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Transient overexpression of the *Miscanthus sinensis* glucose-6-phosphate isomerase gene (*MsGPI*) in *Nicotiana benthamiana* enhances expression of genes related to antioxidant metabolism

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Abstract

We investigated the expression of genes related to antioxidant mechanisms through transient overexpression of the glucose-6-phosphate isomerase (*GPI*) gene of *Miscanthus sinensis* in *Nicotiana benthamiana* leaves. Full-length cDNA was isolated from *M. sinensis* GPI (*MsGPI*). An analysis of the MsGPI amino acid sequence revealed significant similarities to the GPIs of *Festuca ovima* (94%), *Hordium vulgare* (94%), *Triticum aestivum* (94%), and *Zea mays* (97%). RT-PCR showed that *MsGPI* expression was induced significantly by NaCl. Transient *M. sinensis GPI* sense construct overexpression resulted in increased *N. benthamiana* ascorbate peroxidase (*NbAPX*) and phenylalanine ammonia lyase (*NbPAL*) transcript levels in *N. benthamiana* leaves. These observations suggest that *MsGPI* is involved in antioxidant metabolism and that it is a transcriptional regulator of *NbAPX* and *NbPAL*.

Keywords: Antioxidant metabolism; Glucose-6-phosphate isomerase; NbAPX, NbPAL, Transient overexpression.
Abbreviations: ABA_abscisic acid; APX_ascorbate peroxidase; MS_Mmurashige and Skoog; MsGPI-AS_*Miscanthus sinensis* glucose-6-phosphate isomerase-antisense constuction; MsGPI-S_*Miscanthus sinensis* glucose-6-phosphate isomerase-sense construction; PAL_Phenylalanine ammonia lyase.

Introduction

Plants activate various physiological, metabolic, and defense systems to survive and sustain their growth during diverse abiotic environmental stresses (Yi et al., 2004). Abiotic stresses such as salinity, drought, and low temperatures can have adverse effects on crop growth and productivity (Suzuki et al., 2005), including by generating reactive oxygen species (ROS) and inhibiting photosynthesis (Hasegawa et al., 2000). Glucose-6-phosphate isomerase (GPI), also known as phosphoglucose isomerase, is an intracellular enzyme that catalyzes the reversible interconversion of glucose-6-phosphate and fructose-6-phosphate (Fru6P) (Smith and Doolittle, 1992). GPI is a dimeric enzyme, and each subunit consists of two domains (Kugler and Lakomek, 2000). GPIs are involved in many metabolic pathways in plants and may act as messengers that respond to stimulation by environmental factors such as nitrates (Munjral et al., 2007). GPI can also act as an autocrine motility factor (Chiu et al., 2008) and it may be related to the flagellar proteome of *Chlamydomonas reinhardtii* (Pazour et al., 2005). Furthermore, *GPI* is involved in glycolysis, the pentose-phosphate pathway, gluconeogenesis, glycogen synthesis, and the glucuronic acid pathway (Yamamoto et al., 2008), and it is universally distributed among eukaryotes, bacteria, and some Archaea (Grauvogel et al., 2007). Spinach GPI is significantly inhibited by erythrose 4-phosphate, causing limitations in starch synthesis at low Fru6P concentrations (Backhausen et al., 1997). GPI is also involved in the adaptation of plants to high salinity (Cui et al., 2009), glucose metabolism (Benevolensky et al., 1994), and pathogenicity in *Xanthomonas oryzae* pv. oryzae and *X. compestris* pv. citri (Tung and Kuo, 1999). *Miscanthus* is a bioenergy crop that has been studied with the goal of increasing its biomass. The expression of antioxidant-related genes is a major theme in enhancing biomass yields. Phenylalanine ammonia lyase (PAL) is a key enzyme in the phenylpropanoid pathway, which is important for salicylic acid synthesis and the accumulation of phytoalexins (Batz et al., 1998) in response to infection. PAL exerts unique regulatory control over phenylpropanoid biosynthesis (Dixon and Paiva, 1995), and it plays an important role in wound healing, pathogen defense reactions, abscission, stress responses, and secondary metabolism. No reports have been published regarding the transient overexpression of GPI genes in *Nicotiana benthamiana*. GPI is expressed as described below, based on measurements of ascorbate peroxidase (APX) (Cocetta et al., 2012), which is a key inhibitor of oxidative stress in photosynthetic organisms (Xia et al., 2009). No previous report has described a direct interaction between GPI and PAL. In this study, the expression profile of the *M. sinensis* (Ms) *GPI* gene was assayed in *N. benthamiana* leaves using

