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Involvement of G6PDH in heat stress tolerance in the calli from *Przewalskia tangutica* and *Nicotiana tabacum*

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Abstract

Glucose-6-phosphate dehydrogenase (G6PDH) has been implicated in supplying reduced nicotine amide cofactors for biochemical reactions and in modulating the redox state of cells. In this study, the role of G6PDH in thermotolerance of the calli from *Przewalskia tangutica* and tobacco (*Nicotiana tabacum* L.) was investigated. Results showed that *Przewalskia tangutica* callus was more sensitive to heat stress than tobacco callus. The activity of G6PDH and antioxidant enzymes (ascorbate peroxidase, catalase, peroxidase and superoxide dismutase) in calli from *Przewalskia tangutica* and tobacco increased after 40 °C treatment, although two calli exhibited a difference in the degree and timing of response to heat stress. When G6PDH was partially inhibited by glucosamine pretreatment, the antioxidant enzyme activities and thermotolerance in both calli significantly decreased. Simultaneously, the heat-induced H_2O_2 content and the plasma membrane NADPH oxidase activity were also reduced. Application of H_2O_2 increased the activity of G6PDH and antioxidant enzymes in both calli. Diphenylene iodonium, a NADPH oxidase inhibitor, counteracted heat-induced H_2O_2 accumulation and reduced the heat-induced activity of G6PDH and antioxidant enzymes. Moreover, exogenous H_2O_2 was effective in restoring the activity of G6PDH and antioxidant enzymes after glucosamine pretreatment. Western blot analysis showed that *G6PDH* gene expression in both calli was also stimulated by heat and H_2O_2, and blocked by DPI and glucosamine under heat stress. Taken together, under heat stress G6PDH promoted H_2O_2 accumulation *via* NADPH oxidase and the elevated H_2O_2 was involved in regulating the activity of antioxidant enzymes, which in turn facilitate to maintain the steady-state H_2O_2 level and protect plants from the oxidative damage.

Additional key words: antioxidant enzymes, hydrogen peroxide, tobacco.

Introduction

Heat stress is expected to become a more frequent problem in the coming years (Wahid *et al*. 2007). Heat stress affects the structure of plants, especially cell membranes, and many basic physiological processes such as photosynthesis, respiration and water relations (Wahid *et al*. 2007). At the molecular level, the effect of heat stress reflects the temperature dependence of Michaelis-Menten constant of every enzyme (Mitra and Bhatia 2008). To cope with heat stress, plants developed different mechanisms including the maintenance of cell membrane stability, regulation of oxidative stress by a synthesis of antioxidants, osmo-regulation, induction of some kinases or chaperones (Wahid *et al*. 2007).

Glucose-6-phosphate dehydrogenase (G6PDH, EC1.1.1.49) is the first and rate-limiting enzyme of the oxidative pentose phosphate pathway (OPPP). It controls

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Abbreviations: APX - ascorbate peroxidase; CAT - catalase; DPI - diphenylene iodonium; EL - electrolyte leakage; G6PDH - glucose-6-phosphate dehydrogenase; GR - glutathione reductase; GSH - reduced glutathione; H_2O_2 - hydrogen peroxide; OPPP - oxidative pentose phosphate pathway; PM - plasma membrane; POD - peroxidase; ROS - reactive oxygen species; SOD - superoxide dismutase.
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the carbon flow through OPPP and produces reducing equivalents in the form of NADPH to meet cellular needs for biochemical reactions and to modulate the redox state of cells (Kletzien *et al*. 1994, Krüger and Von Schaewen 2003). It has been indicated that G6PDH plays a protective role against oxidative stress (Kletzien *et al*. 1994, Pandolfi *et al*. 1995). G6PDH is also involved in nitrogen assimilation (Oji *et al*. 1985, Bowsher *et al*. 1989, Esposito *et al*. 2003), response to pathogenesis (Šindelář and Šindelářová 2002, Scharte *et al*. 2009), metal toxicity (Esposito *et al*. 1998), salt stress (Nemoto and Sasakuma, 2000, Liu *et al*. 2007, Wang *et al*. 2008) and combination of drought and heat stress (Rizhsky *et al*. 2004). It was found that under salt stress G6PDH is involved in maintenance of intracellular reduced glutathione (GSH) in reed callus (Wang *et al*. 2008) or NADPH content in olive plants (Valderrama *et al*. 2006). These studies demonstrated that G6PDH might be a key enzyme in stress response. Recent results indicated that G6PDH contributes to the expression of pro-oxidative enzymes, including nitric oxide synthase and NADPH oxidase by supplying NADPH to plant cells (Pugin *et al*. 1997, Scharte *et al*. 2009).

