A cytosolic glucose-6-phosphate dehydrogenase gene, ScG6PDH, plays a positive role in response to various abiotic stresses in sugarcane

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As one of the key enzymes in the oxidative pentose phosphate pathway, glucose-6-phosphate dehydrogenase (G6PDH) plays a role in response to abiotic stresses and pathogenesis. Here, a full-length cDNA was obtained, designed as ScG6PDH from sugarcane. The ScG6PDH gene is 1,646 bp long with a 1,524-bp long ORF encoding 507 amino acid residues. Analysis of a phylogenetic tree indicated that this gene is a member of the cytosolic G6PDH gene family, which is consistent with results from a subcellular localization experiment. Based on a real-time quantitative RT-PCR performed under salt, drought, heavy metal (CdCl2) and low temperature (4 °C) treatments, the transcription levels of the ScG6PDH gene were higher compared with transcription levels where these treatments were not imposed, suggesting a positive response of this gene to these environmental stresses. Furthermore, G6PDH activity was stimulated under 4 °C, CdCl2, NaCl and PEG treatments, but the increments varied with treatment and sampling time, implying positive response to abiotic stresses, similar to the transcript of the G6PDH gene. Ion conductivity measurements and a histochemical assay provided indirect evidence of the involvement of the ScG6PDH gene in defense reactions to the above-mentioned abiotic stresses.

When plants grow in the light, photosynthesis in green tissues can generate nicotinamide adenine dinucleotide phosphate (NADPH), which is used in processes that include carbon fixation, fatty acid synthesis and nitrogen assimilation. When plants grow in the dark, however, NADPH in photosynthetic or non-photosynthetic tissues is supplied by the oxidative pentose phosphate pathway (OPPP)1,2. Glucose-6-phosphate dehydrogenase (G6PDH, EC1.1.1.49) is an important regulating enzyme of the OPPP that catalyzes the conversion of glucose-6-phosphate to 6-phosphogluconolactone, provides NADPH for biosynthesis, and supplies pentose for nucleic acid synthesis3,4. G6PDH exists widely in plants, animals and prokaryotes. It is generally accepted that G6PDH can be detected in the cytoplasm or plastids of plants5. Based on this distribution, G6PDH is divided into cytosolic G6PDH and plastidic G6PDH. Two sites, a substrate-binding site (IDHYLG) and NADP-binding site (NEFVIRLQP), are highly conserved in the cytosolic G6PDH gene sequence6.

In addition, two different types of plastidic G6PDH, P1 and P2, which evolved from a common ancestral gene, were discovered by researchers based on the alignment of mature proteins and signal peptides. The difference in signal peptides between P1 and P2 exists in the cleavage site, which is designated as (I/V/L)X(S/T/K) for P1 and (I/V)X(S/T/A) for P27. Furthermore, Wendt et al. observed that the P1 and P2 isoforms showed different gene expression patterns in Solanum tuberosum L.8.

G6PDH is not only a rate-limiting enzyme, but also plays a role in response to biotic and abiotic stresses. The response of plant G6PDH has been examined under different types of environmental stresses. For example, Scharte et al. reported an increase in G6PDH activity in the resistant Nicotiana tabacum Samsun NN cultivar, but not in the susceptible Xanthi cultivar after infection with Phytophthora nicotianae. In addition, the role of the G6PDH isoenzyme in tobacco drought tolerance and flowering was verified using the RNAi method. These results demonstrate that G6PDH may play a crucial role during early-stage drought stress, plant development and pathogen defense. The tolerance of G6PDH to low temperature has been reported by several researchers10,11. A phenotypic difference between wild-type and T1 generation transgenic tobacco plants containing the PsG6PDH gene from Populus suaveolens was observed in a cold treatment experiment12. The results indicated that wild-type
plants suffered earlier and recovered later from cold injury compared with transgenic lines subjected to a temperature change from 25°C to 4°C without cold acclimation. These results were consistent with the trends in peroxidase (POD) activity, malondialdehyde (MDA) content and superoxide dismutase (SOD) activity. Meanwhile, gene expression related to cold stress in transgenic tobacco plants over-expressing PsG6PDH was induced, indicating the role of PsG6PDH in the development of cold tolerance in tobacco. In *Trichitis aestivum* L. subjected to 0.15 M NaCl stress, the transcript level of *G6PDH* was quantified using northern hybridization, which increased over time and reached a maximum at 12 h. Zhang *et al.* discovered the key role of G6PDH in *Oryza sativa* suspension cells under salt stress, which was the result of the coordination of G6PDH and NAPDH oxidase to maintain cell redox balance. The activity of the G6PDH enzyme responsive to heavy metal stresses in *Phaseolus vulgaris* L. and wheat has been reported to be modulated by zinc or aluminum.