reverse transcription-polymerase chain reaction (RT-PCR) in response to various abiotic stresses. Transgenes inserted into T-DNAs can be examined rapidly for expression in plant leaves by *Agrobacterium*-mediated transient infiltration (Fischer et al., 1999). Various genes related to antioxidant metabolism (e.g., *APX* and *PAL*) were induced.

Results

Sequence analysis and multiple alignment of MsGPI

We created an *M. sinensis* cDNA library and analyzed several ESTs and cDNA clones to determine the genes involved in increasing the biomass of this bioenergy crop. We were able to determine the full-length sequences through a sequence analysis of smaller cDNA clones. The precise sequence of *MsGPI* was determined by comparison with cDNA sequences obtained via RT-PCR. This new sequence has been submitted to GenBank under accession number ADI24331. The sequence homologies among five *GPI* genes, including *MsGPI*, were analyzed using the BLAST program from the NCBI. The putative MsGPI amino acid sequence showed high identity with known GPIs from other plants (Figs. 1 and 2). The putative protein encoded by *GPI* shared 94% identity with *Festuca ovima* GPI (GenBank accession no. ABB90111), 94% identity with *Hordium vulgare* GPI (GenBank accession no. ABE41789), 94% identity with *Triticum aestivum* GPI (GenBank accession no. ABE41790), and 97% identity with *Zea mays* GPI (GenBank accession no. NP001105368).

Expression of MsGPI in response to various abiotic and biotic stresses

MsGPI expression in response to various abiotic stresses was analyzed in the leaves of *M. sinensis* by RT-PCR (Fig. 3). Abscisic acid (ABA) is involved in the aging process in plants with ROS-dependent stress signaling. *MsGPI* was induced up to 6 h and then was reduced after 12 h of ABA treatment (Fig. 3B). We next examined the association between *GPI* expression and salt stress by treating *Miscanthus* with NaCl. *GPI* showed gradually increasing expression until 24 h after treatment (Fig. 3C). Mannitol is a signaling chemical related to drought stress, and *GPI* was expressed with repeated reduction and induction (Fig. 3D). Methyl viologen (MV) was used to assess the importance of oxidative stress. The GPI expression pattern was repeated, with a reduction up to 24 h and then an increase, similar to the response to mannitol stress (Fig. 3E). The *MsGPI* transcript levels did not differ significantly during treatment with MV and mannitol (Fig. 3D and E).

Transient expression of NbAPX and NbPAL in N. benthamiana

To demonstrate the expression of genes related to defense and oxidative stress, *Agrobacterium tumefaciens* strain LBA4404, harboring the binary vector pMBP1 with the *MsGPI* coding region under the control of the CaMV *35S* promoter in the sense and antisense orientations, was infiltrated into *N. benthamiana* leaves (Fig. 4A-C). We thus investigated the role of *MsGPI* in the response of *N. benthamiana* genes to abiotic stress signaling (Fig. 5). *APX* expression was induced gradually until 48 h following *MsGPI*-S gene infiltration. In contrast, *MsGPI*-AS expression was abruptly reduced within 48 h following infiltration into *N. benthamiana* leaves (Fig. 5). *NbPAL* expression increased gradually until 12 h and was then maintained at a relatively stable level until 48 h following *MsGPI*-S and *MsGPI*-AS infiltration into the leaves of *N. benthamiana*, indicating its role in antioxidant mechanisms.

