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ORIGINAL RESEARCH PAPER

Functional analysis of *PsG6PDH*, a cytosolic glucose-6-phosphate dehydrogenase gene from *Populus suaveolens*, and its contribution to cold tolerance improvement in tobacco plants

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Abstract A 1,697-bp cDNA sequence, designated as *PsG6PDH*, was amplified from *Populus suaveolens*. Multiple sequence alignment and phylogenetic analysis indicated that *PsG6PDH* encodes a cytosolic G6PDH isoform, with Southern blot analysis demonstrating that the gene is single or low copy in *Populus*. Transgenic tobacco plants over-expressing *PsG6PDH* exhibited enhanced cold tolerance. In both transgenic and wild-type (WT) tobacco plants, cold stress increased leaf malondialdehyde (MDA) content, electrolyte leakage (EL), and peroxide (POD) and superoxide dismutase (SOD) activities; relative to WT, however, transgenic lines had lower MDA content and EL and higher SOD and POD activities. In addition, *PsG6PDH* activated the expression of stress-related genes, including *NtERD10b*, *NtERD10c*, and *NtSOD*, in tobacco plants. Our results provide evidence regarding *PsG6PDH* regulatory function in plants during low temperature stress.

Keywords *Populus suaveolens* · Glucose-6-phosphate dehydrogenase · Oxidative pentose phosphate pathway · Cold resistance

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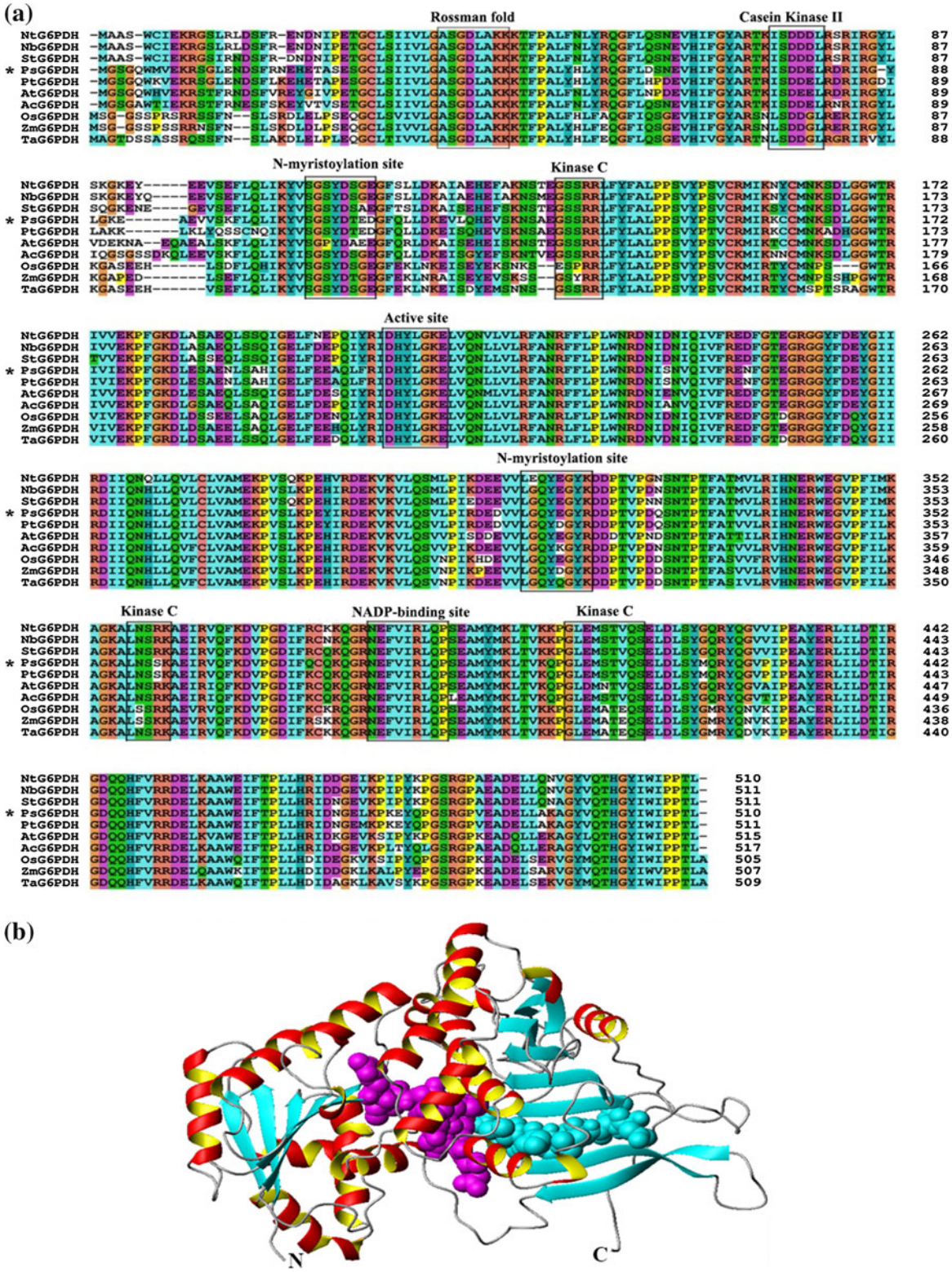
Introduction

