

# A new isoform of thioredoxin h group in potato, *SbTRXh1*, regulates cold-induced sweetening of potato tubers by adjusting sucrose content

Tianjiu He · Botao Song · Jun Liu ·  
Xia Chen · Yongbin Ou · Yuan Lin ·  
Huiling Zhang · Conghua Xie

Received: 12 February 2012/Revised: 21 March 2012/Accepted: 2 April 2012/Published online: 18 April 2012  
© Springer-Verlag 2012

**Abstract** In order to study the molecular mechanism of the cold-induced sweetening (CIS) of potato tubers, a novel isoform of thioredoxin h group, *SbTRXh1*, which was up-regulated early in the 4 °C storage of CIS-resistant potato (*Solanum berthaultii*) tubers, was cloned in present research. The genetic transformation of over-expression (OE) and RNA interference (RNAi) of *SbTRXh1* into potato cv. E-Potato 3 (E3) was carried out to clarify its function in CIS regulation. The results showed that the transcripts of *SbTRXh1* in either OE- or RNAi-tubers were strongly induced in 4 °C storage and quantitatively related to the reducing sugar (RS) accumulation, indicating that *SbTRXh1* is involved in the CIS process of potato tubers. Regression analysis between the transcripts and protein contents of *SbTRXh1* showed a very significant logarithmic relationship implying that the expression of *SbTRXh1* may be mainly regulated at transcriptional level. Further monitoring the variation of the sugar contents in cold-stored tubers demonstrated a linear relationship between RS and sucrose (Suc). Thus, it can be inferred that *SbTRXh1* may function in the Suc–RS pathway for CIS regulation of potato tubers.

**Key message** *SbTRXh1* is primarily demonstrated to be involved in the regulation of cold-induced sweetening (CIS) of potato tubers, and it may function in the Suc–RS pathway for CIS regulation.

**Keywords** Potato · Cold-induced sweetening · *SbTRXh1* · Reducing sugar · Sucrose

## Abbreviations

CIS Cold-induced sweetening  
RS Reducing sugar

## Introduction

Potato (*Solanum tuberosum*) is the fourth most important food crop in the world. Potato tubers are often stored at low temperature in processing industries to prevent sprouting, water loss, and disease infection for prolonging their storage period. However, cold storage leads to accumulation of reducing sugar (RS) in the tubers, known as cold-induced sweetening (CIS) (Greiner et al. 1999). During frying, RS interacts with free amino acids, making the products unsavory and unsightly (Thill and Peloquin 1995). This reaction also generates a neurotoxin and potential carcinogen acrylamide (Shepherd et al. 2010). Therefore, exploring the mechanism of CIS has both scientific and applied importance (Muttucumararu et al. 2008). Changes in carbohydrate metabolism (Blenkinsop et al. 2004) and membrane properties (Wismer et al. 1998) have been postulated to explain CIS, but its regulation mechanism remains incompletely understood.

Thioredoxin h (TRX h) in higher plants is involved in numerous biological processes. TRX h can regulate cereal seed quality by controlling the compounds delivery during seed filling, promoting the mobilization of stored nitrogen and carbon in the endosperm in imbibed-seed, and protection against oxidative stress in the living tissues during seed maturation and germination (Lozano et al. 1996; Yano et al. 2001; Abderrakib et al. 2008; Li et al. 2009). TRX h

Communicated by E. Benvenuto.

T. He · B. Song · J. Liu · X. Chen · Y. Ou · Y. Lin ·  
H. Zhang · C. Xie (✉)  
National Centre for Vegetable Improvement (Central China);  
Key Laboratory of Horticultural Plant Biology (Huazhong  
Agricultural University), Ministry of Education, Huazhong  
Agricultural University, Wuhan 430070, China  
e-mail: xiech@mail.hzau.edu.cn

is also believed to be involved in other biological processes, such as self-incompatibility (Cabrillac et al. 2001), carbon and nitrogen metabolism (Kobrehel et al. 1991), defense responses (Sun et al. 2010) and seed sprouting (Guo et al. 2011). In the last 30 years, more than 400 potential thioredoxin target proteins were identified and some enzymes reported to contribute to CIS were included, such as Suc synthase (Blenkinsop et al. 2004), starch debranching enzyme (Cho et al. 1999) and alcohol dehydrogenase (Pinhero et al. 2007). However, information that directly indicates the association of thioredoxin to potato CIS is limited.

Based on 29 expressed sequence tags (ESTs) identified from a suppression subtractive hybridization (SSH) cDNA library that enriched the CIS-resistance genes of potato tubers (unpublished), an EST showing high similarity to thioredoxin was found to be up-regulated early in potato tubers stored at 4 °C. In present article, a novel TRX h gene, *SbTRXh1*, was cloned from a wild potato species *Solanum berthaultii* with CIS resistance and its involvement in CIS regulation of potato tubers was investigated.