Sugarcane (*Saccharum officinarum* L.) is an important sugar and economic crop. In the past decade, sugarcane has accounted for more than 90% of sugar production in China and 80% of the world sugar production in 2012. The negative impact of abiotic stresses, such as cold, salt and drought, on plant metabolism, physiology and productivity results in yield loss. Among these factors, drought and cold are the two major environmental stresses which can affect or limit sugarcane growth and thus result in a serious decrease in sugarcane production, especially in China. Breeding resistant sugarcane cultivars is one effective strategy to overcome these problems. Considering of modern sugarcane variety is a complex of *S. officinarum* and *S. spontaneum* with a highly complicated genetic background of polyploidy and aneuploid, and with about 120 or more chromosomes, it is not easy to obtain an ideal cultivar by means of traditional crossing and selection due to an adverse linkage of important industrial and agricultural traits. With the development of modern genetic engineering, it is practical to obtain highly resistant cultivars by introducing resistance genes to improve gene expression in modern cultivar derived from traditional crossing. While, unfortunately, sugarcane genome sequencing is just beginning and only very limited genome information is obtained. Therefore, to better understand the molecular mechanism of the stress response and the ability to avoid stress or increase tolerance to adverse growing conditions, it is beneficial to identify stress responsive genes in sugarcane. In recent years, some genes related to environmental stress have been cloned from sugarcane and investigated. For example, Guo *et al.* reported a *ScDir* gene cloned from sugarcane, which was involved in the response to drought, salt and oxidant stresses. Su *et al.* reported two stress-related genes, i.e., *ScGluA1* and *ScGluD1*, and a novel stress-induced *Scdrl* gene, which was isolated from sugarcane and displayed tolerance to drought, salt and oxidative stresses in transgenic tobacco plants. Transgenic tobacco plants containing the gene showed higher tolerance to drought, salinity and oxidative stress. To date, although the *G6PDH* gene has been isolated from several other plant species and investigated, it remains unreported in sugarcane.

In this study, we investigated the role of the *G6PDH* gene in sugarcane (Accession number: KJ620023) subjected to CdCl$_2$, polyethylene glycol (PEG), NaCl and cold treatments. Expression analysis determined using a real-time quantitative RT-PCR (RT-qPCR), and increased enzyme activities showed that ScG6PDH responded positively to environmental stresses. In addition, the role of this enzyme in the hypersensitive response (HR) in tobacco and its sub-cellular location was determined. The amino acid sequence of ScG6PDH shares 77.50%, 91.34%, 77.34% and 83.63% homologies with the cytosolic G6PDH from tobacco (CA049994), rice (CACY09489), potato (CAA52442) and wheat (BA97663), respectively (see Fig. S1). The prediction results did not reveal an N-terminal signal peptide or transmembrane in ScG6PDH (see Supplementary Fig. S2 and Fig. S3). A phylogenetic tree shows that ScG6PDH belongs to the cytosolic G6PDH family (see Fig. 1B).

