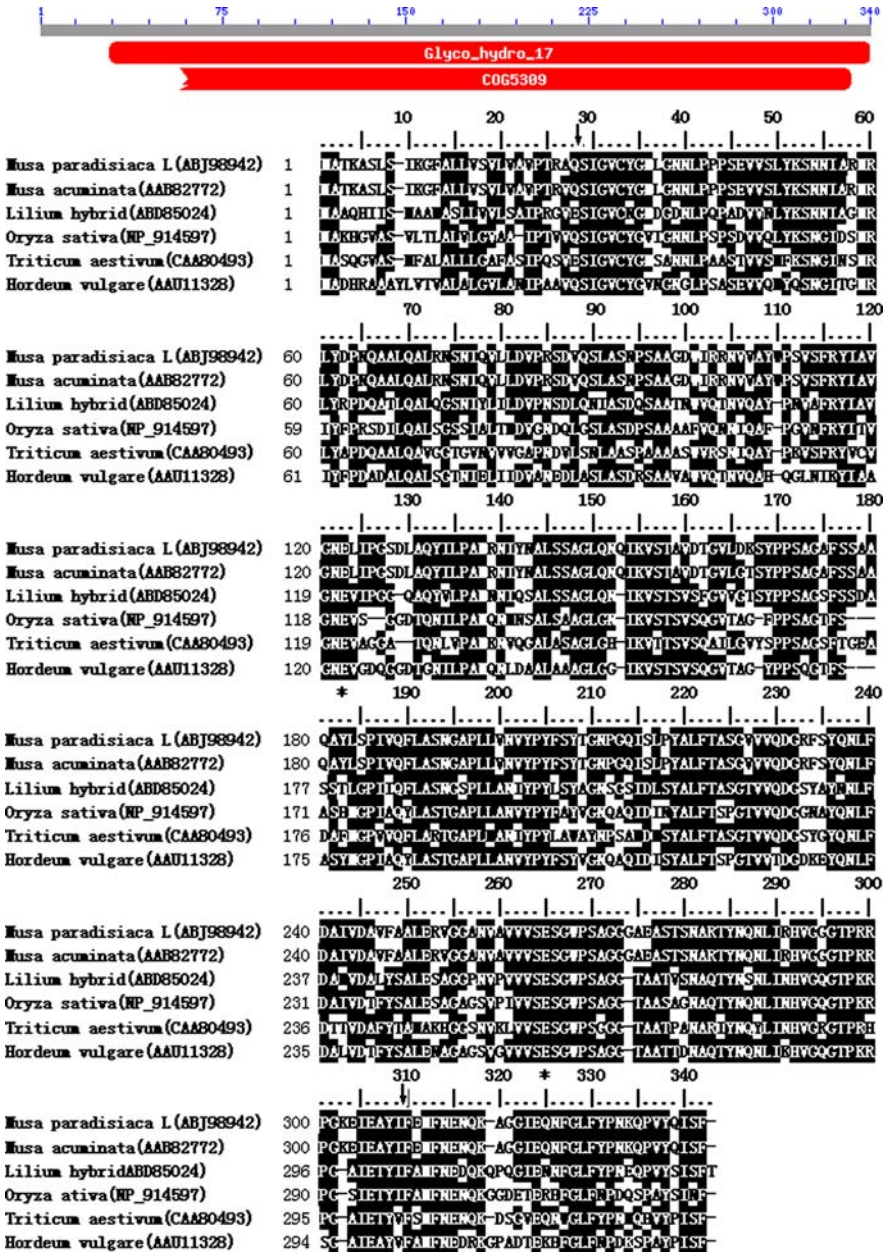

Fig. 1 Deduced amino acid sequence identity of MpGlu with β-1, 3-glucanases of the other monocotyledon plants: *Musa acuminata*, *Lilium hybrid*, *Oryza sativa*, *Triticum aestivum*, and *Hordeum vulgar*. Identical and similar residues are indicated by black and grey shading, respectively. Glyco_hydro_17 indicates the conserved domain of family 17 glycohydrolases and COG5309 indicates an incomplete conserved domain of exo-beta-1,3-glucanase. The asterisks indicate the catalytic central cleft. The arrowheads indicate the *N*-terminal signal peptide and *C*-terminal signal peptide

Expression in different tissues and inoculated by *Fusarium*

Semi-quantitative RT-PCR was performed to investigate the expression of *MpGlu* mRNA in various tissues of mature plantain plants. *MpGlu* mRNA was expressed in the leaves, peel, and pulp, while no *MpGlu* mRNA was detected in other tissues such as roots and corm (Fig. 3).