## Materials and methods

### Cloning, sequencing and bioinformatics analysis of *SbTRXh1*

The *SbTRXh1* cDNA was cloned by rapid-amplification of cDNA ends (RACE) approach using a SMART<sup>TM</sup> RACE cDNA Amplification Kit (Clontech, USA) following the operation manual. Primers 5'-TTCTGAGGAAGGACAAGTGAT-3' (forward, for 3'-RACE) and 5'-TTAATGAAGCAAAGGTAGGCA-3' (reverse, for 5'-RACE) designed according to the EST sequence identified from the SSH cDNA library were used. The 5'- and 3'-fragments were spliced with the primer 5'-TTACGAAGAGCTAAGGATTTG-3' (forward) and Oligod (T)18 through the overlapping PCR way. The single-strand cDNA synthesized from the RNA of *S. berthaultii* tubers stored at 4 °C for 5 days was used as a template. The PCR products were gel-purified and cloned into the pMD18-T vector (TaKaRa, Japan) (the yielded plasmid was denoted as pMDTRX) for sequencing at Shanghai Sangon Biological Engineering Technology & Service Co., Ltd. (Sangon, China). The homologous sequences were analyzed using the National Center for Biotechnology Information (NCBI) blastn (nr/nt) (<http://www.ncbi.nlm.nih.gov>). The conserved domains of the deduced protein sequences were analyzed by the Pfam sequence search (<http://pfam.sanger.ac.uk>). Phylogenetic and molecular evolutionary analyses were conducted using the MEGA version 4. The protein theoretical molecular

weight (MW) was predicted by the Compute pI/Mw ([http://www.expasy.org/tools/pi\\_tool.html](http://www.expasy.org/tools/pi_tool.html)), and the signal peptide of putative proteins was forecasted by the Signal 3.0 server (<http://www.cbs.dtu.dk/services/SignalP-3.0>).

### Subcellular localization of *SbTRXh1*

The tobacco BY-2 cell line (*Nicotiana tabacum* L. cv. Bright Yellow 2) was kindly supplied by professor Mengxiang Sun (Key Laboratory of Plant Developmental Biology, Wuhan University, Ministry of Education) and cultured in darkness in liquid Murashige and Skoog (MS) medium (pH 5.8) containing 1.0 mg/L 2,4-D and 3 % sucrose (Suc) at 26 °C on an incubator at 130 rpm, and sub-cultured every 5 days; 4-day-old cells were used in the experiment.

The open reading frame (ORF) of *SbTRXh1* (70–438 bp) was cloned into pBI121GFP vector (35S CaMV) via *NruI/SmaI* (TaKaRa, Japan), resulted in pBITRX-GFP plasmid. The vector was verified by sequencing (Sangon, China) and transformed into BY-2 cells by particle bombardment as previously described (Von 2007). The transformed cells were incubated at 28 °C in darkness for 24 h. After treating with 0.5 M NaCl for 10 min, fluorescence detection was carried out using a confocal laser scanning microscope (LSM510 Meta, Zeiss, Germany) with excitation 488 nm for green fluorescent protein (GFP).

### Development of RNA interference (RNAi) and over-expression (OE) constructs

A 326-bp fragment of *SbTRXh1* (286–611 bp) was selected for constructing the RNAi vector, and the 372 bp ORF of *SbTRXh1* (70–441 bp) was used in constructing the OE vector. The plasmid pMDTRX was used as template in the DNA fragment cloning. The PCR products amplified using primers 5'-AAAAAGCAGGCTAAGAAATTGCAGAGGATTGGAATGT-3' (forward) and 5'-AGAAAG CTGGTTAGCCTTACACCAGTAAACACCATAT-3' (reverse) were transformed into the pHGRV vector to produce RNAi-construct pHGTRXR (35S CaMV) by the BP clonase pathway using Gateway R BP clonase<sup>TM</sup> II Enzyme mix (Invitrogen) following the operation manual. The OE-recombinant pBITRX was constructed by cloning the PCR products amplified using primers 5'-TTGAGCTCGGATCCTGTGAGAATGGCTACTTCAT-3' (forward) and 5'-TTGTCGACAGCA GTCACAATAGCAGGAG-3' (reverse) into the pBI121 vector (35S CaMV) through double restriction digestion (*Bam*HI/*Sac*I; TaKaRa, Japan) and DNA ligation reaction (T4 DNA Ligase; TaKaRa, Japan). The inserts of both constructs were confirmed by sequencing (Sangon, China).