Glucose-6-phosphate dehydrogenase (G6PDH; EC1.1.1.49) is the first and primary regulatory enzyme of the oxidative pentose phosphate pathway (OPPP). It catalyzes the conversion of glucose 6-phosphate to 6-phosphogluconolactone, and is ubiquitous in prokaryotes, animals, and plants. Because the main physiological function of G6PDH is to provide NADPH for anabolic pathways, including fatty acid synthesis, nitrite reduction, and glutamate synthesis (Dennis et al. 1997), it plays important roles in plant growth and developmental processes. Numerous studies have been carried out regarding G6PDH functionality in response to biotic and abiotic stresses such as metal stress (Slaski et al. 1996), salt stress (Nemoto and Sasakuma 2000), pathogen infection (Ludek and Lenka 1999), drought stress (Scharte et al. 2009), nutrient starvation (Esposito et al. 2005), reactive oxygen species production and cell death response (Asai et al. 2011), and ABA response (Cardi et al. 2011). Recent analysis has provided further evidence for functional roles of one cytosolic (Cyto) and two plastidic (P1 and P2) isoforms, demonstrating that


(a)
Rossman fold
Casein Kinase II
N-myristoylation site
Kinase C
Active site
N-myristoylation site
Kinase C
NADP-binding site
Kinase C
NtG6PDH
NbG6PDH
StG6PDH
* PsG6PDH
PtG6PDH
AtG6PDH
AcG6PDH
OsG6PDH
ZmG6PDH
TaG6PDH
(b)
N
C

◀ **Fig. 1** Multiple sequence alignment and predicted tertiary structure of PsG6PDH protein. **a** Multiple alignment of the deduced amino acid sequences from plant cytosolic G6PDH cDNAs. The deduced amino acid sequence of *P. suaveolens* (GenBank aceession number: AY445917) was aligned with those of *A. thaliana* (AJ010971), *M. sativa* (MSU18238), *N. tabacum* (AJ001769), *O. sativa* (AY078072), *S. tuberosum* (AB029454) and *T. aestivum* (AB029454). **b** The predicted tertiary structure of PsG6PDH protein. *Red spaceball helices area* NADP-binding domain; *blue spaceball sheet area* substrate-binding domain

each isoform appears to have a different regulatory mechanism and plays a distinct role in development and resistance to various stresses (Wakao and Benning 2005). Of particular interest, low temperatures induce increases in G6PDH activity and OPPP capacity (Lin et al. 2005; Honjoh et al. 2007); this increased G6PDH activity may provide sustainable levels of NADPH for continued biosynthesis (Kruger and Schaewen 2003). In woody plants under cold stress, however, the molecular role of G6PDH isoforms is still poorly understood.

Populus suaveolens (poplar), a freeze-resistant arborescent plant from the Great Xing'an Mountains of northeastern China, is an ideal material with which to study mechanisms of freezing resistance in woody plants. In an earlier study, we found that freezing acclimation in poplar seedlings increased SOD, POD, and cytosolic G6PDH activities and decreased MDA content (Lin et al. 2005). Changes in cytosolic G6PDH activity were closely correlated with variations in SOD activity, POD activity, MDA content, and degree of freezing resistance. In this present study, a glucose-6-phosphate dehydrogenase gene (*PsG6PDH*) was isolated from *P. suaveolens* and transformed into tobacco plants for functional profiling. Transgenic tobacco plants containing *PsG6PDH* were subsequently subjected to low temperature treatments, and an ion leakage assay was performed. In addition, SOD and POD activities and MDA content were measured. Finally, *NtSOD*, *NtERD10b*, and *NtERD10c* transcription levels were analyzed by real-time quantitative RT-PCR (qRT-PCR).

Materials and methods

Plant material

Perennial graft cuttings of *Populus suaveolens* were collected as explants from the Nursery of Forestry Genetic and Tree Breeding, Beijing Forestry University, China. Explants were sanitized and cultured on modified MS medium supplemented with 0.5 mg BA l^{-1} and 0.1 mg NAA l^{-1} for 25 days at 25 °C under cool fluorescent light illumination for 16 h per day. Shoots regenerated in vitro were obtained from propagated buds of explants and subcultured on modified MS medium supplemented with 0.1 mg IBA l^{-1} and 0.1 mg NAA l^{-1} for rooting. Young rooting plantlets at the 4–6 leaf stage were used in the experiments.

DNA and RNA extraction and first-strand cDNA synthesis

Genomic DNA was extracted from mature leaves of poplar or tobacco rooting plantlets with a Plant Genomic DNA kit (Tiangen, Beijing, China). Total RNA was extracted from leaf samples using an RNAprep Pure Plant kit (Promega, Madison, WI, USA), and treated with RNAse-free DNAseI to eliminate residual genomic DNA. Genomic DNA and total RNA were then evaluated by agarose gel electrophoresis and spectrophotometric analysis. Total RNA was used to generate first-strand cDNA using a PrimeScript RT Reagent kit (NEB Biolabs, Beijing, China).

Isolation of the *Populus* G6PDH gene *PsG6PDH*

The full-length *PsG6PDH* cDNA sequence was obtained by PCR using the first-strand cDNA as a template and primers 5′-TCATGGATCCGGTTACAGGACCTCTG AGAAACA-3′ and 5′-GCTCAAGCTTCAGGCTACA TTTCTACAATGTAGGAGG-3′ (added restriction sites *Bam*HI and *Sac*I, respectively, are underlined). The resulting PCR product was cloned into a pGEM-T vector (Promega, Madison, WI, USA) and sequenced.