Discussion

We examined the *MsGPI* expression patterns in *M. sinensis* plants treated with various biotic and abiotic stresses, including salt, drought, ABA, and MV. *MsGPI* expression was induced by high-salt stress, similar to the *GPI* gene of *Dunaliella salina* (Cui et al., 2010). During salt stress, glycerol, which is the major compatible solute, accumulates so that the plant can adapt to the stressful environment (Chen and Jiang, 2009). To adapt to salt stress, plants activate biochemical pathways involved in the synthesis of compatible solutes, the maintenance of intracellular ion homeostasis, alterations in the intensity of photosynthesis, the scavenging of reactive oxygen species, and the modification of the expression of membrane structural elements and enzymes related to energy metabolism (Popova et al., 2008). In our study, the *MsGPI* expression level decreased after drought stress. Similarly, Xue et al. (2008) reported that the *GPI* expression level decreased slightly following drought stress in *T. aestivum* (Xue et al., 2008). Drought stress leads to a reduction in carbon fixation, which is ascribed physiologically to the closing of stomata in the leaves and attributed biochemically to a decrease in photosynthesis (Ghannoum et al., 2003). This, in turn, alters the carbohydrate metabolic equilibrium. The downregulation of additional photosynthetic genes has also been reported in barley and other grass species following drought (Talame et al., 2007). *MsGPI* transcription increased in the presence of ABA during the early phase of exposure. Antioxidant enzymes, which play an important role in abiotic stress tolerance in plants, are induced by ABA treatment (Zhou and Gou, 2005). Exogenous ABA treatment induces H_2O_2 and nitric oxide production, which leads to stomatal closure and the increased expression and activity of antioxidant enzymes in plants (Jiang and Zhang, 2003). Wang et al. (2003) reported that the *GPI* gene responded quickly to nitrate stress in *Arabidopsis*. Furthermore, GPI overexpression plays a significant role in glucose metabolism (Benevolensky et al., 1994), starch hydrolysis, and sucrose synthesis in irradiated sweet potato (Ajlouni and Hamdy, 1988). The *NbAPX* and *NbPAL* transcription levels changed significantly when *MsGPI*-S and *MsGPI*-AS were transiently overexpressed in *N. benthamiana*. Cytosolic APX plays a protective role against oxidative stress (Wang and Portis, 1992) and acts as an H_2O_2 scavenging enzyme in higher plants (Asada, 1992). In tobacco, a deficiency in thylakoid-bound APX has adverse effects on plant growth and photosynthetic performance (Yabuta et al., 2002). In addition, APX1 may be directly involved in the scavenging of H_2O_2, which leaks from the chloroplasts or peroxisomes (Davletova et al., 2005). It is possible that APX1 functions as a defensive barrier between the three major ROS-producing organelles in plant cells (Davletova et al., 2005). Enhanced levels of *APX* mRNA have been found in drought-stressed peas (Mittler and Zilinskas, 1994) and ozone-fumigated *Arabidopsis* (Conklin and Last, 1995). Thus, changes in *APX* transcription factors could occur in different plant species in response to different abiotic and biotic stressors. The *PAL* expression levels increased markedly after 12 h under conditions of stress, suggesting that *MsGPI* plays an important role in host-pathogen interactions. Increases in PAL activity are often associated with the progressive incorporation of phenolic

Fig 1. The nucleotide (a) and deduced amino acid (b) sequences of *GPI* gene isolated from *M. sinensis* (red letter). Untranslated regions (UTR) (black letter). This gene has received an accession number ADI24331 from NCBI. One underline was indicated the binding site used to construction of sense and antisense *MsGPI* gene.