Increase in ROS, such as superoxide anion and hydrogen peroxide, has been implicated in response to heat stress in plants (Königshofer *et al*. 2008). Numerous studies have shown that plasma membrane (PM) NADPH oxidase is the primary generator of ROS during biotic and abiotic stresses, including heat stress (Simon-Plas *et al*. 2002, Torres *et al*. 2002, Königshofer *et al*. 2008). The function of NADPH oxidase is to catalyze the extracellular formation of $O_2^{\cdot-}$ from O_2 using NADPH as an electron donor (Sagi and Fluhr 2001). Plants have evolved efficient enzymatic and non-enzymatic systems to counteract ROS, thereby protecting cells from oxidative damage and maintaining cellular homeostasis. Key enzymes include superoxide dismutases (SOD), catalases (CAT), peroxidases (POD), ascorbate peroxidases (APX) and other enzymes implicated in the ascorbate-glutathione pathway (Sairam *et al*. 2000, Chaitanya *et al*. 2002, Mishra *et al*. 2005, Locato *et al*. 2008). G6PDH has been recognized as an antioxidant enzyme, and is involved in protection against ROS under heat stress in yeast (Kim *et al*. 2006, Liao *et al*. 2009). However, the role of G6PDH in plants under heat stress was barely investigated.

Przewalskia tangutica, endemic to China, is mainly distributed in sandy and gritty land in dry grassland of the Qinghai-Tibet Plateau. It has been reported that it is of great medicinal importance (Yang, *et al*. 2002, Ren *et al*. 2008), but the physiological characteristics, especially the resistance to heat stress is unclear. In the present study, we used the calli from *Przewalskia tangutica* and tobacco (as a control) to investigate the physiological role of G6PDH in the tolerance to heat stress.

Materials and methods

Embryogenic callus, derived from leaves of *Przewalskia tangutica* Maxim (family *Solanaceae*), was obtained as described by Xu *et al*. (2008). The callus was subcultured on Murashige and Skoog (1962, MS) solid medium containing 0.5 mg/dm^3 α-naphthaleneacetic acid (NAA), 0.5 mg dm^{-3} benzyladenine (6-BA) and 2.0 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D) at 25 °C in continuous darkness. Embryogenic callus from tobacco (*Nicotiana tabacum* L.) cv. Liuye was obtained as described by Tiburcio *et al.* (1985). The callus was subcultured on MS solid medium containing 0.5 mg dm^{-3} 6-BA and 2 mg dm^{-3} 2,4-D at 25 °C in the dark. Following 4-month sub-cultures, the calli from *Przewalskia tangutica* and tobacco were used for various treatments.

Heat stress was induced by incubation of calli at 40 °C for different times. To inhibit the G6PDH activity, 2 cm^3 of 10 mM glucosamine was added on the surface of the solid MS medium for 24 h after filter sterilization. During 5 h of H_2O_2 treatment, it was replaced by 2 cm^3 of 1 mM H_2O_2 adding on the surface of the solid MS medium every 3 h. To inhibit the ROS production, 2 cm^3 of 25 μM diphenylene iodonium (DPI) was added on the medium surface for 48 h. Following the various treatments, heat stress was applied immediately and the calli were collected at indicated time points, washed for 2 min by distilled water, and excess water was blotted with filter paper. The samples were used immediately or stored at -80 °C for later use.

Electrolyte leakage (EL) was determined according to Sairam and Srivastava (2002) with some modifications. Calli (0.1 g) were collected and washed in de-ionized water for three times in order to remove surface-adhered electrolytes. Then, calli were placed in test tubes and immersed in 10 cm^3 of de-ionized water for 3 h at 25 °C. After the incubation, the conductivity in the bathing solution was determined (C_1), and the conductivity of de-ionized water was also determined (C_0). The samples were heated in boiling water for 1 h before the total conductivity was measured in the bathing solution (C_2). Relative EL was expressed as a percentage of the total conductivity after heating in boiling water [$EL = (C_1-C_0)/(C_2-C_0) \times 100$].

H_2O_2 content was determined by the peroxidase-coupled assay according to Veljovic-Jovanovic *et al*. (2002). Callus was homogenized with 1 M $HClO_4$ in the presence of 5 % insoluble polyvinylpyrrolidone (PVP) at 4 °C. The homogenate was centrifuged at 12 000 *g* for 10 min at 4 °C. The supernatant was neutralized with 5 M K_2CO_3 to pH 5.6 in 0.3 M phosphate buffer (pH 5.6). The

resultant mixture was centrifuged at 12 000 *g* at 4 °C for 1 min and the sample was incubated for 10 min with 1 U ascorbate oxidase (*Sigma*, St. Louis, USA) to oxidize ascorbate prior to assay. The reaction mixture consisted of 0.1 M phosphate buffer (pH 6.5), 3.3 mM 4-dimethyl-aminobenzoic acid (DMAB), 0.07 mM 3-methyl-2-benzothiazolinone hydrazone (MBTH), and 0.3 U POX. The reaction was initiated by adding 0.2 cm^3 sample. The absorbance at 590 nm was monitored.