## Transformation of *SbTRXh1* into potato

The transformations of pHGTRXR (RNAi) and pBITRX (OE) into potato (*S. tuberosum*) cv. E-potato 3 (E3) microtubers were carried out via *Agrobacterium*-mediated transformation as described by Si et al. (2003), except that 50 mg/mL kanamycin was used for the selection of OE-transgenic plants. As described previously (Ni et al. 2010), the confirmation of transformations was performed by PCR analysis with specific primers of *npt II* gene and Southern blot hybridization using the DIG-labeled *npt II* fragment as probe. The transcripts of *SbTRXh1* in three-week-old transgenic plants were detected by RT-PCR analysis using primers 5'-TGCTGCTCTGCTATTGTTGA-3' (forward) and 5'-TATACCACAAATGACCACATTCC-3' (reverse). The transcripts of the *elongation factor 1- $\alpha$*  (*ef1 $\alpha$* ) were used for normalization. Single-copy transgenic plants that showed expected transcripts of *SbTRXh1* were selected for further analysis.

## Storage of potato tubers and sugar content analysis

The RNAi- and OE-transgenic lines and the wild-type (WT) potato cv. E3 were grown in 24 cm  $\times$  24 cm plastic pots in a greenhouse at 20–25 °C. The mature tubers were harvested, placed at room temperature for 10 days for skin setting, and then divided into two groups for storage temperature treatments in darkness: one group was stored at 4 °C and the other at 20 °C. Tubers were sampled at time points of 0, 5, 15, and 30 days during storage. Sampled tubers were cut into small pieces, immediately frozen in liquid N<sub>2</sub> and kept in a –70 °C freezer for the molecular and biochemical analyses as indicated below. Starch, RS and Suc contents were determined as described previously (Song et al. 2005; Liu et al. 2010).

## PCR and qRT-PCR

The PCR programs for RACE, overlapping, and RNAi- and OE-recombinant construction were performed as follows: 94 °C for 3 min, 30 cycles of 94 °C for 45 s, 58 °C for 45 s and 72 °C for 1 min, and then 72 °C for 5 min. For the RT-PCR, the amplification cycles were reduced to 27.

RNA isolation, reverse transcription, and qRT-PCR were performed as described previously (Liu et al. 2010). The transcripts of *ef1 $\alpha$*  were used as an internal control. The specific primers for the qRT-PCR were 5'-GAAGTGGATAGGGTGGTTGG-3' (forward) and 5'-AGCAGCACCATGC TTCTCTA-3' (reverse). The relative gene expression data were analyzed using a comparative Cp method, as described by Roche. The PCR program was set up as: 95 °C for 30 s, 40 cycles of 95 °C for 15 s, 55 °C for 30 s and 72 °C for 5 s.

## Tricine-SDS-PAGE and Western blot hybridization

The total protein of sampled tubers was extracted using the plant total protein extraction kit (Applygen, China) following the operation manual. The total protein concentration was determined by Bradford analysis using the Protein Quantification Kit (Applygen, China) following the instructions of the manufacturer. The polyclonal antibody (rabbit) of SbTRXh1-PepEST “CHKVEDWEVQPQKGV” was produced by Hangzhou HuaAn Biotechnology Co., Ltd. (China). The antibody of  $\beta$ -tubulin (aC-18) was purchased from Santa Cruz Biotechnology, Inc., USA.

