Identification and Characterization of a Glyoxalase I Gene in a Rapeseed Cultivar with Seed Thermotolerance

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Glyoxalase I (GLYI) is a ubiquitous enzyme in all organisms that catalyzes the conversion of the potent cytotoxin methylglyoxal to S-D-lactoylglutathione. Although many reports suggest the importance of GLYI in the plant response to stress, its function in seeds requires further study. Here, we identified a heat-induced GLYI from *Brassica napus* seeds, BnGLYI, using a comparative proteomics approach. Two-dimensional gel analyses revealed that BnGLYI protein expression upon heat treatment was significantly elevated in thermotolerant seeds but was diminished in heat-sensitive seeds. The *BnGLYI*-2 and *BnGLYI*-3 genes from the heat-sensitive and thermotolerant cultivars, respectively, were characterized, and analyzed. Only two amino acid residue variations were found between the amino acid sequences of the two genes. Moreover, overexpressing *BnGLYI*-3 in yeast cells enhanced tolerance to heat and cold stress and significantly increased GLYI activity compared to overexpressing *BnGLYI*-2. In addition, *BnGLYI*-3 transformants showed enhanced superoxide dismutase activities under heat and cold treatment, whereas these activities were diminished for *BnGLYI*-2 transformants. Taken together, these results indicate that overexpression of the *BnGLYI*-3 gene imparts thermotolerance and cold tolerance in yeast and that the variations in *BnGLYI*-3 may play an important role in the responses to temperature stresses.

Keywords: Glyoxalase I gene, seed thermotolerance, proteomics, *Brassica napus* L., yeast

INTRODUCTION

Seeds not only provide the majority of the human food supply but are also an important supply of nutrients in animal husbandry and industrial feedstock; high-quality seeds are of great significance for socioeconomic development (Jannmohammadi et al., 2008). Seed quality is determined by factors such as the rate and uniformity of germination, vigor, and storability of a seed lot. Biotic and abiotic factors affect seed vigor, germination, and storability. High temperature is one abiotic factor often incurred in seed storage, which may reduce seed vigor and decelerate or completely inhibit seed germination (Wahid et al., 2007). Tests using artificial aging induced by high temperature are therefore often used to evaluate seed storability (Jannmohammadi et al., 2008). In addition, heat stress is a major factor that affects plant development (Hall, 1992; Marcum, 1998; Howarth, 1997).

Abbreviations: ROS, reactive oxygen species; GSH, glutathione (reduced); MG, methylglyoxal; GLYI, glyoxalase I; GLYII, glyoxalase II; *BjGLYI*, *B. juncea* GLYI gene; *BnGLYI*, *B. napus* GLYI gene.
Tuomainen et al., and Mustafiz et al., 2014). To date, GLYI activity can also be upregulated under stress conditions and in the presence of developmental cues, and this observation suggests that GLYI plays an important role in the stress response and in development (Ramaswamy et al., 1984; Sethi et al., 1988; Deswal et al., 1993; Yadav et al., 2005a,b; Singla-Pareek et al., 2006; Lin et al., 2010; Hossain et al., 2011). Tomato GLYI was upregulated in response to salt stress, and osmotic and phytohormonal stimuli (Espartero et al., 1995). Transgenic tobacco overexpressing the B. juncea GLYI gene (BjGLYI) showed significantly enhanced tolerance to MG and high salt concentrations compared to non-transgenic plants (Veena et al., 1999; Singla-Pareek et al., 2003). In a recent study, tobacco leaves transiently overexpressing TaGLYI from wheat (T. aestivum L.) showed increased tolerance to ZnCl₂ stress compared to control leaves (Lin et al., 2010). Despite the long-standing interest of plant scientists in the function of GLYI in stress responses, the involvement of plant GLYI genes in the thermotolerance of seeds has not yet been described.

Rapeseed (canola, oilseed rape; Brassica napus L., Brassicaceae family), an important oilseed plants worldwide, often encounters high temperature during storage, which leads to decline or deterioration in seed vigor (Nagel and Börner, 2010). Therefore, it is important to identify thermotolerance-related genes in B. napus seeds to improve their storability and thermotolerance. In this study, we analyzed seed tolerance to an extremely high temperature to screen for thermotolerance-related genes. A B. napus GLYI gene, BnGLYI, was identified by comparative proteomic analysis. Two-dimensional gel analyses showed that the protein level of BnGLYI in thermotolerant seeds was significantly increased in response to heat stress. Heterologous expression of BnGLYI in yeast conferred tolerance to severe temperature stress. In conclusion, BnGLYI is a heat-responsive protein that participates in tolerance to heat stress, and the variations in the BnGLYI protein sequence might affect its function. These results shed light on the applicability of a molecular breeding approach to enhance plant thermotolerance and seed storability.