compounds into the cell wall during incompatible plant-microbe interactions (Umesha, 2006). PAL is a key enzyme in the phenylpropanoid pathway, and lignin biosynthesis may result in increased phenol accumulation and increased lignin synthesis (Umesha, 2006). *PAL* mRNA production and activity is more rapid, higher, and longer lasting during incompatible plant-pathogen interactions (Cui et al., 1996), resulting in an oxidative burst of H_2O_2 in the localized area surrounding sites of pathogen entry (Levine et al., 1994). Therefore, increased *PAL* expression due to *MsGPI* overexpression could increase the amount of accumulating phenol and lignin synthesis to increase resistance against the pathogen and to adapt to the oxidative stress. We investigated *MsGPI* expression during abiotic stress using RT-PCR. The *MsGPI* gene expression patterns differed significantly when plants were exposed to NaCl, drought, ABA, or MV, suggesting that *MsGPI* is related to the abiotic stress response. Thus, *MsGPI* overexpression protects plant tissues from a variety of abiotic and biotic stresses.

Materials and Methods

Construction of sense and antisense GPI genes isolated from M. sinensis

We constructed a cDNA library and performed an EST analysis to study various *Miscanthus* genes (data not shown). The full-length *MsGPI* sequence was determined using nucleotide primers based on sequence alignments. The primer sequences for *MsActin* were 5′-ACCCTCTGTTGTCCCTGGAG-3′ (forward) and 5′-CTCGTCACCCTCGTCATCTG-3′ (reverse). cDNAs encoding two GPIs, designated *MsGPI*-S and *MsGPI*-AS, were isolated and sequenced. Primers were then designed to amplify full-length *MsGPI*-S and *MsGPI*-AS. The sequences were: 5′-GGG TCT ATG GCG TCG CCG GCG CTA-3′ (forward) and 5′-GGG GAG TTA CAG ATG TAC TAG GGA-3′ (reverse) for the sense orientation and 5′-GGG GAG ATG GCG TCG CCG GCG CTA-3′ (forward) and 5′-GGG TCT TTA CAG ATG TAC TAG GGA-3′ (reverse) for the antisense orientation of *MsGPI*. The band detected by PCR was cloned into pMBP1 harboring the *NPTII* gene with kanamycin resistance as a selectable marker under the *35S* promoter. The cloned *MsGPI* sequences were confirmed to match the full-length sequences and were transformed into *Agrobacterium* LBA4404.

Miscanthus sinensis chemical treatments

Miscanthus sinensis were transferred to pots and kept in a greenhouse. Various abiotic elicitors were then applied. The detached leaves were placed in a solution containing 400 mM NaCl, 400 mM mannitol, 0.05 mM MV, and 0.1 mM ABA. Plants were treated with distilled water as a control. In a greenhouse, plants grown from seed germination to about 5-6 weeks were cut with scissors, around 4-5 leaves per abiotic treatment. Samples with detached leaves were exposed to stress treatments for various times. The treated leaves were