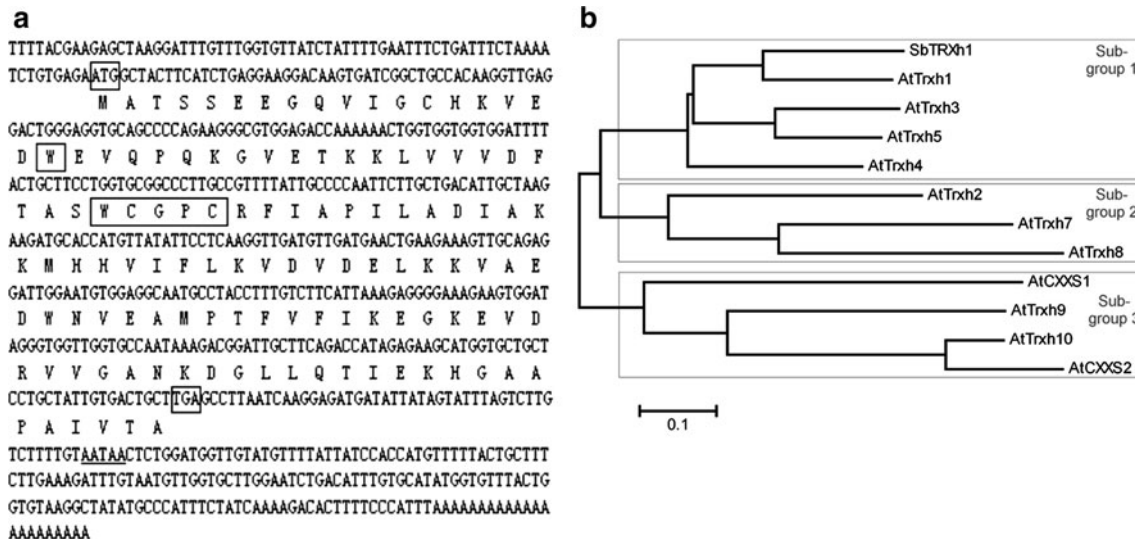
For each sample, approximately 30  $\mu$ g (for RNAi-transgenic lines and WT) or 15  $\mu$ g (for OE-transgenic lines and WT) total protein was subjected to 15 % Tricine-SDS-PAGE (12 % glycerine v/v, 1.83 M acrylamide, 0.023 M methylene diacrylamide, 1 M Tris-HCl pH 8.45, 0.1 % SDS w/v, 0.1 % AP w/v, and 0.05 % TEMED v/v), and then transferred onto a PVDF membrane (0.45  $\mu$ m) in mini-PROTEAN electrophoresis cells (Bio-Rad). Western blot was performed using the PepEST-antibody as the primary antibody and the goat anti-rabbit IgG conjugated with horse radish peroxidase as the secondary antibody. The protein bands were visualized by enhanced chemiluminescence detection reagents (Applygen, China). The protein content of  $\beta$ -tubulin was taken as an internal control.

## Results

### Cloning and characterization of *SbTRXh1* cDNA

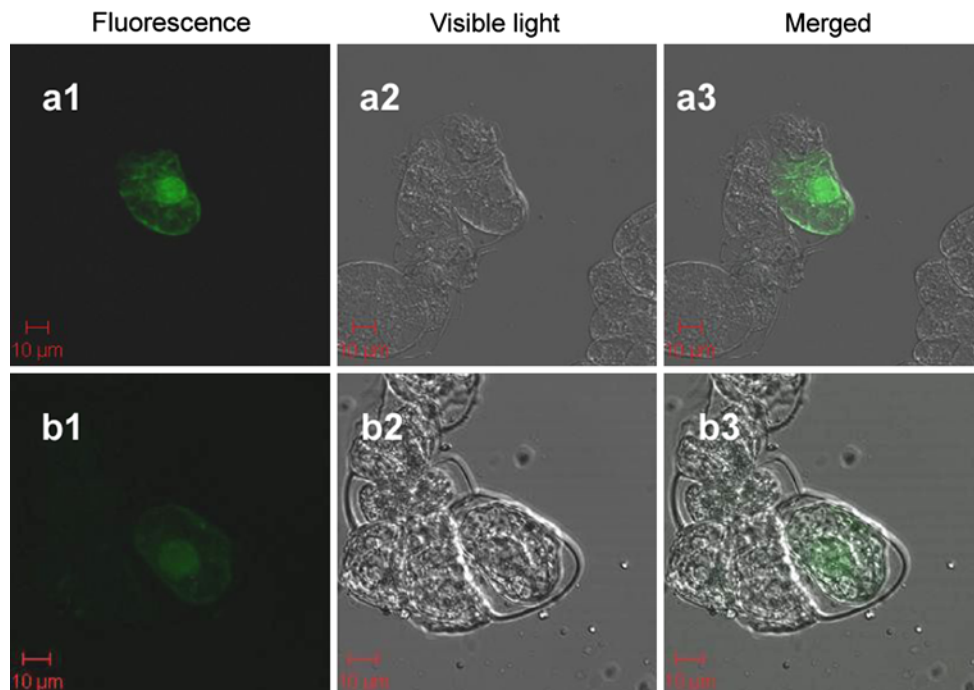
*SbTRXh1* was cloned from the cold-stored tubers of *S. berthaultii*. The 5'-RACE of *SbTRXh1* resulted in a 340 bp fragment, while the 3'-RACE produced a 587 bp fragment. The 258 bp common fragment showed 100 % identity with the EST of *SbTRXh1*. Splicing of the two fragments yielded a 669 bp sequence of *SbTRXh1* gene containing a 72 bp 5'-untranslated region and a 228 bp 3'-untranslated region (Fig. 1a). The ORF of *SbTRXh1* encodes a protein of 123 amino acids (aa) which has an estimated MW of 13.56 kDa and isoelectric point of 5.54 without signal peptide. The *SbTRXh1* gene was submitted to the GenBank database (GenBank ID: DQ413184.1).