MATERIALS AND METHODS

Heat Stress Assay in Rapeseeds and Collection of Plant Materials

A heat-tolerant and a heat-sensitive B. napus cultivar, named 3382 and 2682, respectively, were used in this study. The two cultivars were screened by heat stress treatment from many cultivars of B. napus (data unpublished). The heat stress assay was performed using a previously described protocol (Gao et al., 2010; Chu et al., 2012). Briefly, surface-sterilized mature rapeseed seeds were incubated at 37°C for 2 h and then treated at 65°C for 2 h. Immediately after this treatment, the seeds were moved to room temperature and finally incubated at 28°C to assess their ability to germinate. The heat treatments were performed in the dark. Five sets of 100 seeds were used for each genotype. The treated seeds and the control seeds (maintained at 28°C) were simultaneously collected for RNA and protein isolation. The simultaneously harvested seeds were used for either the control or the heat treatment. The leaves from plants grown in a greenhouse were used for DNA extraction according to previously reported methods (Murray and Thompson, 1980).
Protein Extraction

Protein was extracted from both the treated and control *B. napus* seeds via trichloroacetic acid (TCA)/acetone precipitation as described by Damerval et al. (1986) and Gan et al. (2010) with minor modifications. Briefly, the seeds were ground in a precooled mortar in the presence of liquid nitrogen. The powders were precipitated with 10 volumes of precooled acetone (containing 10% TCA and 0.07% dithiothreitol (DTT)), and the mixtures were incubated at −20°C for 1 h. After centrifuging at 20,000 × g for 30 min and removing the supernatant, the pellet of each sample was rinsed and incubated in ice-cold acetone (containing 0.07% DTT) for 1 h at −20°C. This step was repeated three times. Finally, each pellet was air-dried and resuspended in 500 µl of lysis buffer (7 M urea, 2 M thiourea, 4% 3-[3-cholamidopropyl] dimethylammonio]-1-propanesulfonate (CHAPS), 1 M phenylmethanesulfonyl fluoride (PMSF), 50 mM 3382 and 2682 were designated as Glyoxalase I. An aliquot of 5 µl of the cDNA was used as a template to conduct PCR using full-length primers in a PTC-200 Peltier Thermal Cycler (Bio-Rad, USA); the PCR protocol consisted of an initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min, and a final extension of 10 min at 72°C. The desired PCR products were cloned into the pEASY-T1 vector (TransGen Biotech, China), and clones were selected on plates supplemented with IPTG, X-gal, and ampicillin. The clones were identified by PCR and then sequenced. Sequence comparisons were performed using BLAST. The *BnGLYI* sequences cloned from *B. napus* 3382 and 2682 were designated as *BnGLYI*-3 (KT720495) and *BnGLYI*-2 (KT720496), respectively.

Sequence Analysis

Amino acid (AA) sequence alignment was conducted using the DNAMAN program2. The conserved domains within the GLYI coding nucleotide sequence were searched in the NCBI database3.

Quantitative RT-PCR (qRT-PCR) Analysis

Total RNA was extracted from yeast using an RNeasy Mini Kit (QIAGEN, Germany). RNA pellets were dissolved in DEPC-treated water; the presence of RNA was confirmed by agarose gel electrophoresis; and the RNA concentrations were quantified based on the absorbance of the samples at 260 nm. The RNA samples were reverse-transcribed into first-strand cDNA using a ReverTra Ace-α-TM qPCR RT kit (Toyobo, Japan) and stored at −80°C. Expression of the GLYI genes in transgenic yeast was evaluated using LightCycler 480 SYBR Green I Master mix and a LightCycler 480II real-time PCR system (Roche, Switzerland). Expression of the *BnGLYI* gene was normalized to that of *Actin* according to the 2−ΔΔCT method (Livak and Schmittgen, 2001) using the following formula:

\[
2^{-\Delta\Delta CT} = 2^{-(\text{CT}_{\text{Target}} - \text{CT}_{\text{Actin}}) - (\text{CT}_{\text{Target}} - \text{CT}_{\text{Actin}})_{\text{calibrator}}}
\]

where CT, *Actin* is the CT value of the reference gene *Actin*, and CT, *target* is the CT value of the gene under investigation. The value determined by this formula represents the relative expression of a gene in a sample relative to that in a calibrator. The primers and the amplification conditions used are described in Table 1. Each sample was examined in triplicate.

Ectopic Expression of the *BnGLYI* Gene in Yeast

The coding sequence of the *BnGLYI* gene was amplified from cDNA by PCR using pfu DNA polymerase (Tiangen, China). An *EcoRI* site and a *NotI* site (underlined in Table 1) were introduced at the 5′-end and the 3′-end of the PCR product, respectively. The *EcoRI/NotI* fragment (558 bp) was directly

1http://www.matrixscience.com

2http://www.lynnon.com

3http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi
TABLE 1 | Primer pairs used in the gene-specific amplification of GLYI genes.

| Primer name | Fragment (bp) | Primers (5’–3’) | Tm (°C) | Used for | Reference |
|-------------|---------------|------------------|---------|----------|-----------|
| BnGLYF      | 558 bp        | ATGCGCTCAGAACAGG | 67.9    | Gene cloning | Veena et al., 1999 |
| BnGLYR      | 558 bp        | GGAATTCACATGCGTGGAAAG | 73.5 | Gene expression | This study |
| ACT1-F      | 131 bp        | ATCATGTTCGAGACTTTCAACG | 59.2 | qRT-PCR in yeast | Zhang et al., 2015 |
| ACT1-R      | 131 bp        | AATGGGACACGTTGGTAA | 60.1 | qRT-PCR | This study |
| BnGLYF      | 148 bp        | CAAGCTGCTCAGAATGGCAT | 63.0 | qRT-PCR in yeast | This study |
| BnGLYR      | 148 bp        | ATGTTCGAGGCTGACAOCA | 63.5 | qRT-PCR | This study |