Callus (0.5 g) was ground with 3 cm^3 ice-cold 50 mM phosphate buffer (pH 7.8) containing 0.2 mM EDTA and 1 % PVP. The homogenates were centrifuged at 10 000 *g* for 15 min at 4 °C, and the supernatant was collected for enzyme assays. APX (EC 1.11.1.11) activity was measured according to Nakano and Asada (1981) by monitoring the rate of ascorbate oxidation at 290 nm (coefficient of absorbance, ε = 2.8 mM^{-1} cm^{-1}). CAT (EC 1.11.1.6) activity was measured as described by Durner and Klessing (1996) and calculated according to a decrease in absorbance at 240 nm (ε = 39.4 mM^{-1} cm^{-1}) following decomposition of H_2O_2. POD (EC 1.11.1.7) activity was measured according to Hammerschmidt *et al*. (1982) by monitoring the rate of guaiacol oxidation at 470 nm (ε = 26.6 mM^{-1} cm^{-1}). SOD (EC 1.15.1.1) activity was determined by the method of Beauchamp and Fridovich (1971). One unit of SOD was defined as the amount of enzyme required to cause 50 % inhibition of the reduction of nitroblue tetrazolium as monitored at 560 nm. All activity measurements were carried out at 25 °C in 3 cm^3 reaction mixtures using a spectrophotometer *DU640* (*Beckman*, USA) with no lag period.

G6PDH was extracted according to the method described by Esposito *et al*. (2001) with some modifications. Briefly, 0.5 g of callus was ground in liquid nitrogen and 1 cm^3 of extraction buffer containing 50 mM Hepes-Tris (pH 7.8), 3 mM $MgCl_2$, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM dithiothreitol (DTT) was added. The homogenate was centrifuged at 12 000 *g* for 20 min at 4 °C. A 0.1 cm^3 aliquot of supernatant was added to the total dehydrogenase (G6PDH + 6PGD) assay buffer [50 mM Hepes-Tris (pH 7.8), 3.3 mM $MgCl_2$, 0.5 mM D-glucose-6-phosphate disodium salt, 0.5 mM 6-phosphogluconate, 0.5 mM $NADPNa_2$] and the 6-phosphogluconate dehydrogenase (6PGD) assay buffer [50 mM Hepes-Tris (pH 7.8), 3.3 mM $MgCl_2$, 0.5 mM 6-phosphogluconate, 0.5 mM $NADPNa_2$]. G6PDH activity was calculated as the difference of total dehydrogenase activity and the 6PGD activity (Tian *et al*. 1998).

Plasma membranes were isolated by the method of Qiu and Su (1998) with some modifications. Callus (10 g) was homogenized in 20 cm^3 isolation buffer containing 250 mM sucrose, 25 mM Hepes-Tris (pH 7.6), 1 mM DTT, 1 mM EDTA, 1.5 % PVP, 1 mM PMSF. The entire isolation procedure was carried out at 4 °C. The homogenate was filtered through four layers of cheesecloth and centrifuged at 15 000 *g* for 30 min. The supernatant was centrifuged at 80 000 *g* for 50 min to obtain a microsomal pellet (microsomal membranes), which was resuspended in a buffer containing 250 mM sucrose, 1 mM EDTA, 1 mM DTT, and 2 mM Hepes-Tris (pH 7.2). The microsomal membranes were used for the determination of NADPH oxidase activity.

NADPH oxidase activity assay was performed as described by Yang *et al*. (2003). The determination of the NADPH-dependent O_2^- generation activity in isolated microsomal membranes was carried out by measuring the rate of SOD-inhibited reduction of NBT using NADPH as an electron donor. The reaction mixture consisted of 50 mM Tris-HCl buffer (pH 7.4), 250 mM sucrose, 0.1 mM NBT, 0.02 % (m/v) polyoxyethylenecetyl ether (Brij-58), and 0.03 cm^3 isolated microsomal membranes (containing 50 - 100 μg protein). After 1 min pre-incubation, the reaction was initiated by adding 0.1 mM NADPH, and the absorbance changes at 530 nm were recorded for 5 min. Rates of O_2^- generation were calculated using ε = 12.8 mM^{-1} cm^{-1}. NBT reduction by O_2^- was determined from the difference of NBT reduction rates in the presence and absence of 50 U SOD.

For Western blot analysis, SDS-PAGE was performed as described by Laemmli (1970). About 40 μg proteins were solubilized and separated on an 11.5 % (m/v) polyacrylamide gel containing 6 M urea. After electrophoresis, the separated proteins were transferred to a polyvinylidene difluoride membrane. The membrane was blocked for 90 min with 5 % non-fat milk in 0.5 % (m/v) *Tween 20*, 10 mM Tris-HCl (pH 8.0), and 150 mM NaCl. The polyclonal G6PDH antibody (*Sigma*) was added and incubated with the membrane overnight. After washing, alkaline phosphatase-coupled secondary antibody was added and incubated for 2 h. The chemi-luminescence assays were performed according to the manufacturer's instruction. Protein concentration was determined by the Bradford (1976) method and bovine serum albumin was used as a standard.