Computer-assisted analysis

The online program Compute pI/Mw (http://web.expasy.org/compute_pi/) was used to predict the theoretical isoelectric point (pI) and molecular weight (MW) of the PsG6PDH protein. PsG6PDH and plant homologue G6PDH amino acid sequences retrieved from the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/) were aligned using CLUSTALX1.83 software. The online bioinformatics server of the Swiss Institute of Bioinformatics (http://cn.expasy.

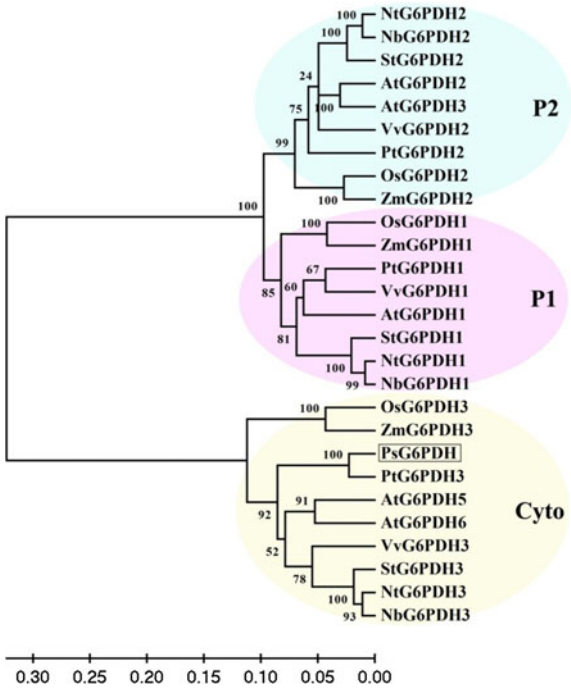


Fig. 2 Phylogenetic tree of the plant G6PDH proteins and their homologs. *P1*, *P2* plastidic isoform. *Cyto* cytosolic isoform. The G6PDH proteins and their homologs employed were as follows: *A. thaliana* AtG6PDH1 (GenBank aceession number: Q43727), AtG6PDH2 (Q9FY99), AtG6PDH3 (Q8L743), AtG6PDH5 (Q9FJI5), AtG6PDH6 (Q9LK23); *N. benthamiana* NbG6PDH1 (BAK22408), NbG6PDH2 (BAK22409), NbG6PDH3 (BAK22 407); *N. tabacum* NtG6PDH1 (CAA04994), NtG6PDH2 (AAF8 7216), NtG6PDH3 (CAA04992); *O. sativa* OsG6PDH1 (AAQ0 2671), OsG6PDH2 (AAL79959), OsG6PDH3 (AAS07054); *S. tuberosum* StG6PDH1 (CAA58775), StG6PDH2 (CAB52708), StG6PDH3 (CAA52442); *P. suaveolens* PsG6PDH (AAR26303), *P. trichocarpa* PtG6PDH1 (ABC74526), PtG6PDH2 (ABC74 527), PtG6PDH3 (EEE82659); *Vitis vinifera* VvG6PDHH1 (XP_ 002276987), VvG6PDH2 (CBI38149), VvG6PDH3 (XP_00226 6527); *Zea mays* ZmG6PDH1 (ACN36479), ZmG6PDH2 (ACG39 996), ZmG6PDH3 (ACG29334)

org/) was employed to predict tertiary structure. Phylogenetic trees were constructed using the neighbor-joining method with 1,000 bootstrap replicates as implemented in MEGA version 4.1.

Southern blot analysis

Poplar genomic DNA was subjected to overnight digestion with restriction enzymes *Xba*I, *Hind*III, *Eco*RI, and *Bam*HI (Promega) at 37 °C. After electrophoresis on a 1 % agarose gel, the DNA was blotted onto a Hybond-N^+ nylon membrane using a standard protocol. A 1,000-bp DNA fragment identical to the 3′- end of the *PsG6PDH* genomic sequence was PCR-amplified using the primer pair 5′-GGAGAGTTGTTTGAAGAAGCAC-3′ and 5′-AGGCGGCCGCGAATTCACTAGTG-3′; the amplified product was excised from the gel and purified using a Qiaex II gel extraction kit (Qiagen, Valencia, CA, USA), and then labeled using a PCR DIG Probe Synthesis kit (Roche, Mannheim, German). Hybridization was performed using a DIG-High Prime DNA Labeling and Detection Starter II kit (Roche).

Vector construction

The full-length ORF of the *PsG6PDH* gene was PCR-amplified from a *PsG6PDH* cDNA clone. The resulting PCR product was digested with *Bam*HI and *Sac*I (Promega) and purified with a QIAquick Gel Extraction kit (Qiagen, Hilden, Germany). This purified product was then fused to the CaMV 35S promoter sequence of a pBI121 vector (Clontech, USA) that had been previously digested with *BamHI* and *SacI* to release the *GUS* reporter gene. The resulting vector was designated as *pBI121- PsG6PDH*.