Gene cloning

cloned into the pEASY-T1 vector (TransGen, China) and was then sub-cloned into the Pichia expression vector pPIC3.5K (Multi-Copy Pichia Expression Kit, Invitrogen, USA) to generate the pPIC3.5K-BnGLYI-2 and pPIC3.5K-BnGLYI-3 plasmids. These plasmids were introduced into Pichia pastoris GS115 cells by electroporation (MicroPulser Electroporation Apparatus, Bio-Rad, USA) according to the manufacturer’s instructions for overexpression of the given fusion protein. Transformants were selected by plating cells on synthetic minimal medium agar lacking histidine, and were then confirmed by PCR using gene-specific primers (Table 1). The cells transformed with empty vector (pPIC3.5K), pPIC3.5K-BnGLYI-2 and pPIC3.5K-BnGLYI-3 were designated as GS3.5K, GSGLYI-2, and GSGLYI-3, respectively.

Heterologous expression of BnGLYI was induced under the transcriptional control of the yeast AOX1 promoter. Expression of BnGLYI in P. pastoris GS115 was induced according to the instructions of the Multi-Copy Pichia Expression Kit as well as the method described by Wang et al. (2013) with slight modification. The transformant expressing the highest abundance of GLYI was selected and used for further studies. Aliquots (1 ml) of cells were harvested at 24, 48, 72, and 96 h and then centrifuged at maximum speed for 2–3 min at room temperature to collect the cells. Total cellular protein samples were electrophoresed via 12% SDS–PAGE, followed by Coomassie blue staining.

Assessment of Yeast Resistance to Temperature Stress

Yeast cultured to the exponential phase in a yeast extract peptone dextrose medium (YPD) was inoculated in buffered glycerol complex medium (BMGY). After induction in buffered methanol complex medium (BMMY), the cultured cells were diluted by 1:10,000. The diluted cells were treated under heat and cold stress according to a previously reported method (Li et al., 2009) with minor modifications. Three aliquots (100 μl each) were treated at 42°C in a water bath for 2.5, 3.5, and 5 h. Simultaneously, three additional aliquots were frozen at −20°C for 10 days (D) and 13 D. The test samples were cultured on a YPD culture plate at 30°C after the treatments. Three aliquots were directly plated on a YPD plate as a control. The survival rate of the treated cells relative to the control cells is expressed as the percentage of colony-forming units (CFU%). The experiments were repeated three times. Tukey’s test was used to analyze all the data, which were presented as the means ± SD (n = 3), to compare the survival rate between the yeast cells harboring BnGLYI-2 and BnGLYI-3 and the control cells under either normal or stress conditions. A P < 0.05 was considered to indicate a significant difference.

Determination of Enzyme Activities

Glyoxalase I activity in total protein extracts from yeast was measured as described previously (Singla-Pareek et al., 2003; Mustafiz et al., 2010). Each of the measurements was performed using three replicates. The enzymatic production of S-lactoylglutathione (extinction coefficient 3.37 mM−1 cm−1) was measured at 240 nm at 15 s intervals for 2 min. Specific activity of GLYI is expressed as μmol S-lactoylglutathione formed/hr/mg protein (Junaid et al., 2004). The yeast protein was extracted using Y-PER Yeast Protein Extraction Reagent (Thermo Scientific, USA). Bradford reagent (Aidlab, China) was used to determine the protein concentrations. ImageJ software was used to determine the relative expression levels of BnGLYI-2 and BnGLYI-3.

Superoxide dismutase (SOD) activity was assessed by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Beauchamp and Fridovich (1971). One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of NBT reduction at 560 nm (Beauchamp and Fridovich, 1971). SOD activity is expressed as units per milligram of protein.

RESULTS

Thermotolerance of Oilseed Seeds

To demonstrate the basal thermotolerance of oilseeds, mature seeds were treated at high temperature. When treated at 65°C for 2 h, the germination of 3382 seeds was not evidently affected, as only a slight decrease in the germination rate was observed; however, only approximately 40% (41.7 ± 1.2%) of the 2682 seeds remained alive (Figure 1). With increasing treatment time, the germination rates of 3382 seeds gradually declined. However, the germination rates of 2682 seeds declined sharply, and up to 100% of the 2682 seeds were dead after 6 h at 65°C. The germination rate of 3382 seeds was significantly higher than that of 2682 seeds under heat treatment.

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GLYI is a Heat-Responsive Protein

The objective of this study was to identify novel genes involved in the development of heat tolerance in *B. napus*. To reveal how plant cells respond to heat stress by modulating protein expression, we performed a two-dimensional gel analysis to screen for heat-responsive proteins. One heat-responsive protein was identified as GLYI (Figures 2A,B, Supplementary Figures S1 and S2). Nine peptides were matched to the predicted GLYI protein sequence with a MASCOT score of 117 (Supplementary S1 and S2). The variation at position 93 may affect the putative metal binding sites and this position may be an active site based on the analysis (Figure 3B).