Each experiment was repeated at least three times. All mean comparisons were done using Duncan's test for independent sample.

Results and discussion

Several studies have provided evidence that cellular membranes are the primary sites of temperature perception (Königshofer *et al*. 2008, Saidi *et al*. 2009). Cell membrane thermostability can be estimated through electrolyte leakage (EL) following exposure to heat stress (Heckman *et al*. 2002, Grigorova *et al*. 2011). Relative

EL significantly increased after 2 h under 40 °C stress in *Przewalskia tangutica* callus, whereas it increased after 3 h in tobacco callus (Table 1). Heat stress caused a significant increase in H_2O_2 content in calli from both *Przewalskia tangutica* and tobacco. The H_2O_2 reached the highest content at 2 h of heat treatment in *P. tangutica* callus and at 3 h of heat treatment in tobacco callus. Longer exposure to heat stress resulted in no further increase in H_2O_2 contents in both calli (Table 1).

Heat stress induces oxidative stress, and several studies showed that G6PDH is involved in oxidative stress in plants (Zaka *et al*. 2002, Liu *et al*. 2007). The G6PDH activity in calli from *P. tangutica* and tobacco was induced by heat stress (Table 1). It reached the maximum in *P. tangutica* callus at 2 h of heat stress (123.4 % of the control), whereas in tobacco callus, it reached the maximum at 3 h of heat stress (129.7 % of the control).

Previous studies have elucidated that heat stress alters the activity of antioxidant enzymes including SOD, CAT, APX, and POD in many plant species such as potato, tomato, wheat and mulberry (Rainwater and Gossett 1996,

Table 1. Time course of changes in relative electrolyte leakage (EL), H_2O_2 content, and activities of G6PDH, APX, CAT, POD and SOD in calli from *Przewalskia tangutica* and tobacco under heat stress (40 °C). Means of three replicates ± SE. Different letters indicate statistically significant differences between treatments at the 5 % level.

Parameter	Species	Treatment time [h] 0	1	2	3	4
Relative EL	*P. tangutica*	35.47±0.95d	36.37±1.49d	42.87±1.16c	47.77±0.99b	54.97±0.90a
[%]	tobacco	40.87±1.31c	41.80±0.96c	43.33±1.57c	46.80±1.31b	49.93±1.28a
H_2O_2 content	*P. tangutica*	1.91±0.08b	1.93±0.06b	2.67±0.12a	1.84±0.11b	1.58±0.07c
[umol mg^{-1}(d.m)]	tobacco	1.78±0.06c	1.80±0.12c	2.16±0.09b	2.58±0.12a	1.88±0.07c
G6PDH activity	*P. tangutica*	145.59±1.80c	158.19±2.09b	179.68±2.99a	162.96±3.11b	126.41±3.20d
[µmol(NADP) mg^{-1}(prot.) min^{-1}]	tobacco	118.80±1.36c	120.54±1.85c	146.40±2.27b	154.12±2.07a	145.30±1.77b
APX activity	*P. tangutica*	4.12±0.15c	4.52±0.18b	4.87±0.24a	4.16±0.06c	4.09±0.11c
[µmol(asc.) mg^{-1}(prot.) min^{-1}]	tobacco	3.94±0.10d	4.94±0.21c	6.84±0.11b	7.78±0.15a	6.67±0.19b
CAT activity	*P .tangutica*	57.34±1.55c	61.17±2.09bc	75.20±0.92a	65.20±1.55b	58.27±2.05c
[nmol(H_2O_2) mg^{-1}(prot.) min^{-1}]	tobacco	86.50±1.86e	128.13±1.33c	154.23±2.13a	143.53±2.35b	101.43±1.72d
POD activity	*P. tangutica*	0.57±0.04c	0.63±0.08b	0.87±0.03a	0.89±0.13a	0.64±0.09b
[µmol(gua.) mg^{-1}(prot.) min^{-1}]	tobacco	5.38±0.17d	6.42±0.16c	8.52±0.10	9.01±0.15a	8.56±0.20b
SOD activity	*P. tangutica*	16.27±0.73d	29.07±0.66a	26.80±0.67b	19.63±0.66c	13.43±0.52e
[U mg^{-1}(prot.) min^{-1}]	tobacco	22.03±0.93d	23.27±1.08d	41.73±1.02b	46.13±1.09a	33.60±0.64c

Table 2. Effect of glucosamine (Glucm) on G6PDH activity, relative electrolyte leakage, relative growth rate and activity of APX, CAT, POD and SOD in calli from *Przewalskia tangutica* and tobacco. 2 cm^3 10 mM of Glucm was added to the surface of the medium and after 24 h, calli were subjected to heat treatment (40 °C for 2 h). After treatment, calli were collected for determination of enzymes activity and EL. Relative growth rate was determined after calli were recovered for 7 d under control conditions. Means of three replicates ± SE. Different letters indicate statistically significant differences between treatments at the 5 % level.