**Subcellular localization.** The ScG6PDH gene was fused with the pCAMBIA 2300 vector between the 35S promoter and GFP to generate the subcellular localization vector pCAMBIA 2300-ScG6PDH-GFP (see Fig. S4 and Fig. S5). When Agrobacterium-mediated transformation of *Nicotiana benthamiana* leaves had been initiated, green fluorescence was evident and appeared in the cytoplasm and cell nucleus under an laser scanning confocal microscope, while green fluorescence in the pCAMBIA 2300-ScG6PDH-GFP group was only observed in the cytoplasm (see Fig. 2 and Fig. S6).

**Tissue specific expression analysis of *ScG6PDH*.** The transcript level of *ScG6PDH* gene in different tissues in sugarcane variety Yacheng 05-179 was investigated with the GAPDH being used as an internal control. As showed in Fig. 3, the *ScG6PDH* transcript was observed in all the six types of tissues, including leaf, bud, root, stem epidermal, stem pith and leaf sheath. The *ScG6PDH* transcript level in the stem pith and bud was obviously higher than the other four tissue types, which was 2.9 times and 2.7 times higher respectively compared with sheath. The transcript level of the *ScG6PDH* in sheath was also significantly lower than leaf (1.4 times), bud (2.1 times) and root (1.6 times).

Expression of *ScG6PDH* gene in *E. coli* and its growth under abiotic stresses. The results of recombinant protein, the blank and the mock induced for 2 h using isopropyl β-D-1-thiogalactopyranoside (IPTG) were shown in Fig. 4A. Target protein of ScG6PDH expressed successfully in *E. coli* Rosetta cells was obviously observed with the band approximate 60 kDa molecular mass (Lane 6 in Fig. 4A). Due to the presence of 6-HIS tag in the PET28a vector, the molecular mass of the specific protein showed in Fig. 4A was higher than that of the estimated 57 kDa.

The growth curves of Rosetta strain cells in liquid culture under different stresses were shown in Fig. 4. Interestingly, when NaCl, CdCl$_2$ or PEG was added in the media respectively, Rosetta cells expressed ScG6PDH protein showed decreased growth in cell quantity than those without ScG6PDH. It seemed the protein ScG6PDH expressed in Rosetta cells didn’t increase the growth ability in these abiotic stresses.

Expression profiles of the *ScG6PDH* gene under environmental stresses in sugarcane. To investigate the role of the *ScG6PDH* gene in response to environmental stresses in sugarcane plantlets of Yacheng05-179, the expression profiles were detected using RT-qPCR under CdCl$_2$ (500 μM), NaCl (250 mM), PEG 8000 (25%), and 4°C treatments. As shown in Fig. 5, the up-regulation expression trends of *ScG6PDH* were similar after exposure to different environmental stresses. However, the *ScG6PDH* transcript was strongly induced by exogenous CdCl$_2$ and low temperature stresses, and was 43 times higher after 12 h (CdCl$_2$ stress) and 129 times higher after 24 h (4°C stress) compared with the control. Although the increase in *ScG6PDH* transcript levels induced by...
Figure 1 | The cDNA sequence and coding amino acid sequence of ScG6PDH (A) and phylogenetic tree of the deduced amino acid sequences of G6PDHs from Saccharum officinarum and other plant species (B). The substrate-binding site and NADP-binding site are underlined in (A). The plant species in (B) include Oryza sativa, Nicotiana tabacum, Solanum tuberosum, Petroselinum crispum, Triticum aestivum, Medicago sativa, and Spinacia oleracea.
NaCl and PEG was significant, the increment was considerably lower than those induced by CdCl₂ and 4°C stresses.

Changes in enzyme activity of G6PDH under various environmental stresses. Based on the different degrees of increased transcript levels of ScG6PDH gene under the four stress treatments, the activity of G6PDH was measured to investigate changes in protein abundance. As shown in Fig. 6, G6PDH activity peaked after 12 h (175.9% of the control level) and remained higher than that of the control though with a little of decrease after treatment with 500 μM CdCl₂, while its activity remained enhanced during the time course of 4°C stress and reached a peak of 269.2% of the control on 24 h, showing the same trends with the transcript levels. The salt treatment on seedlings resulted in only a little change before 24 h, and a sharp enhancement was observed at 24 h to 48 h (193.2% of the control level at 48 h), also showing its positive role in response to salt stress. Meanwhile, a similar change tendency was observed under the treatment 25% PEG 8000 and the maximum at 48 h was 163.6% of that of the control.