**Characterization of GLYI cDNA Clones from *B. napus***

Based on the sequences of *BjGLYI* (GenBank accession no. Y13239) and *A. thaliana AtGLYI* (AT1G08110.1), full-length cDNAs of *BnGLYI*, encoding proteins sharing 99 and 100% AA identity with the *BjGLYI* sequence, respectively, were cloned from the 3382 and 2682 cultivars. The open reading frame (ORF) of *BnGLYI* cDNA consisted of 558 bp encoding a protein of 185 amino acids. The calculated molecular mass and pI were 20.8 kDa and 5.42, respectively, and these values were consistent with the apparent molecular mass and pI of the target protein spot on the two-dimensional gels.

Proteins containing a glyoxalase domain (PF00903) and having a putative lactoylglutathione lyase function have been classified as GLYI proteins (Mustafiz et al., 2011). In the two deduced protein sequences, two glyoxalase domains were found (AA 29–171, E-value 18.e-25) based on Pfam 23.0, and this result showed that the gene corresponding to both proteins was *BnGLYI*. Multi-alignment analysis of the deduced AA sequences from the *BnGLYI* gene revealed that the *BnGLYI* gene exhibited significantly high similarity to other GLYI genes (Figure 3A). For example, the *BnGLYI*-3 protein (from *B. napus* 3382) shared 79, 94, 99, 98, and 99% AA identities with GLYI from *Solanum lycopersicum* (Accession no. Z48183), *A. thaliana* (Accession no. AT1G08110.1), *B. juncea* (Accession no. Y13239), *Arachis hypogaea* (Accession no. DQ989209.2), and *B. napus* 2682 (*BnGLYI*-2), respectively (Figure 3A). These results suggested that this protein has been evolutionarily conserved. AA residues probably involved in metal and GSH binding sites were also conserved among the GLYI sequences (Figure 3B). Moreover, we found only two variations (Pro/Ala at position 9 and Ala/Val at position 93) in the AA sequence between *BnGLYI*-2 and *BnGLYI*-3. The variation at position 93 may affect the putative metal binding sites and this position may be an active site based on the analysis presented in Figure 3B.

**Overexpressing BnGLYI-3 in P. pastoris Enhances Tolerance to Temperature Stresses**

To determine whether the variations between the two *BnGLYI* protein sequences affected the function of the protein, we overexpressed the two *BnGLYI* genes in *P. pastoris* cells. Each full-length *BnGLYI* coding sequence was subcloned into the pPIC3.5K vector, and the resulting plasmids were transformed into yeast. The qRT-PCR results showed that the transformants harboring *BnGLYI*-2 and *BnGLYI*-3 displayed significantly increased *BnGLYI* expression, but that *BnGLYI* expression was not detected in the transformants carrying the empty vector or in the wild-type cells (Supplementary Figure S3). In addition, the soluble proteins extracted from *P. pastoris* cells were analyzed by SDS-PAGE. A distinct band with a molecular mass of ~21 kDa was visible in the protein extract obtained from the GSGLYI-2 and GSGLYI-3 cells (Figure 4A). Moreover, the GSGLYI-2 and GSGLYI-3 cells exhibited a similar *BnGLYI* protein level for the same amount of total protein (Figure 4A). These results indicated that the two sequence variations did not affect *BnGLYI* protein stability. Serial dilutions (10−4, 10−5, 10−6) of cell cultures plated on a YPD plate showed that the transformants had similar growth rates to GS3.5K and GS115 cells when incubated at 30°C (data not shown).

http://pfam.sanger.ac.uk/
A thermotolerance assay was conducted on yeast cells transformed with the recombinant plasmids pPIC3.5k-BnGLY-2 and pPIC3.5k-BnGLY-3, and cells harboring the empty pPIC3.5K vector were used as a control. Compared with the GS3.5K cells, the GSGLYI-3 cells showed increased tolerance to high temperatures; however, the GSGLYI-2 transformant did not show any thermotolerance under heat stress (Figures 4B,C). Within the first 2.5 h of heat treatment, loss of cell viability was observed. The survival rates of GS3.5K, GSGLYI-2, and GSGLYI-3 cells were approximately 70.2, 66.7, and 99.1%, respectively. The viability of GSGLYI-3 cells was significantly different from that of GS3.5K and GSGLYI-2 cells (P < 0.05). After 3.5 h of heat treatment at 42°C, 40.0 and 75.9% of the cells overexpressing BnGLYI-3 and BnGLYI-2, respectively, had survived. After 5 h of heat treatment at 42°C, 70.1% of the GSGLYI-3 cells remained alive, but most of the GS3.5K and GSGLYI-2 cells were dead (Figures 4B,C). Taken together, the survival rate of the BnGLYI-3-transformed cells was significantly higher than that of the BnGLYI-2 and empty vector transformants. However, the survival rate of GSGLYI-2 cells was not significantly different from that of GS3.5K cells.