Plant transformation

Tobacco leaf disc transformation was performed using the *Agrobacterium*-mediated method. After selection of T_0 and T_1 generation transgenic lines on 100 mg kanamycin l^{-1}, putative transgenic plantlets were confirmed by RT-PCR. The verified transgenic tobacco plants were then propagated and synchronized (using vegetative stem cutting containing an axillary bud) from primary transformants in MS medium. The 30 day old, in vitro-grown T1 plantlets were transferred to soil and cultivated in a growth chamber at 25 °C under a 16/8 h light/dark photoperiod for 1 month, and then used for subsequent cold tolerance assays and gene expression analyses.

Cold-resistance assay of transgenic tobacco plants

Cold treatment experiments were carried out on transgenic and wild-type plants in a DP-200 low-temperature biochemical culture apparatus. Two cold conditions were tested: (1) no cold acclimation, with the ambient temperature directly reduced from 25 to 4 °C; and (2) cold acclimation in which the ambient

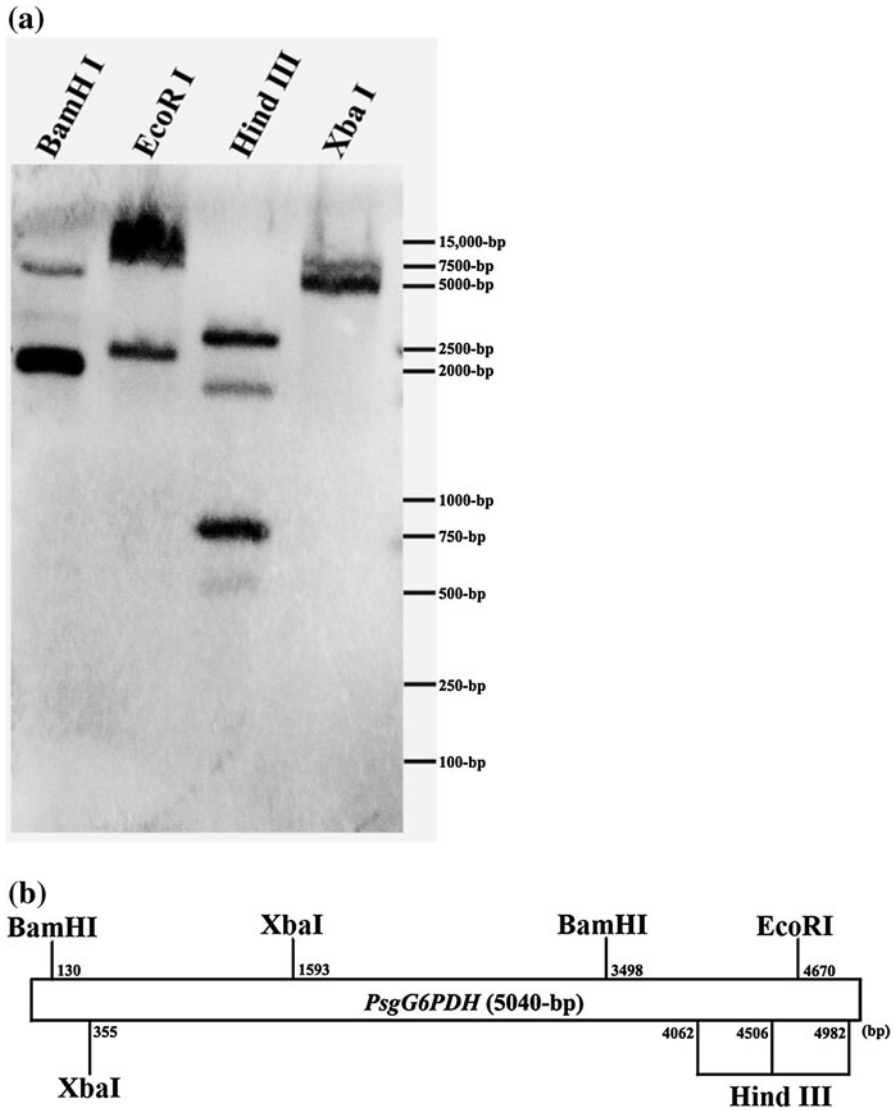


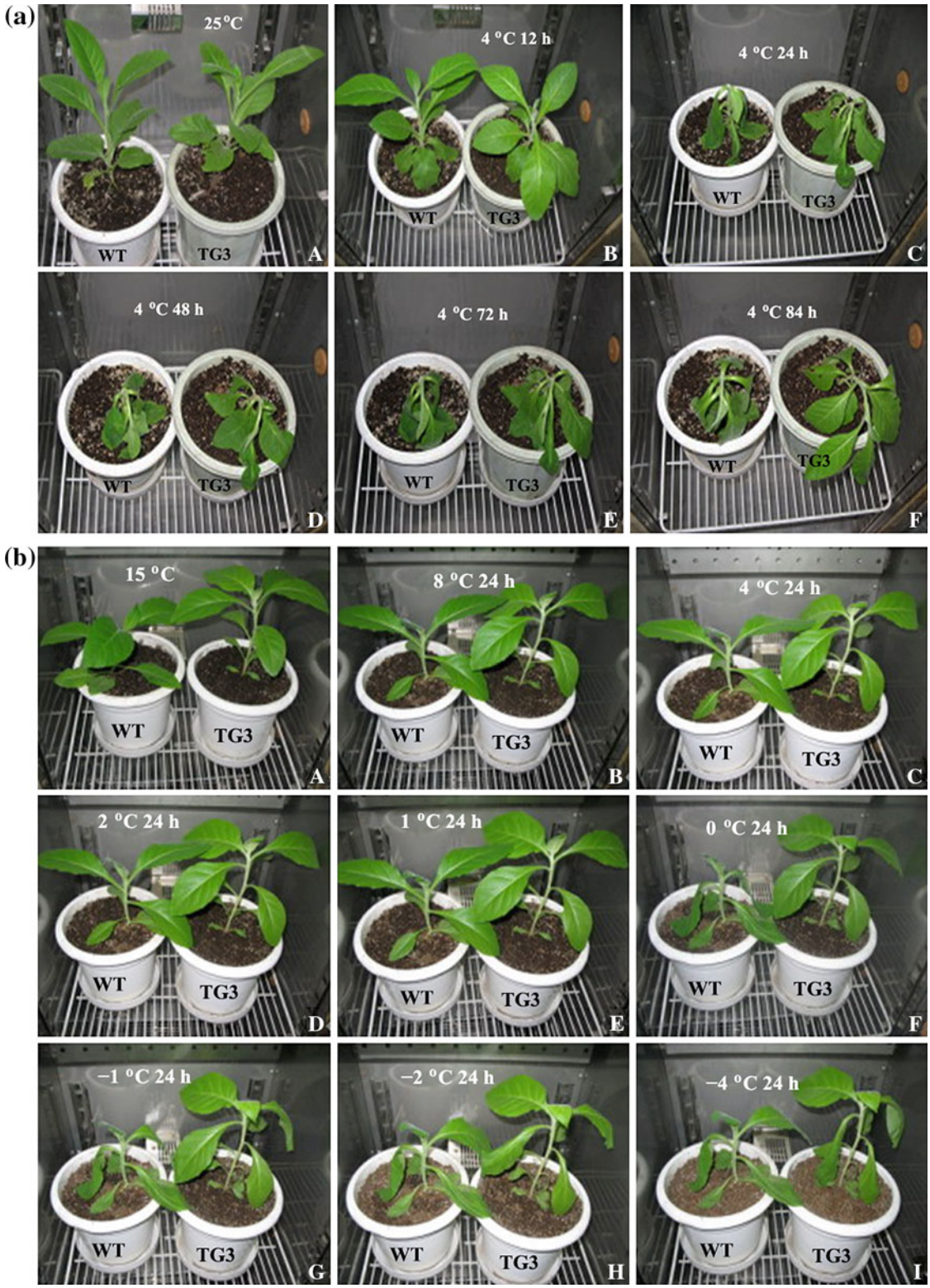
Fig. 3 **a** Southern analysis of *PsG6PDH* gene with *P. suaveolens* genome. The reference molecular weights are marked in bp on the *right*. **b** Restriction map of *PsG6PDH* genomic DNA sequence. Total length was 5,040 bp

temperature was gradually decreased from 15 to −4 °C. A 16/8 h light/dark photoperiod was used.

An electrolyte leakage assay was performed using the method described by Lin et al. (2005). After exposure to different cold temperatures (4, 0, −2, or −4 °C) for 2 h, 10 leaf disc samples (5 mm diam. by stiletto) were washed and immersed in 5 ml deionized water. Following vacuum infiltration, electrical conductivity of the supernatant (S1) was measured with a SevenEasy-S30 detector (Mettler, Switzerland). Samples were then boiled to measure ultimate maximum conductivity (S2). Electrolyte leakage (EL) was calculated from the ratio S1/S2.