Transient expression of ScG6PDH induces a defense response in tobacco. To investigate the role of ScG6PDH in the hyper-sensitive response and immunity in plants, an overexpressed pCAMBIA 1301-ScG6PDH vector was constructed and an Agrobacterium-mediated transient expression method was performed to identify the effect of ScG6PDH expression on the induction of the defense response in tobacco leaves. The expression of H₂O₂ can be used as an early signal molecule of the plant-pathogen interaction that reflects the extent of plant hypersensitive cell death. Here, H₂O₂ was observed as a brown leaf color using the 3,3’-diaminobenzidine solution (DAB) staining method. As shown in Fig. 7, a typical hypersensitive response with enhanced ion conductivity (see Fig. 7A) and deeper DAB staining color (see Fig. 7B) in leaves expressing ScG6PDH was observed in 48 h after infection. This was significantly higher than that of the control.

Figure 2 | Localization of pCAMBIA 2300-GFP and pCAMBIA 2300-ScG6PDH-GFP in Nicotiana benthamiana protoplasts. Scale bar = 10 μm.

Figure 3 | Tissue-specific expression analysis of the ScG6PDH in different tissues in sugarcane variety Yacheng05-179. All data are normalized to the GAPDH transcript level and a data point is the mean ± SE (n = 3) Different lowercase letters indicate a significant difference, as determined by the least-significant difference test (p-value < 0.05).
control without *ScG6PDH*. Both the ion conductivity and histochemical assays demonstrated that the *ScG6PDH* gene may play a role in hypersensitive cell death response and defense reaction.

**Discussion**

As a key enzyme of the pentose phosphate pathway, G6PDH can catalyze the synthesis of 6-phosphogluconolactone and the production of NAPDH. Because of the connection between protein location and function, knowledge of the G6PDH location is useful to determine its role in plants. In previous reports, G6PDH was classified into two types based on location, i.e., plastidic and cytosolic G6PDH, both encoded by the nuclear gene and with similar molecular mass (approximately 56 kDa). One report provided further evidence for the functional role of one cytosolic and two plastidic (P1 and P2) isoforms, suggesting each isoform appeared to have a different regulatory mechanism and played distinct roles in growth and stress tolerance. In this study, we isolated the *ScG6PDH* gene (Accession number: KJ620023) from sugarcane. This gene contains two conserved sites, i.e., a substrate-binding (IDHYLG) and a NADP-binding site (NEFV IRLQP) (see Fig. 1A), sharing high homology (77.34%–91.34%) in amino acid sequences with cytosolic G6PDH from other plant species (see Fig. S1). In addition, according to the phylogenetic tree (see Fig. 1B), ScG6PDH was classified as part of the cytosolic G6PDH family and bearing the closest relationship to cytosolic OsG6PDH (Accession number: CAC09489) (http://www.ncbi.nlm.nih.gov/prote in/CAC09489). This agrees with our prediction of no signal peptide or transmembrane, indicating that the ScG6PDH presented here may belong to the cytosolic isoform. Based on the subcellular location results of the recombinant protein that exists in the cytoplasm, we suggest that ScG6PDH belongs to the cytosolic G6PDH family.