To investigate the role of the BnGLYI protein in the response to cold shock, the GSGLYI-2 and GSGLYI-3 transformants as well as the empty vector control transformants were treated at −20°C. After being frozen for 10 D, approximately 60% of the GSGLYI-3 cells and 30% of the GSGLYI-2 and GS3.5K cells remained alive. After being frozen for 13 D, clearly more surviving cells (approximately 50%) remained in the GSGLYI-3 cell lines than in the GSGLYI-2 and control GS3.5K cells. Statistical analysis revealed that the survival rate of the GSGLYI-3 cells was 50.5±13.1%, whereas the survival rate of GSGLYI-2 and GS3.5K was approximately 17.4±1.5 and 12.9±2.5%, respectively (Figures 4B,C). These results indicated that overexpressing the BnGLYI-3 gene significantly enhanced the freeze tolerance of the host cells.

Furthermore, we measured GLYI activity in the crude protein samples extracted from GSGLYI-2, GSGLYI-3, and GS3.5K cells. As shown in Figure 4D, GS3.5K, GSGLYI-2, and GSGLYI-3 cells exhibited similar GLYI activity under normal conditions without induction. After induction, the GSGLYI-3 and GSGLYI-2 cells displayed approximately 55 and 80% higher activity, respectively, than the GS3.5K cells. GSGLYI-3 cells showed significantly higher GLYI activity than the GS3.5K and GSGLYI-2 cells after treatment at 42°C for 3.5 and 5 h, as well as after treatment at −20°C (Figure 4D). GSGLYI-3 cells showed approximately 1.4-fold and 0.9-fold higher GLYI activity than GS3.5K and GSGLYI-2 cells, respectively, after the 5-h treatment. In addition, GSGLYI-3 cells displayed approximately twofold and 1.3-fold higher GLYI activity than GS3.5K and GSGLYI-2 cells, respectively, after 10 D of treatment at −20°C. These results suggested that overexpressing GSGLYI-3 may improve heat and cold stress tolerance in P. pastoris.

**SOD Activity in the Transformed Cells**

To further explore the physiological traits by which the GSGLYI-3 cells exhibited enhanced tolerance to temperature stress, the activity of the antioxidant enzyme SOD was examined (Figure 5). SOD showed different activity patterns among the different
cells under heat and cold stress. SOD activity in GSGLYI-3, GSGLYI-2 and GS3.5K cells was similar under heat and cold stress treatments. However, compared to the same cells under control conditions, SOD activity was decreased by 8.6% in GSGLYI-2 cells treated at 42°C for 5 h, but was increased by 2.1 and 57.1% in GS3.5K and GSGLYI-3 cells, respectively (Figure 5). After treatment at −20°C for 13 D, SOD activity was decreased by 56.5 and 28.9% in GS3.5K and GSGLYI-1 cells, respectively, but was increased by 6.4% in GSGLYI-3 cells relative to the levels in the corresponding control-treated cells (Figure 5).

**DISCUSSION**

In the present study, two *BnGLYI* genes were isolated from *B. napus* seeds via a proteomic analysis of thermotolerant (3382) and thermosensitive (2682) seeds. The coding sequences of *BnGLYI-1* and *BnGLYI-2* were cloned and characterized from the thermotolerant and thermosensitive *B. napus* cultivars, respectively. A previous study found that the *BjGLYI* sequence showed significant homology with other GLYI sequences from plant and microbial species at the nucleotide and AA levels (Veena et al., 1999). In our study, the AA sequences of *BnGLYI*...
exhibited up to 79–98% identity to the AA sequences of GLYI from other seed plants. The GLYI coding sequence encodes for a 185 AA protein that contains zinc and GSH binding domains similar to the other reported GLYI proteins. Most interestingly, we found only two variations (Pro/Ala at position 9 and Ala/Val at position 93) between the AA sequences of BnGLYI-2 and BnGLYI-3.

There are 11 members of the GLYI family in both Arabidopsis and rice (Mustafiz et al., 2011). B. napus is an allotetraploid that may harbor many GLYI genes (at least two). After the release of the B. napus genomic sequence data5, BLAST searches were performed against the B. napus database. The results showed that the top hit was the assembled CDS of accession no. GSBRNA2T00156270001 using the AA sequence of BjGLYI (the peptides detected by MALDI-TOF MS analysis) as a query. When the corresponding CDS sequence was used as a query, the top hit was also GSBRNA2T00156270001. These results indicated that we cloned the gene identified by two-dimensional gel analysis. To fully understand the relationship between the two BnGLYI variants and resistance to temperature stresses in vitro, we expressed the two BnGLYI genes in P. pastoris, a relatively simple system, and assessed their resistance to stresses.

Although glyoxalases have been highly conserved throughout evolution, they exhibit a significant structural variation in terms of active site position and number (Mustafiz et al., 2014). In the case of yeast (possessing a monomeric molecular structure), the AA residue mutation E163Q/E318Q of GLYI substantially reduced its activity, and this result confirmed that the yeast GLYI contains two active sites (Fricket et al., 2001). Human glyoxalase I is a homodimeric protein (Ridderström et al., 1998). It has been proposed that residue 172 in the human enzyme, a glutamate, acts as the base for catalysis of the reaction (Ridderström et al., 1998; Cameron et al., 1999). Others have argued that residue 157 of human GLYI has no direct role in catalysis, but is rather involved in forming the substrate-binding site (Ridderström et al., 1997). Met157 was changed into Ala, His or Glu, which resulted in either sharply decreased activity or the same high specific activity as wild-type GLYI (Ridderström et al., 1997). In the present study, of the two variations, only residue 93 was in the metal binding site, according to the structure of GLYI. Converting Ala93 to Val93 caused the cells to lose their resistance to heat and cold stress. This variation might lead to structural changes that could be related to the catalytic activity of GLYI.