β-1,3-Glucanase was previously found in ripening banana fruit (Clendennen and May 1997) and an abundant, catalytically-active β-1,3-glucanase has been isolated from the pulp of ripe bananas (Receveur-Bréchot et al. 2006). *MpGlu* was also expressed in the pulp and peel of plantains, suggesting the possible involvement of MpGlu in ripening and/or softening processes. Northern blot analysis suggested that *MpGlu* mRNA was clearly up-regulated two days

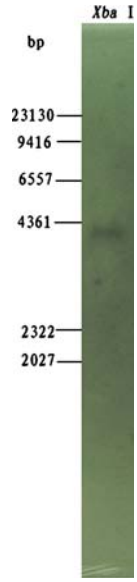

Fig. 2 Southern blot analysis of the *MpGlu* gene in plantain. Plantain genomic DNA was extensively digested with *Xba* I, which produced 2,322 bp and 4,361 bp fragment. Blot probed with a radiolabeled *MpGlu* partial cDNA (482 bp)

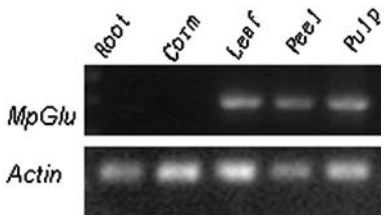

Fig. 3 Semi-quantitative RT-PCR analysis of *MpGlu* mRNA in different plantain tissues. A *MpGlu* fragment was amplified by 25 PCR cycles. As control, an *Actin* fragment was simultaneously amplified

after inoculating plantains with *Fusarium*, while there was no clear expression in uninoculated plantains and in plantains one day after inoculation by *Fusarium* (Fig. 4).

Functional expression of *MpGlu* in *E. coli*

For prokaryotic expression, the mature portion of the β-1,3-glucanase-coding sequence was amplified by PCR and cloned into the *E. coli* vector pGEX-4T-1. A 65 kD protein was expressed after IPTG induction, which was purified and subjected to SDS-PAGE (Fig. 5). The specific activity of the *E. coli*-expressed

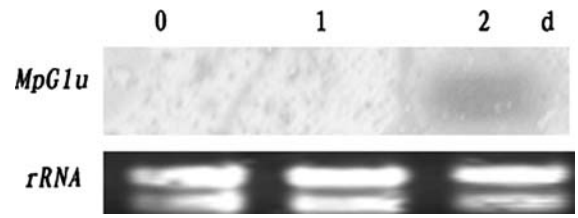

Fig. 4 Northern blot analysis of *MpGlu* expression in plantain leaves inoculated by *Fusarium*. RNA isolation from plantain leaves that was collected at the time points (d) indicated in the top of the figure after inoculating plantain leaves against *Fusarium.* Total RNA (20 μg/lane) was separated in a formaldehyde/agarose gel, transferred onto a nylon membranes and hybridized with a ^{32}P-labelled cDNA fragment. Lower panels show the ethidium bromide staining of the RNA samples analysed .The 18S and 28S rRNA bands visualized after staining the gel with ethidium bromide (EB) demonstrate that the RNA samples were loaded equally

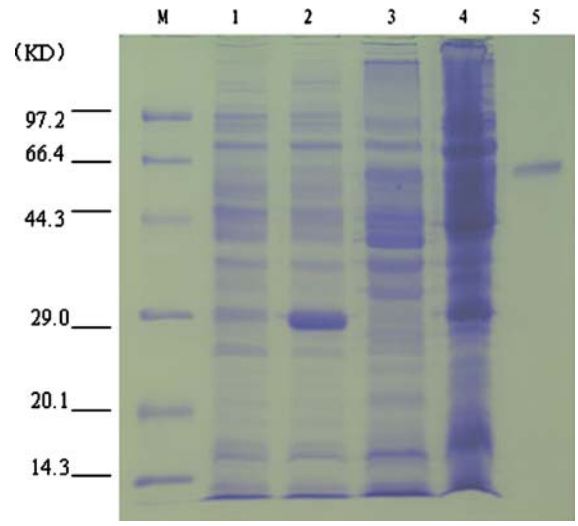

Fig. 5 SDS–PAGE analysis of purified GST-MpGlu recombinant protein expressed in *E. coli*. Soluble proteins extracted from IPTG-induced and non-induced E. coli were separated by SDS–PAGE on a 12% polyacrylamide gel and stained with Coomassie Brilliant Blue R250. Lane 1, soluble fraction of non-induced *E. coli* containing empty pGEX-4T-1 (pGST) vector; lane 2, soluble fraction of induced *E. coli* containing empty pGST; lane 3, soluble fraction of non-induced *E. coli* containing pGST with the MpGlu insert; lane 4, soluble fraction of induced *E. coli* containing pGST with the MpGlu insert; lane 5, pGST-MpGlu fusion protein purified by a glutathione–Sepharose 4B column. M, molecular mass markers: phosphorylase b, 97.2 kDa; bovine serum albumin, 66.4 kDa; ovalbumin, 44.3 kDa; carbonic anhydrase, 29 kDa; trypsin inhibitor, 20.1 kDa; and lysozyme, 14.3 kDa

recombinant was determined using *Laminaria digitata* (1→3),(1→6)-β-glucan as a substrate and found to be 2,200 nkat (nanokatals) per mg protein.