In recent years, studies on G6PDH have focused on its response to abiotic stresses. Transgenic tobacco lines carrying *PsG6PDH* from poplar exhibited an increasing cold tolerance compared with wild plants, and a previous hypothesis that increased cytosolic G6PDH activity might involve the induction of freezing resistance in poplar cuttings was also confirmed. The overexpression of Cvcg6pdh from Chlorella vulgaris can lead to improved freezing tolerance in Saccharomyces cerevisiae, indicating that G6PDH would be an indirect factor in the development of freezing tolerance. In the present study, the *ScG6PDH* transcript level was significantly increased when sugarcane was subjected to 4 °C stress. Consistent with these previous reports, G6PDH activity increased at a protein level, indicating that *ScG6PDH* may be involved in the response to cold stress in sugarcane.

In comparison, reports on the role of the G6PDH gene in response to metal stress are limited. Based on a previous study, when the metal (zinc and cadmium) content of bean leaves exceeded a toxic threshold value, an increase in the activity of the G6PDH enzyme was measured. Moreover, a rapid increase in G6PDH activity in aluminum-resistant wheat cultivars was observed during the first 10 h of treatment with 100 μM aluminum, while no change was detected in sensitive cultivars during 24 h exposure, suggesting that G6PDH may be associated with the mechanism of aluminum tolerance through regulation of the OPPP. In addition, changes in G6PDH transcript levels were investigated, showing a 43-fold increase after...
12 h exposure to CdCl2 treatment compared with the control; these high levels were maintained until 24 h. In our study, the G6PDH activity also showed an increase and reached a maximum level after 12 h. Further, the higher transcript levels (in comparison to the control) were maintained for the duration of the investigation period, implying possible involvement of G6PDH in response to metal stress in sugarcane, similar to previous research.

Several studies have shown that G6PDH plays a key role in resistance to salt stress. For example, G6PDH played a role in maintaining cell redox balance in rice suspension cells under salt stress, indicating the coordination of G6PDH and NADPH oxidase\(^4\). In Phragmites communis Trin subjected to salt stress, G6PDH played a crucial role in maintaining the glutathione (GSH) pool\(^21\). In addition, in wheat treated with high salt levels, there was a significant increase in either the G6PDH transcript or its enzymatic activity\(^13\). However, in salt-stressed Arabidopsis, the G6PDH transcript was down-regulated based on a global analysis of expression profiles\(^22\). Interestingly, in all rice plants treated with high salinity, cold temperatures, PEG or abscisic acid (ABA) under experimental conditions, the transcript of the G6PDH gene remained constant, unlike the G6PDH genes in wheat (up-regulated) and Arabidopsis (down-regulated). Therefore, the role of G6PDH in salt stress tolerance is unclear based on the distinct expression patterns in different plant species. In the current study, we performed RT-qPCR to investigate the ScG6PDH expression pattern in sugarcane subjected to salt stress. At 12 h after treatment, there were indications of up-regulation of the transcript of the ScG6PDH gene (see Fig. 5), which is a similar response to the G6PDH gene in wheat. In addition, a distinct increase in the ScG6PDH enzymatic activity measured at 24 to 48 h, and which corresponded to the transcript of the ScG6PDH gene under salt treatment, implied a positive response to salt stress in sugarcane.

Drought is a common abiotic stress limiting plant growth and crop production. To date, many reports have shown the positive role of the G6PDH gene to drought stress. G6PDH could provide GSH and ascorbate by utilizing NADPH to participate in the regulation of root development after drought stress. This was recently confirmed in Glycine max roots subjected to drought stress\(^23\). Further, the role of G6PDH in the cytosol as a key factor in determining plant development, drought stress and early pathogen defense has been demonstrated\(^9\). In sugarcane, we observed an increase in both the ScG6PDH transcript and its enzymatic activity after drought stress (stimulated by PEG) (see Fig. 5, Fig. 6), which was consistent with the G6PDH increase in soybean roots\(^24\), indicating the likely involvement of the ScG6PDH gene in drought stress in sugarcane.