The glyoxalase system, a ubiquitous detoxification system, is considered to play an important role in the removal of MG, a cytotoxic compound whose abundance increases during various stresses (Yadav et al., 2005a). In plants, this pathway is considered to be associated with tolerance to various abiotic stresses. GLYI in B. juncea was reported to confer resistance to salt stress (Veena et al., 1999; Singla-Pareek et al., 2003). In onions, upregulation of GLYI activity was observed in response to various stresses (Hossain and Fujita, 2009). In addition to MG detoxification, the physiological significance of the glyoxalase system is the maintenance of redox homeostasis via the regeneration of GSH. In our study, overexpressing BnGLYI-3 gene from the heat-tolerant B. napus cultivar in yeast conferred resistance to heat and cold stress and significantly elevated GLYI activity compared to the control. These results suggest the protective role of enhanced BnGLYI-3 expression. Moreover, reduced GLYI activity results in MG accumulation (Shamsi et al., 2000). Thus, we speculated that an increase in activity of BnGLYI-3 could protect plants from heat and cold damage to a certain extent.

Furthermore, abiotic stress can stimulate ROS production and induce oxidative stress in plants (Hoque et al., 2012). During oxidative stress, the activity of antioxidant enzymes such as SOD generally increases in plants, and this elevated antioxidant enzyme activity correlates with increased stress tolerance (Gill and Tuteja, 2010). In a previous study, overexpression of a B. campestris HSP70 in tobacco clearly conferred enhanced tolerance to heat stress, and transgenic tobacco plants exhibited significantly higher SOD activity than wild-type plants. These results indicated that elevated SOD activity helps to alleviate the damage to the membrane system caused by heat stress (Wang et al., 2015). In our study, compared with the same cells under control condition, the SOD activity of GSGLYI-2 cells was decreased, but that of GSGLYI-3 cells was significantly increased after treatment at 42°C for 5 h. After treatment at −20°C for 13 D, GS3.5K and GSGLYI-2 cells exhibited decreased SOD activity, but GSGLYI-3 cells exhibited increased SOD activity. These results suggested that GSGLYI-3 cells exhibit less accumulation of ROS and more effective growth than GS3.5K and GSGLYI-2 cells under temperature stress.

http://BrassicaDB.org/brad/
CONCLUSION

We have identified a BnGLYI gene, and the overexpression of a variant of this gene in yeast conferred thermotolerance. BnGLYI may also possess a similar function in B. napus seeds. Additionally, the BnGLYI activity level may be affected by the AA variation at position 93. The results of our study may shed light on the mechanisms by which plants cope with heat stress.

AUTHOR CONTRIBUTIONS

Designed the experiments: GY and XW. Conducted the experiments: GY, XL, and XX. Analyzed the data: GY, XL, LW, and XX. Contributed materials/reagents/analysis tools: GY, XL, XX, GG, FL, JQ, JL, BC, KK, LW, and XW. Wrote the paper: GY.

REFERENCES

Beauchamp, C., and Fridovich, I. (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. 44, 276–287. doi: 10.1016/0003-2697(71)90370-8

Cameron, A. D., Riddetström, M., Olin, B., Kavara, M. J., Creighton, D. I., and Mannervik, B. (1999). Reaction mechanism of glyoxalase I explored by an X-ray crystallographic analysis of the human enzyme in complex with a transition state analogue. Biochemistry 38, 15480–15490. doi: 10.1021/ bi990696c

Candiano, G., Bruschi, M., Musante, L., Santucci, L., Ghiggeri, G. M., Carmelloli, B., et al. (2004). Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis 25, 1327–1333. doi: 10.1002/elps.200305844

Chu, P., Chen, H., Zhou, Y., Li, Y., Ding, Y., Jiang, L., et al. (2012). Proteomic and functional analyses of Nelumbo nucifera annexins involved in seed thermotolerance and germination vigor. Planta 235, 1271–1288. doi: 10.1007/s00425-011-1573-3

Clugston, S. L., Daub, E., and Honek, J. F. (1998). Identification of glyoxalase I sequences in Brassica oleracea and Sporobolus stapfianus: evidence for gene duplication events. J. Mol. Evol. 47, 230–234. doi: 10.1007/PL0006380

Damerval, C., Vienne, D. D., Zivy, M., and Thiellement, H. (1986). Technical improvement in two-dimensional electrophoresis increase the level of genetic variation detected in wheat-seedling protein. Electrophoresis 7, 53–54. doi: 10.1002/elps.1150070108

Deswal, R., Chakaravarty, T. N., and Sopory, S. K. (1993). The glyoxalase system in higher plants: regulation in growth and differentiation. Biochem. Soc. Trans. 21, 527–530. doi: 10.1042/bst021527

Espartero, J., Sánchez-Aguayo, I., and Pardo, J. M. (1995). Molecular characterization of glyoxalase-I from a higher plant: up regulation by stress. Plant Mol. Biol. 29, 1223–1233. doi: 10.1007/BF00020464

Ferguson, G. P., Tötemeyer, S., MacLean, M. J., and Booth, I. R. (1998). Methyglyoxal production in bacteria: suicide or survival? Arch. Microbiol. 170, 209–218. doi: 10.1007/s002030050635

Gao, G., Wu, X. M., Lv, X. D., Chen, B. Y., Xu, K., and Yan, G. X. (2010). Genotype differences of seed viability in rapeseed during storage at different temperature. Chin. J. Oil Crop Sci. 32, 495–499.