Parameter	Species	Treatments control	heat	Glucm	heat+Glucm
G6PDH activity	*P. tangutica*	144.43±3.01b	177.65±2.01a	123.99±3.04c	149.33±3.12b
[nmol(NADP) mg^{-1}(prot.) min^{-1}]	tobacco	112.07±3.73c	143.45±2.3a	95.06±2.84d	122.16±2.73b
Relative EL	*P. tangutica*	35.47±0.95c	42.87±1.16b	37.01±0.91c	48.21±1.21a
[%]	tobacco	40.87±0.81b	43.33±0.73b	42.50±1.21b	47.01±1.21a
Relative growth rate	*P. tangutica*	100.00±2.66a	86.73±2.18b	90.43±3.20b	76.54±2.79c
[%]	tobacco	100.00±3.95a	98.47±3.09a	92.54±2.92b	83.95±1.48c
APX activity	*P. tangutica*	4.08±0.17d	4.95±0.17a	4.28±0.26c	4.56±0.21b
[µmol(asc.) mg^{-1}(prot.) min^{-1}]	tobacco	3.82±0.23d	6.95±0.24a	4.45±0.10c	6.01±0.12b
CAT activity	*P .tangutica*	57.49±2.21c	78.87±2.43a	48.53±1.32d	62.23±2.12b
[nmol(H_2O_2) mg^{-1}(prot.) min^{-1}]	tobacco	85.47±3.45c	152.66±2.11a	60.00±1.88d	100.01±2.79b
POD activity	*P. angutica*	0.57±0.05c	0.87±0.33a	0.54±0.04c	0.67±0.03b
[µmol(gua.) mg^{-1}(prot.) min^{-1}]	tobacco	5.29±0.35c	8.58±0.35a	3.46±0.40d	6.98±0.35b
SOD activity	*P. tangutica*	16.20±1.12c	26.14±0.79a	13.64±1.32d	22.11±1.23b
[U mg^{-1}(prot.) min^{-1}]	tobacco	22.89±1.23c	41.28±0.91a	17.25±1.43d	37.03±1.81b

Sairam *et al*. 2000, Chaitanya *et al*. 2002, Vacca *et al*. 2004). In this study, the activity of APX, CAT, POD and SOD in calli from both *P. tangutica* and tobacco increased rapidly under heat stress. The activity of APX, CAT and POD in *P. tangutica* callus reached the maximum at 2 h of heat stress (increased by 18.4, 31.2 and 47.7 %, respectively), while the SOD activity reached the maximum at 1 h of heat stress (increased by 78.7 %). In tobacco callus, the activity of APX, POD and SOD reached the maximum at 3 h of heat stress. They were 197.5, 167.5 and 209.4 % of the control, respectively. The CAT activity reached the maximum at 2 h of heat stress (Table 1).

To examine the injury of callus cells caused by heat stress, both *P. tangutica* and tobacco calli were treated at 40 °C for 1 h to 4 h, and then transferred to the fresh solid medium for a week at 25 °C in dark. Results showed that the *P. tangutica* callus became brownish or dead after heat stress for 3 h or longer; the tobacco callus turned to slight brownish after heat stress for 4 h. The tobacco callus could recover to normal if exposure to heat stress was less than 4 h (data not shown). Based on changes in relative EL, H_2O_2 content, enzyme activity and recovery test, 2 h heat stress treatment was used in further experiments.

To elucidate the role of G6PDH in calli thermotolerance, the G6PDH activity was inhibited by glucosamine. Glucosamine 6-phosphate (N-Glc-6-P) is a well-known competitive inhibitor of G6PDH (Glaser and Brown 1955). Glucosamine can be taken up by plant cells and is rapidly phosphorylated to N-Glc-6-P (Pugin *et al*. 1997). The G6PDH activity in application of glucosamine decreased by 13.7 and 11.6 % in *P. tangutica* and tobacco calli, respectively (Table 2). The G6PDH activity in glucosamine + heat treatment decreased by 14.2 and 15.2 % in *P. tangutica* and tobacco callus, respectively, compared to that in heat stress alone.

The relative EL in glucosamine + heat treatment increased by 12.5 and 8.5 % in *P. tangutica* and tobacco callus, respectively, in comparison with that in the heat treatment alone (Table 2), whereas it did not change under glucosamine treatment alone. The growth rate of *P. tangutica* and tobacco callus after glucosamine pretreatment decreased by 9.6 and 7.5 %, compared to that in control condition. However, the growth rate was reduced by 11.7 and 14.7 % after heat + glucosamine treatment in *P. tangutica* and tobacco callus, respectively, compared to that under heat treatment alone. These data showed that inhibition of G6PDH reduced the thermotolerance of both calli.