It has been shown that reactive oxygen species (ROS) accumulate under unfavourable conditions that include cold temperatures, salt
and drought\textsuperscript{21,24,25}. Lower levels of ROS could be related to signal transduction pathways, while high levels of ROS accumulation may affect cell redox states and result in oxidative damage\textsuperscript{26}. Plant defense reactions under oxidative stress rely on the balance between ROS production and scavenging. There are two efficient antioxidative defense systems in plants to scavenge excessive ROS. One system is composed of enzymes (e.g., SOD, catalase (CAT), ascorbate peroxidase (APX) and POD) involved in hydrogen peroxide and superoxide radical scavenging; the other system is comprised of non-enzymatic antioxidants, including a variety of secondary metabolites such as ascorbate and GSH. These systems maintain appropriate oxidative and reductive states in plants exposed to various

**Figure 6** | Changes of G6PDH activity in seedlings subjected to 500 µM CdCl\textsubscript{2}, low temperature (4°C), 250 mM NaCl and 25% PEG. Data points represent the means ± SE (n = 3).

**Figure 7** | The transient expression of ScG6PDH in tobacco. (A): Ion conductivity to assess the cell death response in leaf discs infiltrated by Agrobacterium tumefaciens strain EHA105 for 48 h (n = 5). (B): DAB staining of ScG6PDH in Nicotiana benthamiana leaves infiltrated by Agrobacterium tumefaciens strain EHA105 for 48 h to assess H\textsubscript{2}O\textsubscript{2} production (n = 5). Images were captured using a Canon camera.
types of stress25,27–29. NADPH, which is an important reductant molecule, is required in the ascorbate-glutathione cycle involved in oxidative stress3. This molecule plays a vital role in maintaining the GSH content in cells. Therefore, NADPH is considered as the most important molecule to determine the potential antioxidant capacity of the cell. Furthermore, the OPPP, with G6PDH as the key enzyme, is the major source of NADPH production. When plants suffer oxidative stress, more NADPH is required to maintain a normal redox state. This may result in an increase of G6PDH, which was confirmed by our results. Therefore, we can conclude that G6PDH plays a vital role in various oxidative stresses by supplying sustainable levels of NADPH to maintain oxidative-reductive balance.

Our conclusion of the role of G6PDH in abiotic stresses was also supported by the ion conductivity and DAB staining results. Hypersensitive response cell death can restrict pathogen growth as well as stimulate the defense reaction of nearby tissues and systemic acquired resistance31,32 of the entire plant. Here, DAB staining showed a deep brown color in the presence of H2O2 in tobacco leaves after 48 h infiltration, and increased electrolyte leakage, suggesting the ScG6PDH gene was associated with HR cell death, which is consistent with a previous report33. The production of H2O2 through oxidative burst has been reported to be closely connected with HR cell death34. We deduce that the transient over-expression of the ScG6PDH gene may be related to HR, providing indirect evidence that ScG6PDH is involved in the plant defense reaction.

Wend et al. ever demonstrated that in contrast with the plasticic P1 isoform, which mRNA amount was accumulated in the leaves, stolons harvested from tissue growing above ground and roots harvested from tissue growing hydropionically in northern blots in potato3, the P2 was also expressed ubiquitously more or less by stolons harvested from tissue growing above ground and roots harvested from tissue growing above ground and roots harvested from tissue growing hydropionically in northern blots in potato3. Our conclusion of the role of G6PDH in abiotic stresses was also supported by the ion conductivity and DAB staining results. Hypersensitive response cell death can restrict pathogen growth as well as stimulate the defense reaction of nearby tissues and systemic acquired resistance31,32 of the entire plant. Here, DAB staining showed a deep brown color in the presence of H2O2 in tobacco leaves after 48 h infiltration, and increased electrolyte leakage, suggesting the ScG6PDH gene was associated with HR cell death, which is consistent with a previous report33. The production of H2O2 through oxidative burst has been reported to be closely connected with HR cell death34. We deduce that the transient over-expression of the ScG6PDH gene may be related to HR, providing indirect evidence that ScG6PDH is involved in the plant defense reaction.