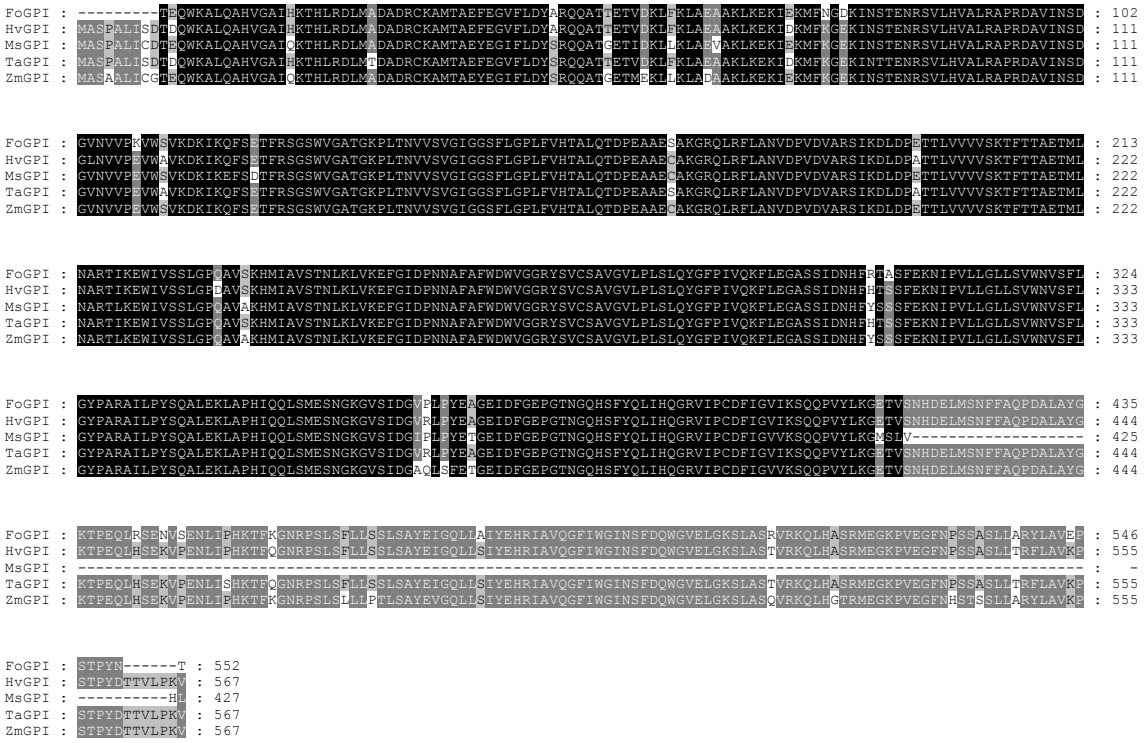

Fig 2. Comparative analysis of derived amino acid sequences of *MsGPI* gene. Residues shaded in black are identical between the five proteins. The GenBank and NCBI accession numbers of peptide sequences are: *Festuca ovima* (*FoGPI*, ABB90111), *Hordium vulgare* (*HvGPI*, ABE41789), *Triticum aestivum* (*TaGPI*, ABE41790), *Zea mays* (*ZmGPI*, NP001105368).

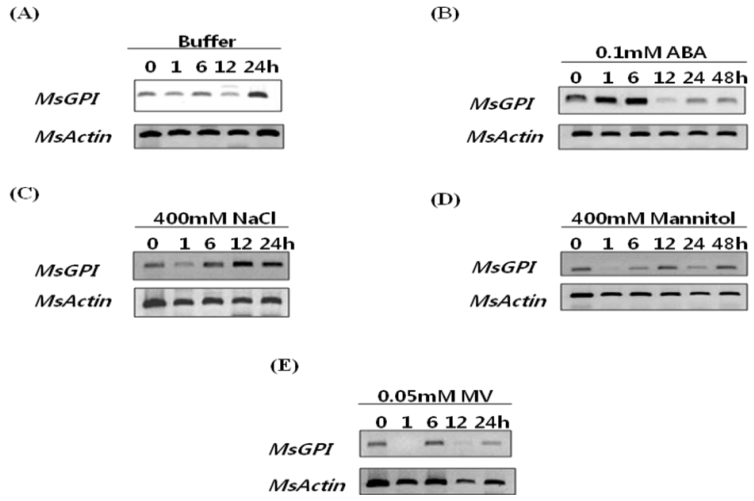

Fig 3. Expression patterns of *MsGPI* gene by abiotic stresses. Leaves of *Miscanthus sinensis* were exposed to (A) Buffer: Distilled water, (B) 0.1 mM ABA (aging stress), (C) 400 mM NaCl (salt stress) and (D) 400 mM mannitol (drought stress), (E) 0.05 mM methyl viologen (MV, oxidative stress). The expressions of response to abiotic stresses were confirmed by RT-PCR. *MsActin* was used as a positive control. The experiment was repeated at least three times and a representative result is shown.

snap-frozen in liquid nitrogen and stored at -70°C until they were used for RNA extraction.