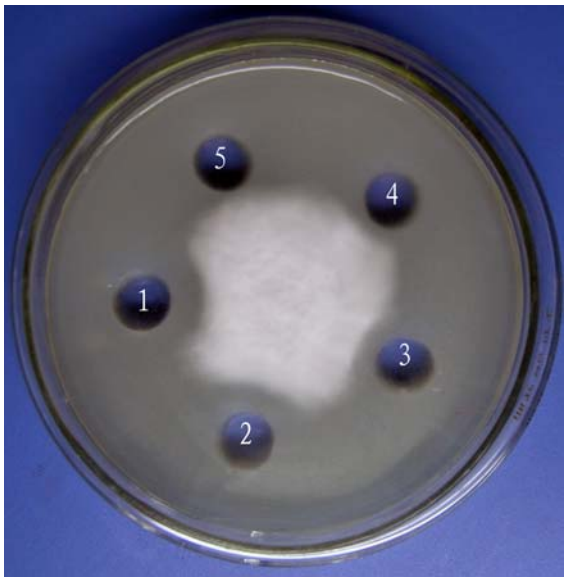

Fig. 6 Inhibitory effect of GST-MpGlu on *Fusarium*. Well 1, 50 μg GST-MpGlu; well 2, 40 μg GST-MpGlu; well 3, 30 μg GST-MpGlu; well 4, 20 μg GST-MpGlu; well 5, 0 μg GST-MpGlu

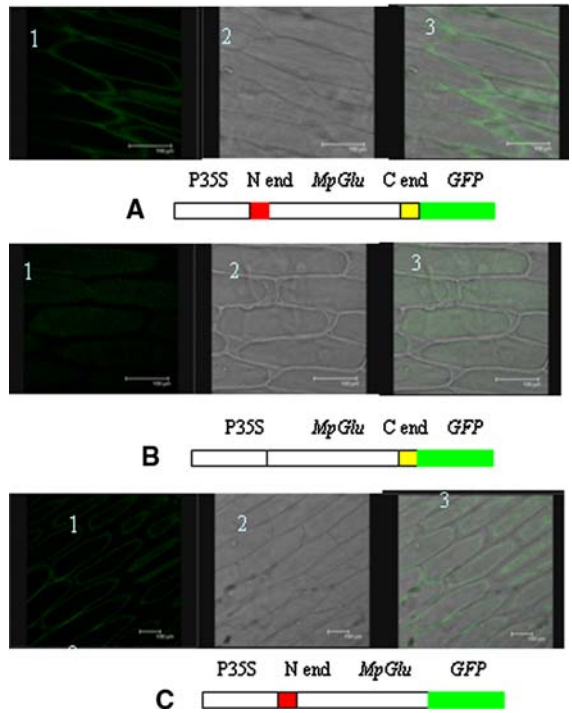

Fig. 7 Subcellular localization of the MpGlu protein. MpGlu-GFP fusion proteins and GFP alone were each expressed transiently in onion epidermal cells and observed under a confocal microscope. A, Subcellular localization of GFP fused with full-length MpGlu; B, Subcellular localization of GFP fused with MpGlu lacking the N-terminal end; C, Subcellular localization of the GFP fused with MpGlu lacking the C-terminal end. (1) fluorescence image; (2) bright field image; (3) overlay of both. The scale bar is indicated in each photo

The growth of *Fusarium* was clearly inhibited by the purified recombinant GST-MpGlu at 40 μg and 50 μg (Fig. 6) indicating that MpGlu probably plays an important role in plantain defense against *Fusarium*.

Subcellular localization of MpGlu

MpGlu has an *N*-terminal secreting signal peptide of approximately 28 residues as predicted by the SignalP Server, and a highly probable cleavage site was identified between Ser-28 and Ile-29 of the amino acid sequence deduced from the *MpGlu* cDNA. There are two conserved amino acids Phe309 and Glu310 at the *C*-terminal end, which were predicted to target the protein into vacuoles. To study the actual localization of MpGlu, full-length MpGlu without the *C*-terminal end and without the *N*-terminal end were individually fused with the GFP protein, and the reconstructed plasmids were transformed into onion epidermic cells. Green fluorescence was observed in the vacuoles and extracellular space of cells transformed with the fusion protein MpGlu-GFP containing the full-length MpGlu. Green fluorescence was observed only in the vacuole when the *N*-terminal end was removed, while green fluorescence was observed only in the extracellular space when the *C*-terminal end was removed (Fig. 7).

Protein processing is important in the intracellular transport and secretion of plant proteins. β-1,3-glucanases can have different spatial distributions, accumulating in extracellular spaces and cell walls (Burketová et al. 2003), or secreted into vacuoles and the extracellular space (Benhamou et al. 1989; Mauch and Staehelin 1989). Therefore, we conclude that the 28 residues signal peptide at the *N*-terminal end is necessary for extracellular secretion and the 32 residues at the *C*-terminal end are necessary for targeting the protein into vacuoles.

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