Methods

Materials. The sugarcane variety Yacheng05-179 was provided by the key laboratory of Sugarcane Biology and Genetic Breeding, Ministry of Agriculture (Fuzhou, China). The Escherichia coli rosette, prokaryotic expression vector PET28a, the subcellular localization vector pCAMBIA 2300, and the plant expression vector pCAMBIA 1301 were obtained from Abmart, Inc. (Tokyo, Japan). The restriction enzymes SacI, Xhol, Nhel, T4 DNA ligase, Ex-Tag enzyme, PrimeScript RT-PCR Kit, TaKaRa LA PCR System, QIAquick Plasmid Purification Kit, DNA and protein molecular marker were purchased from TaKaRa (Tokyo, Japan). RQ1 RNase-Free DNase was purchased from Promega Corporation (Beijing, China), the SYBR Green PCR Master Mix Kit was provided by Roche (Shanghai, China), and the NADPH2 and D-glucose 6-phosphate disodium salt were purchased from Sigma (San Francisco, CA, USA).

Cloning and sequence analysis of the ScG6PDH cDNA. Using the cDNA library of sugarcane variety Yacheng05-179, the sugarcane EST database was searched. The sugarcane EST database was searched and the data were compared. The same operations were carried out using a new established EST database and a new established EST database. The ESTs were obtained from the sugarcane EST database. These ESTs were clustered and spliced. Then, the sugarcane ESTs were predicted and used as a new EST database. The ESTs were used to identify a new EST database. Using this method, a putative novel gene sequence was obtained. In order to isolate this gene from sugarcane, the primers ScG6PDH-F: 5’TCTCGTCCTGGGCGAGAATCTCG-3‘ and ScG6PDH-R: 5’AGGGACTACATCC TAGATAGTAGAT-3‘ were designed according to the assembled sequence. The 25 μL PCR system contained 2.5 μL 10X PCR buffer, 2.0 μL deoxynucleotide triphosphates (dNTPs, 2.5 mM), 1.0 μL each of forward and reverse primers (10 μM), 2.0 μL cDNA of the sugarcane variety Yacheng05-179 (100 ng·μL−1), 0.125 μL Ex-Tag enzyme (5.0 U·μL−1) and 17.375 μL ddH2O. The PCR program included pre-denaturation for 4 min at 94 ºC, denaturation for 30 s at 94 ºC, annealing for 45 s at 58 ºC, extension for 90 s at 72 ºC, for 35 cycles, and final extension for 10 min at 72 ºC.

The open reading frame (ORF) of the full-length cDNA sequence of the ScG6PDH gene was predicted using the ORF Finder online tool (http://www.ncbi.nlm.nih.gov/projects/orflnfl). The signal 4.1 server program (http://www.cbs.dtu.dk/services/SignalP/) and TMHMM server 2.0 software (http://www.cbs.dtu.dk/services/TMHMM-2.0/) were used to analyze signal peptide and transmembrane segments. The homology alignment among the ScG6PDH and other G6PDHs was performed using DNASIS software. The phylogenetic tree of the deduced amino acid sequences of G6PDHs was constructed according to the neighbor-joining method using 4.1 MEGA software.

Subcellular localization. The ScG6PDH gene was sub-cloned with SacI and SalI sites into the pCAMBIA 2300 vector, and then transformed into the Agrobacterium tumefaciens strain EHA105. First, the EHA105 cells were inoculated into LB medium containing kanamycin (50 μg·mL−1) and rifampicin (34 μg·mL−1), shaken overnight at 200 rpm and 28 ºC, and then 1.0 mL LB medium was inoculated into a new LB medium containing kanamycin (50 μg·mL−1) and rifampicin (34 μg·mL−1). After culturing at 28 ºC with shaking (200 rpm) for approximately 8 h, the OD600 of the culture was measured and diluted to OD600 = 0.8 using Murashige and Skoog (MS) liquid medium (containing 200 μM acetoxyresorcinol). A syringe was used to infiltrate the diluted bacterial suspension into tobacco leaves. Injected plants were cultured under 12 h light/12 h dark photoperiod. After two days, the leaves were