Transient expression of MsGPI-S and MsGPI-AS in N. benthamiana

Nicotiana benthamiana seeds were surface-sterilized by immersing them in 70% ethyl alcohol for 1 min, followed by two rinses in sterilized distilled water, soaking in a solution of 5% (v/v) sodium hypochlorite, and gentle shaking for 15 min. This procedure was followed by three successive washes with sterile distilled water. The seeds were plated on Murashige and Skoog (MS) medium (MS salts, 3% sucrose, and 0.8% agar, pH 5.8) at 25°C under a 16-h light/8-h dark photoperiod. After 2 weeks, the germinated plants were transferred to pots and maintained in a growth chamber at 28°C under 150 μmol/m^{2}s illumination and a 16-h photoperiod for 4 weeks. *MsGPI*-S and *MsGPI*-AS were inserted into the *Xba*I-*Sac*I sites of pMBP1 for agroinfiltration experiments. The binary plasmid was transformed into *A. tumefaciens* strain LBA4404. *Agrobacterium* cells were harvested by centrifugation and

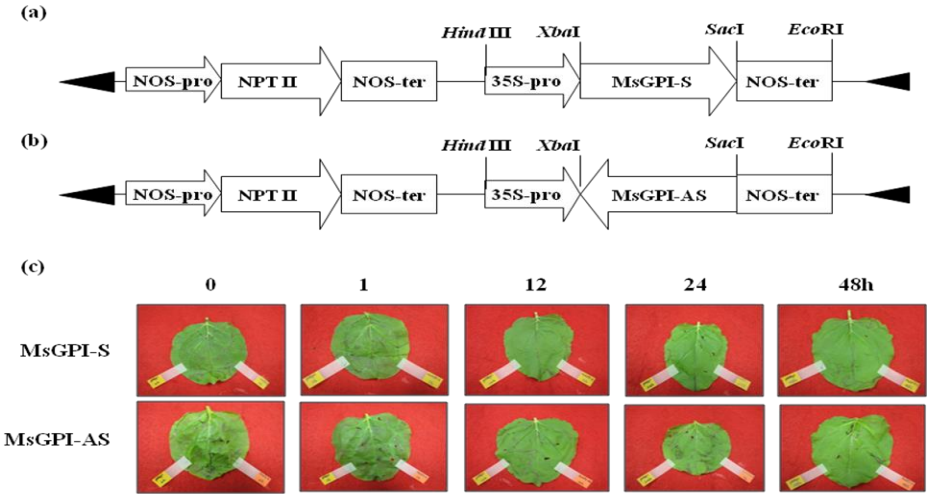

Fig 4. Vector constructions to transient over-expression of *MsGPI* gene in leaves of *N. benthamiana.* Constructions of *MsGPI*-S (a) and *MsGPI*-AS (b) in the pMBP1 vector. (C) *N. benthamiana* leaves that were infiltrated with constructions of overexpressed *MsGPI*-S and *MsGPI*-AS by *Agrobacterium*-mediated transient transformation.

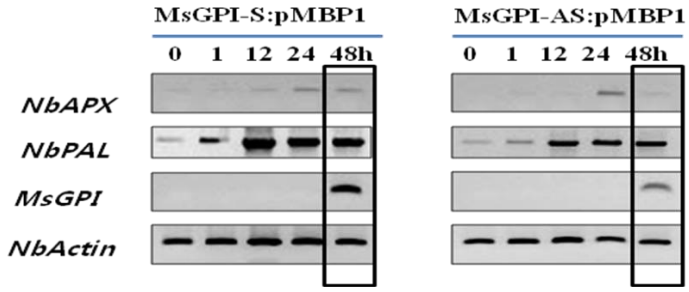

Fig 5. Expression patterns of various genes activated by in *N. benthamiana* leaves infiltrated by transient *MsGPI*-S and *MsGPI*-AS over-expression. The expression of pathogen-related genes (*NbPAL*) and an oxidative stress-related gene (*NbAPX*) were compared by RT-PCR. *NbActin* was used as a positive control. The experiment was repeated at least three times and a representative result is shown.