The blastn (nr/nt) analysis of *SbTRXh1* in NCBI showed that the full-length cDNA of the gene shared 91 % identity with an isoform of thioredoxin h group in *Capsicum annuum* (GenBank ID: EF371503.1) and 88 % identity with the thioredoxin from *N. tabacum* (GenBank ID:  $\times$ 58527.1). The sequence search analysis in Pfam showed that the deduced protein sequence of *SbTRXh1* contained a conserved region of thioredoxin family from the first to the 104th aa, with a characteristic tryptophan (W) of h1



**Fig. 1** Characterization of SbTRXh1 cDNA. **a** Nucleotide sequence of SbTRXh1 cDNA and deduced amino acid sequence. The deduced protein sequence is *underneath* the corresponding nucleotide sequence. The putative polyA signal (AATAA) is *underlined*. The conserved active site (WCGPC), characteristic tryptophan (W), and the beginning and stop codons are framed. **b** The evolutionary relationship of SbTRXh1 to isoforms of thiooxidin h group from *Arabidopsis thaliana*. The phylogenetic tree was constructed by

neighbor-joining method with amino acid sequences. The GenBank accession numbers of the genes used in the construction of the phylogenetic tree are AtTrxh1 (P29448.1), AtTrxh2 (Q38879.2), AtTrxh3 (Q42403.1), AtTrxh4 (Q39239.2), AtTrxh5 (Q39241.1), AtTrxh7 (Q9XIF4.1), AtTrxh8 (Q9CAS1.1), AtTrxh9 (Q9C9Y6.1), AtTrxh10 (Q9LXZ8.2), AtCXXS1 (AF144390) and AtCXXS2 (ATU35639)

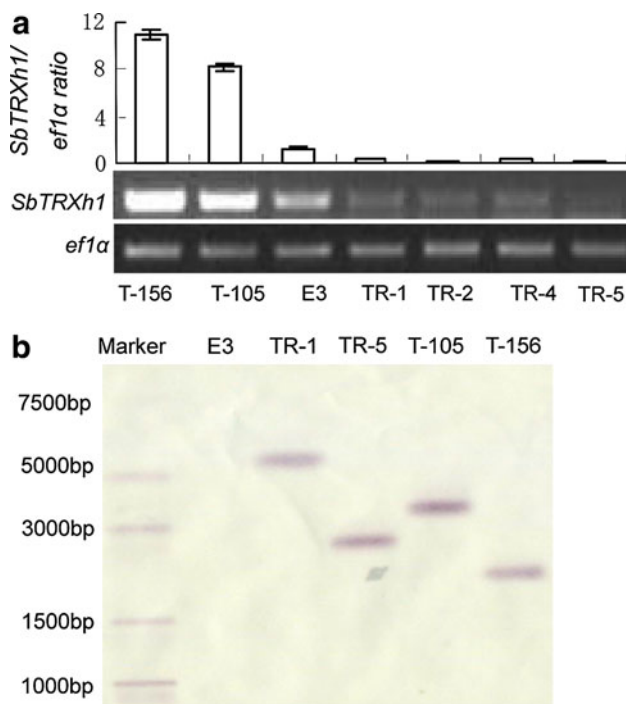


**Fig. 2** Subcellular localization of SbTRXh1 BY-2 cell line was transformed by particle bombardment with pBI121-GFP and pBITRX-GFP plasmids respectively. **a1–a3** Transient expression of pBI121-GFP shows *green* fluorescent protein (GFP) signals in whole

cell. **b1–b3** Transient expression of SbTRXh1-GFP shows GFP signals mainly in nucleus and cytoplasm. The *scale bars* stand for 10  $\mu$ m (color figure online)

subgroup at the 19th aa and a conserved active site (WCGPC) (Gelhaye et al. 2004) from the 41st aa (Fig. 1a). *SbTRXh1* was assigned to the thiooxidin h1 subgroup by

phylogenetic analysis with the TRX h isoforms from *Arabidopsis thaliana*, showing a close relationship with *AtTrxh1* (P29448.1) (Fig. 1b).