In order to understand whether there was a relationship between G6PDH and antioxidant enzymes, the activity of antioxidant enzymes was determined when G6PDH was inhibited. Results showed that the activity of antioxidant enzymes under glucosamine + heat treatment markedly decreased compared to that under heat treatment alone in both calli, although not all antioxidant enzyme activity reduced in glucosamine treatment alone compared to that in control condition (Table 2).

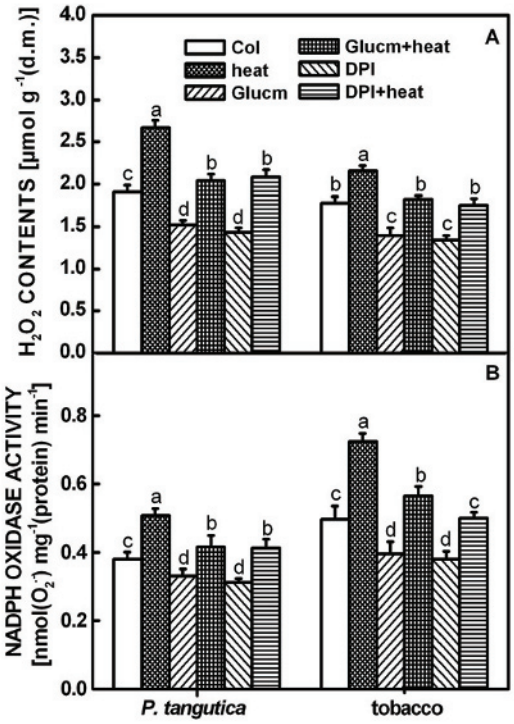

Fig. 1. Changes in H_2O_2 content (*A*) and NADPH oxidase activity (*B*) in calli from *P. tangutica* and tobacco. Calli were cultured on MS solid medium and 10 mM Glucm or 25 μM DPI were added to the surface of the medium for 24 or 48 h, respectively. After that, calli were subjected to heat treatment (40 °C, 2 h) and then H_2O_2 content and NADPH oxidase activity were measured (Col - control). Means ± SE from three independent experiments. Different letters indicate statistically significant differences between treatments at the 5 % level.

When the G6PDH activity was inhibited by glucosamine, the H_2O_2 content decreased by 20.3 and 21.8 % in *P. tangutica* and tobacco callus, respectively (Fig. 1*A*). Under heat + glucosamine treatment, H_2O_2 contents in both calli from *P. tangutica* and tobacco decreased by 23.6 and 15.7 %, respectively, compared to that under heat stress alone. These results suggested G6PDH may play an important role in H_2O_2 accumulation under heat stress. Application of diphenylene iodonium (DPI), a potent inhibitor of PM NADPH oxidase, partially reduced the heat-induced H_2O_2 accumulation in both calli (Fig. 1*A*), suggesting that the NADPH oxidase is involved in H_2O_2 accumulation under heat stress, which was consistent with previous reports (Königshofer *et al*. 2008, Miller *et al*. 2009).

PM NADPH oxidase activity increased by 33.0 and 46.1 % under heat stress in *P. tangutica* and tobacco callus, respectively, whereas it decreased significantly after glucosamine or DPI pretreatment (Fig. 1*B*). It also decreased significantly under glucosamine + heat or DPI