Determination of G6PDH, SOD, and POD activities and MDA content

Two-month-old tobacco plantlets were cold-acclimated at 15 °C for 3 days, and then treated at 4 °C for 1, 3, or 5 days. To determine G6PDH, SOD, and POD activities and MDA content, 1 g leaves was ground in extraction buffer (Lin et al. 2005). One unit of G6PDH enzyme activity was defined as the amount of enzyme giving a 0.01 increase in A_{340} per min under G6PDH enzyme assay conditions (Lin et al. 2005). One unit of SOD activity was defined as the amount of enzyme inhibiting photochemical reduction of Nitrotetrazolium Blue


(a)
25°C
WT
TG3
A
4 °C 12 h
WT
TG3
B
4 °C 24 h
WT
TG3
C
4 °C 48 h
WT
TG3
D
4 °C 72 h
WT
TG3
E
4 °C 84 h
WT
TG3
F
(b)
15 °C
WT
TG3
A
8 °C 24 h
WT
TG3
B
4 °C 24 h
WT
TG3
C
2 °C 24 h
WT
TG3
D
1 °C 24 h
WT
TG3
E
0 °C 24 h
WT
TG3
F
−1 °C 24 h
WT
TG3
G
−2 °C 24 h
WT
TG3
H
−4 °C 24 h
WT
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I

◀ **Fig. 4** Cold treatment experiment on wild type tobacco (*WT left*) and transgenic tobacco (*TG3 right*). **a** Without cold-acclimation. *A* Before cold treatment (25 °C), *B–F* cold treatment at 4 °C for 12, 24, 48, 72, 84 h, respectively. **b** With cold-acclimation. *A* Before cold treatment (15 °C), *B–I* cold treatment for 24 h at 8, 4, 2, 1, 0, −1, −2, −4 °C, respectively

chloride (NBT) by 50 % (Lin et al. 2005). One unit of POD activity was defined as the amount of enzyme causing a 0.01 increase in A_{470} per min under assay conditions (Lin et al. 2005). MDA content was measured by the thiobarbituric acid reaction according to Lin et al. (2005). All determinations were performed three times.