**Fig. 3** Selection of transgenic lines for further study. **a** RT-PCR-based transcript analysis of *SbTRXh1* gene in transgenic and WT potato plants. RNA from 3-week-old transgenic plants was used in the analysis. The transcripts of the elongation factor-1 $\alpha$  were used for normalization. The electrophoretic bands were quantized using Quantity One 1-D analysis software (Bio-Rad, USA), and the *SbTRXh1*/*ef1 $\alpha$*  ratio was calculated by dividing the value of *SbTRXh1* bands by that of *ef1 $\alpha$*  in each sample. The error bar indicates the SE of the three replications. **b** Southern blot analysis on genomic DNA from transgenic and WT potato plants. The genomic DNA was restriction digested with *EcoRI*. The DIG-labeled 676 bp *npt II* fragment was taken as probe. Single-copy transgenic lines TR-1, TR-5, T-105 and T-156, which showed expected transcriptional expression of *SbTRXh1*, were chosen for further study. *E3* WT of E-potato 3, *TR-1* *SbTRXh1*-Ri-E3-1, *TR-2* *SbTRXh1*-Ri-E3-2, *TR-4* *SbTRXh1*-Ri-E3-4, *TR-5* *SbTRXh1*-Ri-E3-5, *T-105* *SbTRXh1*-OE-E3-105, *T-156* *SbTRXh1*-OE-E3-156

Transient expression of plasmids pBI121GFP and pBITRX-GFP in BY-2 cell was carried out to figure out the subcellular localization of *SbTRXh1*. As shown in Fig. 2, fluorescent signals could be detected in whole of the cells for GFP alone, while *SbTRXh1* were mainly localized in cytoplasm and nucleus. However, whether the GFP signal in nucleus is due to the transient expression or the physiological significance of *SbTRXh1* will be further studied.

#### Development and selection of transgenic plants

Five RNAi-transgenic and 33 OE-transgenic lines were obtained from 20 and 170 regenerated plants, respectively. According to the results of the RT-PCR (Fig. 3a) and Southern blot analysis (Fig. 3b), the RNAi-transgenic lines *SbTRXh1*-Ri-E3-1 (TR-1) and *SbTRXh1*-Ri-E3-5 (TR-5),

and the OE-transgenic lines *SbTRXh1*-OE-E3-105 (T-105) and *SbTRXh1*-OE-E3-156 (T-156) with single copy of the insertion were chosen for further study. Among them, the transcripts of *SbTRXh1* were decreased by 66.1 % in TR-1 and by 88.2 % in TR-5, whereas the transcripts were increased to 6.3- and 8.5-fold in T-105 and T-156, respectively (Fig. 3a), representing expected transcriptional expression of *SbTRXh1*.

#### Transcripts of *SbTRXh1* and *SbTRXh1* protein contents in stored potato tubers

During the storage, both transcripts and protein contents of *SbTRXh1* in the RNAi-transgenic tubers were significantly lower than those in the WT tubers at each time point, the reverse was true for the OE-transgenic tubers (Fig. 4).

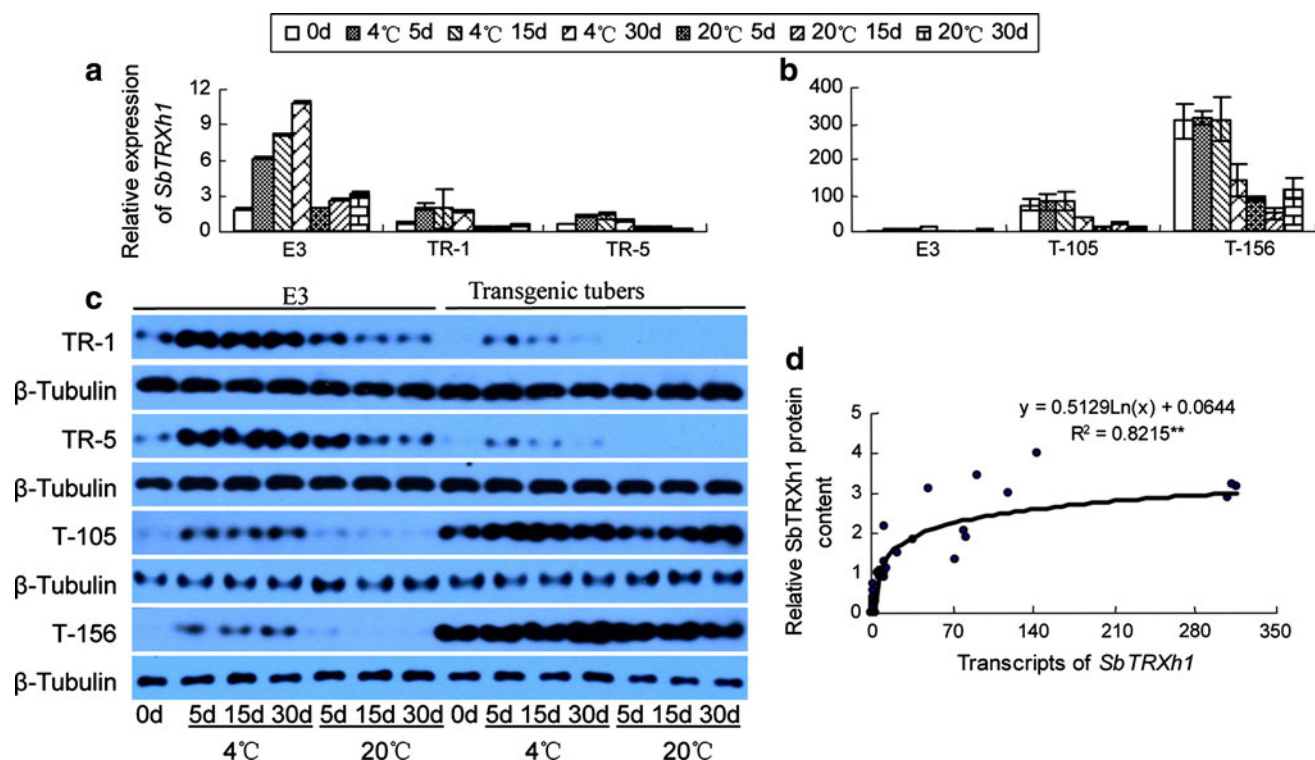
Compared with WT tubers at each time point, transcripts of *SbTRXh1* were suppressed by 62.5–89.4 % in TR-1 and by 65.7–92.7 % in TR-5 (Fig. 4a), while they were increased to 3.4- to 39.4-fold in T-105 and to 13.2- to 166.4-fold in T-156 (Fig. 4b). The results also showed that low temperature (4 °C) induced a dramatically higher expression of *SbTRXh1* when compared to the tubers stored at 20 °C, suggesting *SbTRXh1* is cold-inducible.