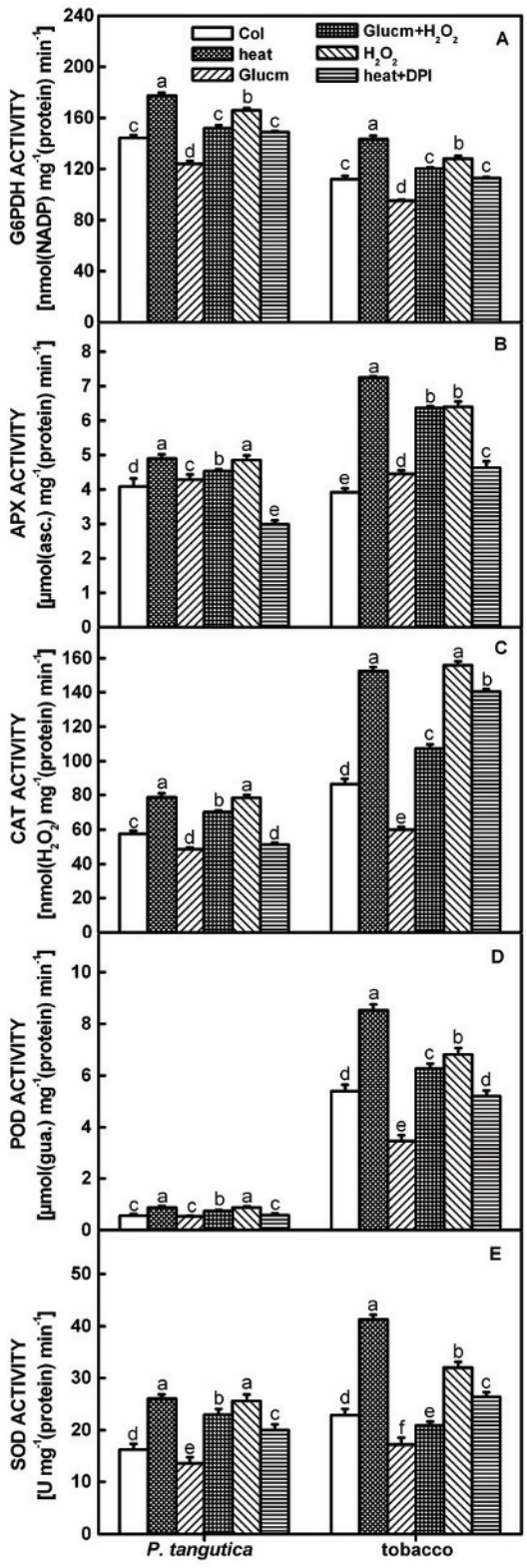

Fig. 2. Effects of H_2O_2 on the activities of G6PDH (*A*), APX (*B*), CAT (*C*), POD (*D*) and SOD (*E*) in calli from *Przewalskii tanguticae* and tobacco. Calli were cultured on MS solid medium and 1 mM H_2O_2 was added to the surface for 5 h. Calli were treated by DPI and Glucm and heat stress as in Fig. 1 (Col - control). Means ± SE from three independent experiments. Different letters indicate statistically significant differences between treatments at the 5 % level.

+ heat treatment compared to that under heat stress alone in both calli (Fig. 1*B*). These results demonstrated that G6PDH play a key role in the regulation of the NADPH oxidase activity and the H_2O_2 accumulation in both calli under heat stress.

Effect of exogenous H_2O_2 on the activity of G6PDH and antioxidant enzymes was determined. The G6PDH activity was induced by H_2O_2 in both calli (Fig. 2). Similarly, the activity of four tested antioxidant enzymes also increased in the presence of H_2O_2. Pretreatment with DPI reduced the heat-induced G6PHD activity in both calli. The heat-induced antioxidant enzyme activity also markedly decreased after DPI pretreatment in both calli. Furthermore, H_2O_2 was effective in rescuing the activity of G6PDH and antioxidant enzymes in glucosamine-treated calli. These results suggested that H_2O_2 might act as a signalling molecule in regulating the activity of G6PDH and antioxidant enzymes under heat stress.

G6PDH has been shown to respond to various oxidative stresses and viral infection at the level of mRNA abundance or enzyme activity (Batz *et al*. 1998, Nemoto and Sasakuma 2000, Šindelář and Šindelářová 2002). To clarify whether the change of the G6PDH activity under heat stress results from elevated protein content or specific activity, Western-blot analysis was carried out. Results showed that the G6PDH protein content in both *P. tangutica* and tobacco callus increased under heat stress (Fig. 3). DPI and glucosamine significantly inhibited the heat-induced expression of *G6PDH* in both calli although the inhibition degree was less in tobacco callus than that in *P. tangutica* callus. Application of H_2O_2 stimulated the *G6PDH* expression and restored the decrease of *G6PDH* expression after glucosamine treatment (Fig. 3). These results demonstrated that the change of G6PDH activity under heat stress and other treatments were consistent with its protein abundance.

Heat stress induces oxidative injury and alters the activity of antioxidant enzymes. Previous studies have elucidated the importance of maintaining a favorable antioxidative level in plant adaptation to heat stress (Rainwater *et al*. 1996, Sairam *et al*. 2000, Chaitanya *et al*. 2002, Vacca *et al*. 2004). The aim of the present study is to investigate the roles of G6PDH in mediating intracellular redox homeostasis in calli from *P. tangutica* and tobacco under heat stress.

It has been found that the endogenous cellular antioxidant capability was crucial in determining the ability of plants to cope with stress condition (Locato *et al*. 2009). In this study, except APX, the activity of other antioxidant enzymes were higher in tobacco callus

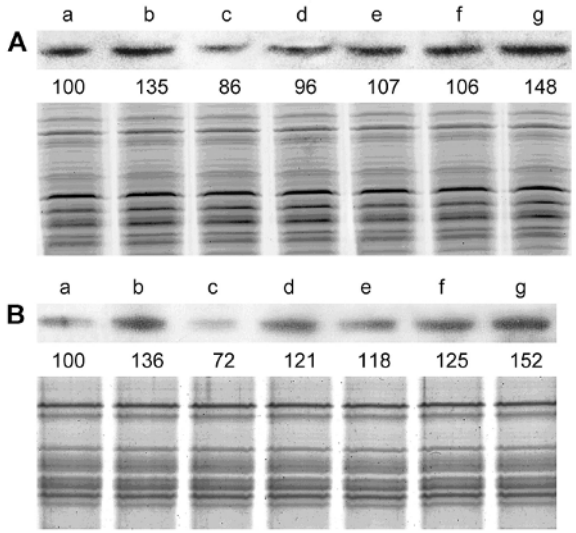

Fig. 3. Western-blot analysis of *G6PDH* gene expression in calli from *Przewalskia tangutica* (*A*) and tobacco (*B*). In the experiment, 40 °C for 2 h, 1 mM H_2O_2, 25 μM DPI, and 10 mM Glucm were used for different treatments. *Lanes*: a - control, b - heat, c - Glucm, d - Glucm + heat, e - DPI + heat, f - Glucm + H_2O_2, g - H_2O_2. Coomassie Brilliantt Blue-stained gel and the lane numbers show the relative amount of G6PDH protein in comparison with the control. Three independent experiments were performed.