The expression of G6PDH in sugarcane (Accession number: EF100284317) as the query probe, several highly homologous sugarcane expressed sequence tags (ESTs) were obtained from the sugarcane EST database. These ESTs were clustered and spliced. Then, the sugarcane ESTs were predicted and used as a new EST database. The ESTs were used to identify a new EST database. Using this method, a putative novel gene sequence was obtained. In order to isolate this gene from sugarcane, the primers ScG6PDH-F: 5’TCTCGTCCTGGGCGAGAATCTCG-3‘ and ScG6PDH-R: 5’AGGGACTACATCC TAGATAGTAGAT-3‘ were designed according to the assembled sequence. The 25 μL PCR system contained 2.5 μL 10X PCR buffer, 2.0 μL deoxynucleotide triphosphates (dNTPs, 2.5 mM), 1.0 μL each of forward and reverse primers (10 μM), 2.0 μL cDNA of the sugarcane variety Yacheng05-179 (100 ng·μL−1), 0.125 μL Ex-Tag enzyme (5.0 U·μL−1) and 17.375 μL ddH2O. The PCR program included pre-denaturation for 4 min at 94 ºC, denaturation for 30 s at 94 ºC, annealing for 45 s at 58 ºC, extension for 90 s at 72 ºC, for 35 cycles, and final extension for 10 min at 72 ºC.

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collected and the subcellular localization was observed using laser scanning confocal microscopy (Leica, Wetzlar, Germany). The protoplast isolation and transient expression of ScG6PDH-GFP fusion gene were also carried out. Choose well-expanded leaves from 4-week-old tabacco plants before fixing. Cut 1 mm leaf strips from the middle part of a leaf using a fresh sharp razor blade without tissue crushing at the cutting site. The leaf strips were transferred quickly into a 0.1 m NaCl, 0.1% w/v gelatin solution containing 20 mM Mes-KOH (pH 5.7, 3 mM MgCl2, 1 mM ethylene diamine tetraacetic acid (EDTA)), 1 mM phenylmethanesulfonyl fluoride, and 1 mM diithiothreitol. The homogenates were centrifuged (12,000 rpm) at 4°C for 20 min. Then, a 100-µl aliquot of extract was added to the assay buffer containing 50 mM Hepes-Tris (pH 7.8), 3.3 mM MgCl2, 0.5 mM-D-glucose 6-phosphate and 0.5 mM NADP+, and the reduction of NADP to NADPH was measured as the change rate of the absorbance at 340 nm for the initial 5 min. One unit (U) of enzyme activity was defined as the amount of enzyme that increased a 0.01 of absorbance at 340 nm per minute under the assay condition. Significant differences in gene expression levels were assessed using Student’s t-test (p-value < 0.05).

**Ion conductivity measurement.** Cell death was quantified by measuring ion leakage as described previously. Ion conductivity was determined by washing six discs/ leaves (10 mm diameter) in 20 mL of double distilled water followed by incubation without gentle shaking for 1 h at room temperature. Conductivity was measured using a conductivity meter (Mettler Toledo, Shanghai, China). Five biologic replications were carried out.

**Histochemical assay.** DAB was used to stain H2O2-producing leaves. The leaves were incubated in DAB solution (1.0 mg mL⁻¹, pH = 5.8) overnight (dark conditions). Then, the leaves were placed into 95% alcohol and treated with boiling water for 5 min until the green color faded. The leaves were rinsed in 95% alcohol, photographed and examined. Five biologic replications were carried out.

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Author contributions

Conceived and designed the experiments: Y.Y. and L.X. Performed the experiments: Y.Y., Z.F., Y.S., X.Z. and G.L. Analyzed the data: Y.Y., Z.F. and Y.S. Wrote the paper: Y.Y. and L.X. Revised the final version of the paper: L.X., J.G. and Y.Q. Approved the final version of the paper: L.X.

Additional information

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