Similar tendency was found in *SbTRXh1* protein content. As shown in Fig. 4c, the RNAi-transgenic tubers had remarkably lower protein contents, whereas the OE-transgenic tubers yielded higher protein contents in comparison with the WT tubers. The storage temperature had obvious effect on *SbTRXh1* protein content; a relatively higher protein content was detected at each time point of 4 °C storage compared with the tubers stored at 20 °C. The analysis further indicated that the protein content (y) showed a high correlation with the abundance of the transcripts (x) represented by a function of  $y = 0.064 + 0.512 \ln x$  ( $r^2 = 0.821$ ,  $n = 56$ ) (Fig. 4d), implying that the function of *SbTRXh1* could be mainly regulated at transcriptional level.

#### Content changes of RS, Suc and starch in stored tubers

It is expected that the low temperature (4 °C) storage led to a continuous increase in RS and Suc content, whereas they were not obviously changed in the tubers stored at 20 °C (Fig. 5a, b). Interestingly, there were no significant shifts in starch content observed in the tubers stored either at 4 or 20 °C (Fig. 5c), leading to a speculation that Suc might be more responsive to CIS than starch in potato tubers.

In comparison with the WT tubers, the RNAi-transgenic tubers exhibited a lower RS content showing 33.7–65.2 % reduction (Fig. 5a) when tubers are stored at 4 °C. Similar results were obtained when measuring the Suc content of these tubers (Fig. 5b), demonstrating that suppression of



**Fig. 4** Analysis of transcripts of *SbTRXh1* and *SbTRXh1* protein content of stored potato tubers. **a, b** Expression patterns of *SbTRXh1* in stored tubers analyzed by RT-qPCR approach. The transcripts of the elongation factor 1- $\alpha$  was set to be 100 and used as normalization in RT-qPCR analysis. The error bar indicates the SE of the three replications. **c** Analysis of *SbTRXh1* protein content in tubers stored at 4 and 20 °C by Western blot. The PepEST-specific polyclonal antibody and  $\beta$ -tubulin-specific monoclonal antibody were used for the detection of *SbTRXh1* and  $\beta$ -tubulin, respectively. **d** Relationship

between relative expression of *SbTRXh1* and relative *SbTRXh1* protein content. Protein bands in Fig. 3c were quantized using Quantity One 1-D analysis software (Bio-Rad, USA), and protein content in E3 tubers stored at 4 °C for 5 days (E3-4-5) was set to be 1, the relative *SbTRXh1* protein content was calculated by dividing the value of protein bands by that of E3-4-5 in the same membrane. \*\*Significant at the  $p < 0.01$  level. E3 WT of E-potato 3, TR-1 *SbTRXh1*-Ri-E3-1, TR-5 *SbTRXh1*-Ri-E3-5, T-105 *SbTRXh1*-OE-E3-105, T-156 *SbTRXh1*-OE-E3-156

the *SbTRXh1* gene had impact on accumulation of Suc and RS in potato tubers subjected to low temperature. However, much difference in RS content between the OE- and WT tubers was not detected at 4 °C after 15 days but a lower Suc content in the former than in the latter was observed, which may reflect a high functional potential of the endogenous *SbTRXh1* gene or its homologue in the recipient plant.