than that in *P. tangutica* callus under both control as well as heat stress conditions. The change of relative EL is insignificant in tobacco callus in comparison to that in *P. tangutica* callus under heat stress (Table 1). Together with the injury experiment of heat stress in both calli (data not shown), our results showed that *P. tangutica* callus had lower thermotolerance than tobacco callus. These results also indicated that plant tolerance to heat stress is related to their habitat because tobacco originally grows in warm region whereas *P. tangutica* originally grows in cold region. The G6PDH activity and expression were also induced in both calli under heat stress (Table 1, Fig. 3). The relative EL significantly increased and the relative growth rate decreased in both calli after the heat-induced G6PDH activity and expression were inhibited by glucosamine (Table 2). This result demonstrated that G6PDH is involved in heat tolerance in both calli.

H_2O_2, primarily generated by PM NADPH oxidase, is rapidly accumulated upon high temperature and other stresses (Laloi *et al*. 2004, Königshofer *et al*. 2008). Our experiment demonstrated that the level of H_2O_2 significantly increased in both calli under heat stress (Table 1). Similarly, the endogenous H_2O_2 level in mustard seedlings subjected to heat stress increased by 65.0 % in comparison with plants grown at 24 °C (Dat *et al*. 1998). In order to further identify the ROS-generating mechanism in both calli upon heat stress, we tested the effect of DPI, a potent inhibitor of PM NADPH oxidase.

Our results demonstrated that DPI inhibited the heat-induced NADPH oxidase activity and H_2O_2 production in both calli (Fig. 1), suggesting that NADPH oxidase is involved in heat-induced H_2O_2 production in both calli. Several reports have shown that immediate and sustained oxidative burst of NADPH oxidase relies on effective NADPH regeneration in the cytosol, and G6PDH is a key determinant for providing reducing equivalents to the NADPH oxidase (Pugin *et al*. 1997, Scharte *et al*. 2009). In this study, changes of the PM NADPH oxidase activity and H_2O_2 content followed a similar pattern with the G6PDH activity, which increased under heat stress and decreased by application of glucosamine (Fig. 1). These results suggested that G6PDH plays a key role in the accumulation of H_2O_2 by providing reducing equivalents to the NADPH oxidase.

H_2O_2 is considered as a central signalling molecule in plant responses to biotic and abiotic stresses (Foyer and Noctor 2005). It activates the protective mechanisms against high temperature in mustard seedlings (Dat *et al*. 1998), chilling in maize (Prasad *et al*. 1994), and photoinhibition in *Arabidopsis* leaves (Karpinski *et al*. 1999). Heat stress-induced H_2O_2 accumulation is required for effective expression of heat shock genes and APX genes (Volkov *et al*. 2006). Heat stress evoked H_2O_2 accumulation, stimulated the activity of antioxidant enzymes and G6PDH in both *P. tangutica* and tobacco calli (Table 1). Application of H_2O_2 induced the activity of antioxidant enzymes and G6PDH, and application of DPI decreased the heat-induced activity of the above enzymes (Fig. 2). It suggested that NADPH oxidase-driven H_2O_2 is involved in regulating the heat-induced activity of G6PDH and antioxidant enzymes. Furthermore, our results showed that glucosamine pretreatment not only inhibited the heat-induced G6PDH activity and H_2O_2 content (Figs. 1,2), but also reduced the antioxidant enzymes activities, increased the relative EL and decreased the relative growth rate in both calli (Fig. 2). Application of H_2O_2 was effective in restoring the activity of G6PDH and antioxidant enzymes after glucosamine pretreatment in both calli (Fig. 2). Taken together, G6PDH is involved in regulating heat-induced activation of antioxidant enzymes and heat tolerance by NADPH oxidase-driven ROS.

In conclusion, our data showed that G6PDH has a role in H_2O_2 production *via* NADPH oxidase under heat stress, thus is related to thermotolerance in *P. tangutica* and tobacco calli, although the two calli have different thermotolerance. Under heat stress, G6PDH and PM NADPH oxidase are induced, and then, the accumulation of H_2O_2 *via* PM NADPH oxidase stimulates the activity of G6PDH and antioxidant enzymes. The elevated G6PDH provides more NADPH for H_2O_2 generation. The enhanced antioxidant enzymes can scavenge H_2O_2, maintaining a steady-state level of H_2O_2 for cell signalling and thermotolerance.

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