Real-time quantitative RT-PCR analysis of downstream genes activated by PsG6PDH

Real-time quantitative RT-PCR (qRT-PCR) assays were conducted according to Lin et al. (2013). To quantify expression of target genes [*PsG6PDH* (GenBank accession number: AY445917), tobacco *NtSOD* (X14482), *NtERD10b* (AB049336), and *NtERD10c* (AB049337)], the tobacco *ACTIN* gene (U60491) was used as an endogenous control. qRT-PCR primers were as follows: *PsG6PDH* forward primer 5′-CGGTCAATGGATGGTAGAG-3′ and reverse primer 5′-GGGAAAGTCTTCTTCTTGGC-3′; tobacco *NtSOD* forward primer 5′-AGTGAGCAGACGGACCTTAG-3′ and reverse primer 5′-TGGTGATTCTGGTGGTGGAG-3′; tobacco *NtERD10b* forward primer 5′-CACTGGCATACTTGGTGGGGA-3′ and reverse primer 5′-AGTTGAATGAGTCTGATCGTC-3′; tobacco *NtERD10c* forward primer 5′-GAAGGTTGAAGAGGGTAGCG-3′ and reverse primer 5′-AACAAACTCAGAGGAAATAG-3′; tobacco *NtACTIN* forward primer 5′-CTGCTGGAATTCACGAAACA-3′ and reverse primer 5′-GCCACCACCTTGATCTTCAT-3′. Each PCR assay consisted of three biological replicates, with each assay technologically replicated twice separately.

Results and discussion

Identification of the *Populus suaveolens* G6PDH gene

The sequenced *PsG6PDH* gene was 1,697 bp in length, and was predicted to encode a protein comprising 510 amino acids with a calculated MW of 58,439 and a predicted pI of 5.88. Two canonical G6PDH sequence motifs found in PsG6PDH, the substrate-binding site (RIDHYLG) and the NADP-binding site (NEFVIRLQP), were similar to those of G6PDH from *Solanum tuberosum*, *Medicago sativa*, *Nicotiana tabacum*, *Triticum aestivum*, *Oryza sativa*, and *Arabidopsis thaliana* (Fig. 1a). Tertiary structure prediction indicated that there was one β-sheet domain at both *N* and *C* terminals, containing six β-sheets and nine β-sheets, respectively, and that the two β-sheet domains were connected by 24 α-helixes, which were mainly distributed near the *N*-terminal (Fig. 1b).

Phylogenetic analysis of PsG6PDH and its homologues classified PsG6PDH into a group of G6PDH cytosolic isoforms previously identified by Wakao and Benning (2005) (Fig. 2). We also used the software programs ChloroP and TargetP to search for a transit peptide in the translated protein and to predict the subcellular localization of PsG6PDH. The translated protein of *PsG6PDH* was predicted to be a cytosolic isoform, consistent with results of the phylogenetic analysis. These characteristics suggest that the gene of interest, *PsG6PDH*, encodes a cytosolic isoform of G6PDH. The Southern blot of genomic DNA displayed strongly hybridizing bands with *Bam*HI, *Eco*RI, *Hin*dIII, and *Xba*I digests (Fig. 3a). The *PsG6PD*H genomic DNA probe strongly hybridized with two *Bam*HI, two *Eco*RI, three *Hin*dIII, and two *Xba*I fragments, corresponding to expected patterns based on restriction mapping of *PsG6PD*H genomic DNA (Fig. 3b). There were a few faint bands on the blot, suggesting that *PsG6PDH* is present in the genome in single or low copies, similar to the situation in wheat (Nemoto and Sasakuma, 2000), tobacco (Knight et al. 2001) and *Chlorella vulgaris* (Honjoh et al. 2007).

Cold resistance in transgenic tobacco plants

To study cold stress response of the transgenic lines, two different cold treatment experiments were conducted on different groups of wild-type (WT) and T1 generation transgenic tobacco plants. Before cold treatment, no visible phenotypic differences were observed between transgenic lines and WT. In the first experiment, in which no cold acclimation was applied, WT plants suffered earlier and recovered later from cold injury compared with transgenic lines (Fig. 4a).