Gill, S. S., and Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol. Biochem. 48, 999–930. doi: 10.1016/j.plaphy.2010.08.016

Hajducz, M., Casteel, J. E., Tang, S., Hearne, L. B., Knapp, S., and Thelen, J. J. (2007). Proteomic analysis of near-isogenic sunflower varieties differing in seed oil traits. J. Proteome Res. 6, 3232–3241. doi: 10.1021/pr07 70149a

Hall, A. E. (1992). Breeding for heat tolerance. Plant Breed. Rev. 10, 129–168.

Hoque, T. S., Uraji, M., Ye, W. X., Hossain, M. A., Nakamura, Y., and Murata, Y. (2012). Methyglyoxal-induced stomatal closure accompanied by peroxidase-mediated ROS production in Arabidopsis. J. Plant Physiol. 169, 979–986. doi: 10.1016/j.jplph.2012.02.007

Hossain, M. A., and Fujita, M. (2009). Purification of glyoxalase I from onion bulbs and molecular cloning of its cDNA. Biosci. Biotechnol. Biochem. 73, 2007–2013. doi: 10.1271/bbb.90194

Hossain, M. A., Hasanuzzaman, M., and Fujita, M. (2011). Coordinate induction of antioxidative defense and glyoxalase system by exogenous proline and glycinebetaine is correlated with salt tolerance in mung bean. Front. Agric. China 5:1–14. doi: 10.1007/s11703-010-1070-2

Howarth, C. J. (2005). Genetic Improvements of Tolerance to High Temperature. New York, NY: Howarth Press.

Jannamohammedi, M., Fallahnejhad, Y., Golsha, M., and Mohammadi, H. (2008). Controlled ageing for storability assessment and predicting seedling early growth of canola cultivars (Brassica napus L.). J. Agric. Biol. Sci. 23, 26–32.

Junaid, M. A., Kowal, D., Barua, M., Pullarkat, P. S., Sklower, B. S., and Pullarkat, R. K. (2004). Proteomic studies identified a single nucleotide polymorphism in glyoxalase I as autism susceptibility factor. Am. J. Med. Genet. A 131, 11–17. doi: 10.1104/pp.105.062257

Leprince, O., Atherton, N. M., Deltour, R., and Hendry, G. A. F. (1994). The involvement of respiration in free radical processes during loss of desiccation tolerance in germinating Zea mays L. (An electron paramagnetic resonance study). Plant Physiol. 104, 1333–1339. doi: 10.1104/pp.104.4.1333

Li, D. D., Tai, F. J., Zhang, Z. T., Li, Y., Zheng, Y., and Wu, Y. F. (2009). A cotton gene encodes a tonoplast aquaporin that is involved in cell tolerance to cold stress. Gene 438, 26–32. doi: 10.1016/j.gene.2009.02.023

Lin, F., Xu, J., Shi, J., Li, H., and Li, B. (2010). Molecular cloning and characterization of a novel glyoxalase I gene TaGLY I in wheat (Triticum aestivum L.). Mol. Biol. Rep. 37, 729–735. doi: 10.1007/s11033-009-9578-3

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 25, 402–408. doi: 10.1006/meth.2001.1262

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.00150
Marcum, K. B. (1998). Cell membrane thermostability and whole plant heat tolerance of Kentucky bluegrass. Crop Sci. 38, 1214–1218. doi: 10.2135/cropsci1998.0011183X003800050017x

Murray, M. G., and Thompson, W. F. (1980). Rapid isolation of high-molecular-weight plant DNA. Nucleic Acids Res. 8, 4321–4325. doi: 10.1093/nar/8.19.4321

Murray, M. G., and Thompson, W. F. (1980). Rapid isolation of high-molecular-weight plant DNA. Nucleic Acids Res. 8, 4321–4325. doi: 10.1093/nar/8.19.4321

Nagel, M., and Börner, A. (2010). The longevity of crop seeds stored under ambient conditions. Seed Sci. Res. 20, 1–12. doi: 10.1017/S0960258509990213

Paulus, C., Köllner, B., and Jacobsen, H. J. (1993). Physiological and biochemical characterization of glyoxalase I, a general marker for cell proliferation, from a soybean cell suspension. Planta 189, 561–566. doi: 10.1006/plta.1998.0620

Ramaswamy, O., Pal, S., Guha-Mukherjee, S., and Sopory, S. K. (1984). Correlation of glyoxalase I activity with cell proliferation in Datura cutulus calyx. Plant Cell Rep. 3, 121–124. doi: 10.1007/BF00244105

Riddetström, M., Cameron, A. D., Jones, T. A., and Mannervik, B. (1997). Mutagenesis of residue 157 in the active site of human glyoxalase I. Biochem. J. 328, 231–235. doi: 10.1042/bj3280231