suspended in 10 mM MES-NaOH (pH 5.6), 10 mM $MgCl_2$, and 100 µM acetosyringone to an optical density (600 nm) of 0.7, incubated at room temperature for 2 h, and used to infiltrate *N. benthamiana* leaves with a needle-free syringe.

RNA preparation

Total RNA was isolated from stress-treated and control materials according to a previously described procedure (Yi et al., 2004) with some modifications. Trizol reagent (5 mL; Invitrogen, Carlsbad, CA, USA) was added to 1 g of plant material that had been frozen in liquid nitrogen and ground to a powder. The mixture was incubated for 5 min at room temperature, and 0.2 mL of chloroform-isoamyl alcohol (24:1) was added. The samples were then centrifuged (12,000×*g*, 10 min, 4°C) after mixing, and the supernatant was transferred to a new tube. This process was repeated. Precipitation was performed with isopropanol at -20°C for 1 h. After centrifugation (12,000×*g*), the pellet was washed with 70% ethanol and redissolved in diethylpyrocarbonate-treated water (30 µL). The integrity of the RNA was checked by 1.0% (w/v) agarose/formaldehyde gel electrophoresis.

RT-PCR analysis

Isolated total RNA was separated on formaldehyde-containing agarose gels. The first strand was synthesized from 1 µg of DNase-treated total RNA (M-MLV RT; Invitrogen). The RT-PCR primers were as follows: 5′-GGG TCT ATG GCG TCG CCG GCG CTA-3′ (forward) with 5′-GGG GAG CTC TTA CAG ATG TAC TAG GGA-3′ (reverse) and 5′-GGG GAG CTC ATG GCG TCG CCG GCG CTA-3′ (forward) with 5′-GGG TCT AGA TTA CAG ATG TAC TAG GGA-3′ (reverse) for *MsGPI*-S and *MsGPI*-AS, respectively. The other gene-specific primers used were: *NbActin* forward primer, 5'-CAGCTCATCCGTGG AGAAGA-3'; *NbActin* reverse primer, 5'-AGGATACGGGGAG CTAATGC-3'; *NbAPX* forward primer, 5'-GTCCATTCGGAACAATGAGG-3'; *NbAPX* reverse primer, 5'-GTGGGCACCAGATAAAGC-3'; *NbPAL* forward primer, 5'-TCGAGTTGCAGCCTAA GG-3'; and *NbPAL* reverse primer, 5'-TCTTCCAAATGCCTCAAGTC-3'. All reactions consisted of an initial denaturation for 5 min at 94°C; 25 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min; and a final 7-min extension at 72°C. Aliquots (12 µL) of the reaction products were separated on 1% agarose gels and visualized by ethidium bromide staining. All experiments were performed in triplicate.

Conclusions

We examined the full-length genes obtained from a cDNA library of *M. sinensis* with the goal of increasing the biomass

of this bioenergy crop. The *GPI* gene of *Miscanthus* showed the highest homology to the equivalent gene from *Z. mays* (97% similarity based on a sequence analysis). Expression of the *GPI* gene was induced markedly in response to salt stress; the relationship between *GPI* and salt should thus be considered in future studies. The function of the full-length gene was investigated through the overexpression and downregulation of *GPI* via transient expression using *Agrobacterium*-mediated infiltration into *N. benthamiana* leaves. Our results indicate that *GPI* expression may control antioxidant-related genes such as *APX* and *PAL*. This study indicates a major role for glucose metabolism in the function of *GPI*. This effect and the antioxidant actions of *GPI* should be examined in future studies.

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