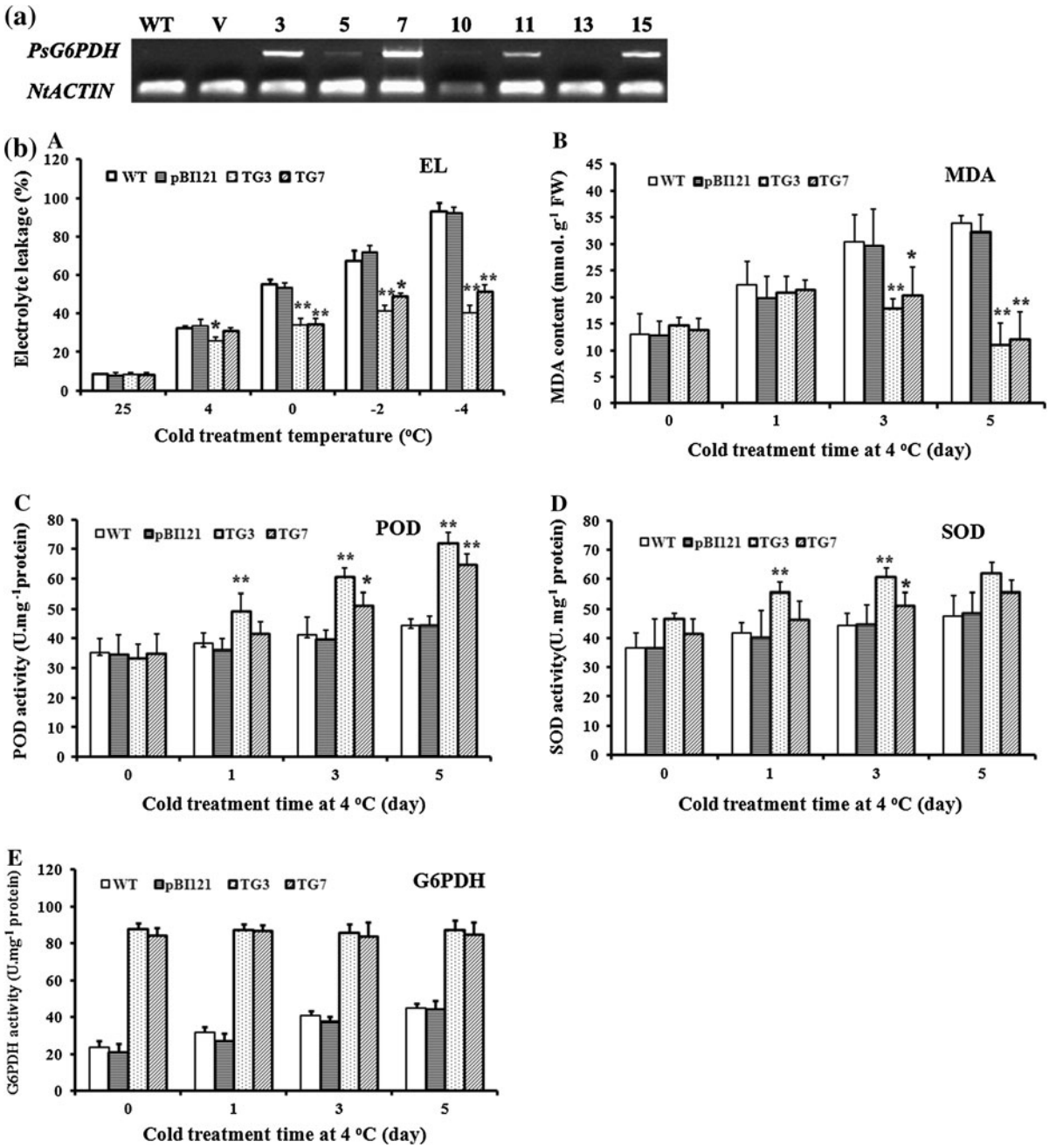

Fig. 5 Biochemical Changes displayed by transgenic plants. **a** Semi-quantitative RT-PCR analysis of *PsG6PDH* expression. *WT* wild-type tobacco lines, *V* tobacco transformed by vector pBI121; 3,5,7,10,11,13,15: transgenic tobacco lines. **b** Effects of *PsG6PDH* over-expression in tobacco plants under cold stress. *A* Variation of electrical conductivity of tobacco leaves after serial treatments at 25, 4, 0, −2, −4 °C, respectively. *B–E* MDA content, SOD activity, POD activity and G6PDH activity of wild type and transgenic tobaccos during cold treatment at 4 °C for 0, 1, 3, 5 days, respectively. *WT* wild-type tobacco lines, *TG3*, *TG7* Transgenic tobacco lines. pBI121: tobacco transformed by vector pBI121. Data was mean with standard deviations obtained from three independent biological experiments. The significance of the differences in gene expression levels between transgenic and wild-type tobacco plants was assessed using Student's t test (**$P < 0.01$ and *$P < 0.05$)