Riddetström, M., Cameron, A. D., Jones, T. A., and Mannervik, B. (1998). Involvement of an active-site Zn2+ ligand in the catalytic mechanism of human glyoxalase I. J. Biol. Chem. 273, 21623–21628. doi: 10.1074/jbc.273.34.21623

Sethi, U., Basu, A., and Guha-Mukherjee, S. (1988). Control of cell proliferation and differentiation by regulating polyamine biosynthesis in cultures of Brassica and its correlation with glyoxalase-I activity. Plant Sci. 56, 167–175. doi: 10.1016/0168-9452(88)90031-3

Shamsi, F. A., Sharkey, E., Creighton, D., and Nagaraj, R. H. (2000). Maillard reactions in lens proteins: methylglyoxal-mediated modifications in the rat lens. Exp. Eye Res. 70, 369–380. doi: 10.1016/exer.1999.0800

Singla-Pareek, S. L., Reddy, M. K., and Sopory, S. K. (2003). Genetic engineering of the glyoxalase pathway in tobacco leads to enhanced salinity tolerance. Proc. Natl. Acad. Sci. U.S.A. 100, 14672–14677. doi: 10.1073/pnas.2034667100

Singla-Pareek, S. L., Yadav, S. K., Pareek, A., Reddy, M. K., and Sopory, S. K. (2006). Transgenic tobacco overexpressing glyoxalase pathway enzymes grow and set viable seeds in zinc-spiked soils. Plant Physiol. 140, 613–623. doi: 10.1104/pp.105.073734

Skipsey, M., Andrews, C., Townson, J. K., Jepson, L., and Edwards, R. (2000). Cloning and characterization of glyoxalase I from soybean. Arch. Biochem. Biophys. 374, 261–268. doi: 10.1006/abbi.1999.1596

Suzuki, N., and Mittler, R. (2006). Reactive oxygen species and temperature stresses: a delicate balance between signaling and destruction. Physiol. Plant. 126, 45–51. doi: 10.1111/j.1399-3054.2005.00582.x

Thornalley, P. J. (1990). The glyoxalase system: new developments towards functional characterization of a metabolic pathway fundamental to biological life. Biochem. J. 269, 1–11. doi: 10.1042/bj2690001

Tuomainen, M., Ahonen, V., Kärenlampi, S. O., Schat, H., Paasela, T., and Svanas, A. (2011). Characterization of the glyoxalase I gene TcGLX1 in the metal hyperaccumulator plant Thlaspi caerulescens. Planta 233, 1173–1184. doi: 10.1007/s00425-011-1370-7

Veena, Reddy, V. S., and Sopory, S. K. (1999). Glyoxalase I from Brassica juncea: molecular cloning, regulation and its over-expression confer tolerance in transgenic tobacco under stress. Plant J. 17, 385–395. doi: 10.1046/j.1365-313X.1999.00390.x

Wahid, A., Gelani, S., Ashraf, M., and Foolad, M. R. (2007). Heat tolerance in plants: an overview. Environ. Exp. Bot. 61, 199–223. doi: 10.1016/j.enex.2007.05.011

Wang, J. Q., Yin, X., Wu, M. C., Zhang, H. M., Gao, S. J., Wei, J. T., et al. (2013). Expression of a family 10 xylanase gene from Aspergillus usamii E001 in Pichia pastoris and characterization of the recombinant enzyme. J. Ind. Microbiol. Biotechnol. 40, 75–83. doi: 10.1007/s10295-012-1201-2

Wang, W., Vinocur, B., Shoseyov, O., and Altman, A. (2004). Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends Plant Sci. 9, 244–252. doi: 10.1016/j.tplants.2004.03.006

Xu, X., Yan, B., Shi, M., Zhou, W., Zekria, D., Wang, H. Z., et al. (2015). Expression of a Brassica campestris HSP70 in tobacco confers enhanced tolerance to heat stress. Proteomics 15, 3567–3576. doi: 10.1002/pmic.201400483

Yadav, S. K., Singla-Pareek, S. L., Ray, M., Reddy, M. K., and Sopory, S. K. (2005a). Methylglyoxal levels in plants under salinity stress are dependent on glyoxalase I and glutathione. Biochem. Biophys. Res. Commun. 337, 61–67. doi: 10.1016/j.bbrc.2005.08.263

Yadav, S. K., Singla-Pareek, S. L., Reddy, M. K., and Sopory, S. K. (2005b). Methylglyoxal detoxification by glyoxalase system: a survival strategy during environmental stresses. Physiol. Mol. Biol. Plants 11, 1–11.

Zhang, T., Ge, C., Deng, L., Tan, T., and Wang, F. (2015). C4-dicarboxylic acid production by overexpressing the reductive TCA pathway. FEBS Microbiol. Lett. 362, 5052. doi: 10.1079/femsle/fov052

Zhou, Y., Chen, H., Chu, P., Li, Y., Tan, B., Ding, Y., et al. (2012). NnHSP17.5, a cytosolic class II small heat shock protein gene from Nelumbo nucifera, contributes to seed germination vigor and seedling thermotolerance in transgenic Arabidopsis. Plant Cell Rep. 31, 379–389. doi: 10.1007/s00299-011-1173-0

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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