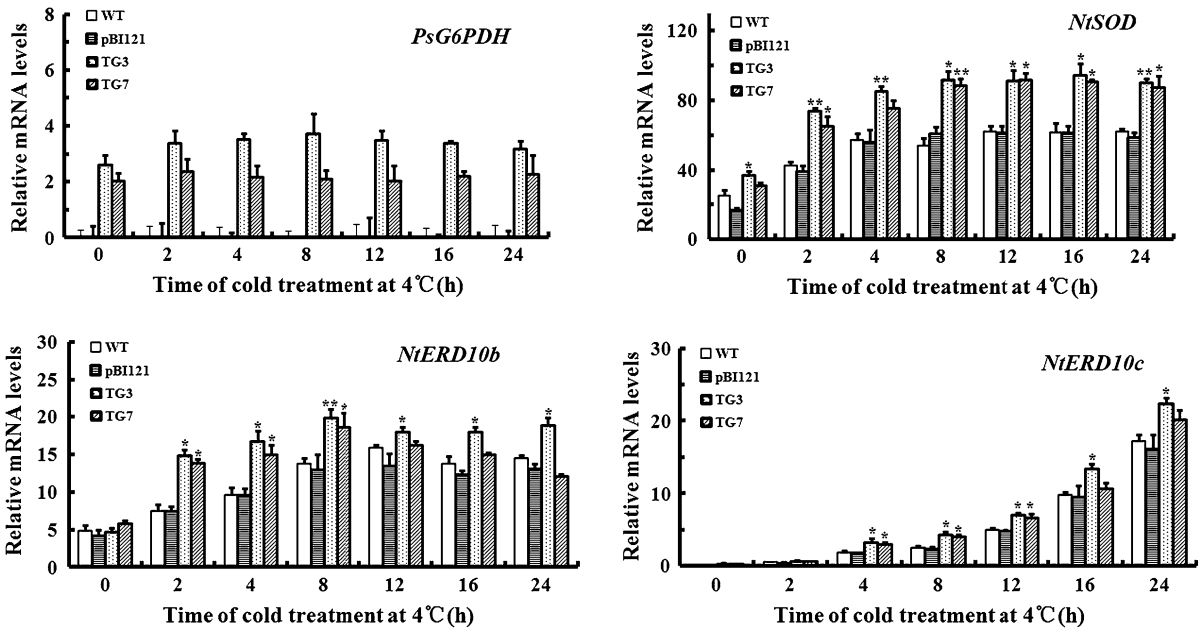

Fig. 6 Increased transcription of cold-stress related genes in tobacco plants over-expressing *PsG6PDH*. The transgenic tobacco lines included TG3 and TG7. *WT* wild-type plants (tobacco). *pBI121* tobacco transformed by vector pBI121. Tobacco *ACTIN* gene (GenBank aceession number: U60491) was used as a reference gene. Data was mean with standard deviations obtained from three independent biological experiments. The significance of the differences in gene expression levels between transgenic and wild-type tobacco plants was assessed using Student's t test (**$P < 0.01$ and *$P < 0.05$)

In the second experiment, in which plants were first subjected to cold acclimation, transgenic lines maintained a greater cold resistance than WT (Fig. 4b). When the ambient temperature was lowered to 0 °C, WT plants suffered overall while transgenic lines seemed to be normal. Transgenic lines even survived at −4 °C. These results confirmed that transgenic tobacco lines carrying poplar *PsG6PDH* exhibited a greater cold tolerance than WT plants.

Biochemical changes displayed by transgenic plants

To further study mechanisms conferring increased stamina on transgenic tobacco plants, we measured electrolyte leakage (EL), SOD and POD activities, and MDA content. Upon temperature reduction from 25 to −4 °C, EL of transgenic lines increased from ∼5 to 40 %, while that of WT increased from ∼5 to 90 % (Fig. 5b—A); especially below 0 °C, EL of both transgenic lines was significant lower than that of WT. After cold stress, SOD and POD activities increased in both WT and transgenic lines; SOD and POD activities in transgenic lines were higher (Fig. 5b—C, D), however, with these levels significantly higher than those of WT after 1 days at 4 °C. In addition, MDA content of WT plants increased as cold treatment time was prolonged, whereas MDA content of transgenic lines decreased, with significantly lower values than those of WT plants after 3 days at 4 °C (Fig. 5b—B). The trend in MDA content was negative with respect to SOD and POD activities in both transgenic lines after 3 days at 4 °C, indicating less severe cold injury in transgenic lines.

The trends in SOD activity, POD activity, and MDA content observed in transgenic lines further confirm our previous hypothesis that increased cytosolic G6PDH activity might be involved in POD and SOD activation and induction of freezing resistance in poplar cuttings (Lin et al. 2005).

Increased transcription of cold-stress related genes in tobacco plants over-expressing *PsG6PDH*

Because poplar cytosolic G6PDH is involved in SOD and POD activation (Lin et al. 2005), we monitored transcript levels of the stress-related genes *NtSOD*, *NtERD10b*, and *NtERD10c* by qRT-PCR. *PsG6PDH*

gene over-expression up-regulated various cold responsive genes, including *NtSOD*, *NtERD10b*, and *NtERD10c*, in transgenic tobacco plants compared with WT plants (Fig. 6). Although over-expression of *PsG6PDH* led to improved cold tolerance in tobacco plants (Figs. 4, 5), cold responsive genes *NtSOD*, *NtERD10b*, and *NtERD10C* were strongly induced after cold stress; this indicates an indirect role for PsG6PDH in the development of tobacco cold tolerance, similar to that observed for *Chlorella* cytosolic G6PDH (Honjoh et al. 2007). In addition, Scharte et al. (2009) reported that cytosolic G6PDH is needed to supply NADPH for a defense response in tobacco. Low temperature stress produces reactive oxygen species (ROS), including superoxide, H_2O_2 and hydroxyl radicals. SOD and POD are important enzyme scavengers of ROS, with NADPH required as a reducing agent in these scavenging reactions (Gapper and Dolan 2006). It is thus possible that over-expression of cytosolic PsG6PDH improves cold tolerance by supplying sustainable levels of NADPH for SOD and POD scavenging reactions under cold stress. Our results reveal that the *PsG6PDH* gene may be responsible for defense responses of transgenic plants to low temperature stress.

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