In stored RNAi-transgenic tubers, the RS (or Suc) contents were parallel to the transcripts of *SbTRXh1* and could be shown by a linear function (Fig. 5d, e). These results indicated a close relationship between *SbTRXh1* expression and RS (or Suc) accumulation in WT and RNAi-transgenic tubers. However, when the data of OE-transgenic tubers were added, the correlations between the transcripts of *SbTRXh1* and RS (or Suc) contents lost their significance because magnificent abundance of *SbTRXh1* transcripts in OE tubers were accompanied by limited accumulation of RS and Suc.

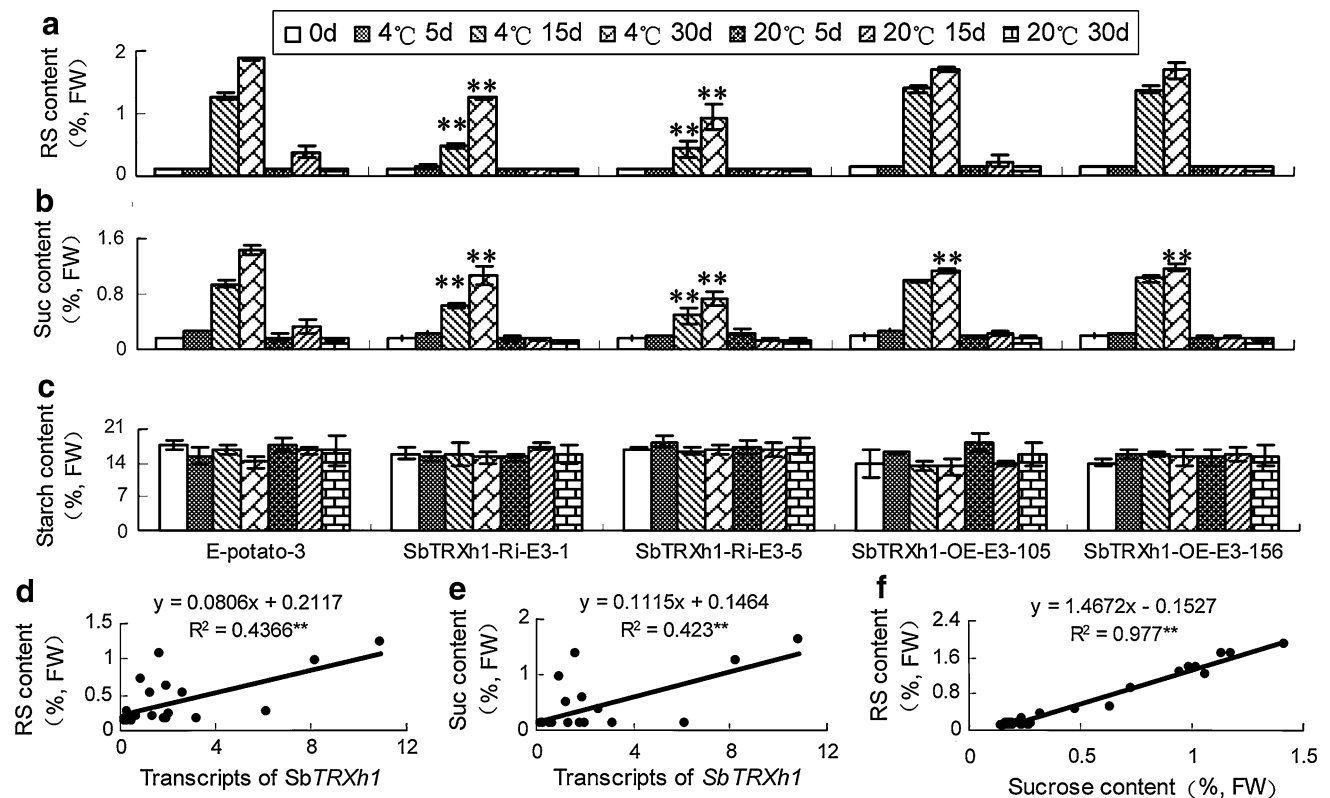
Our results also demonstrated a very significant positive linear correlation between RS and Suc content (Fig. 5f),

implying that the degradation of Suc may be a main source of RS accumulation in cold-stored potato tubers.

## Discussion

In present study, the 5'- and 3'-fragments of the *SbTRXh1* gene were cloned separately. The overlapping sequence showed 100 % identity with the corresponding EST from the SSH library, which confirmed correct cloning of the gene. Results of blastn (nr/nt) analysis in NCBI, sequence search in Pfam, and phylogenetic analysis (Fig. 1) demonstrate that *SbTRXh1* is a gene that encodes a new protein of thioredoxin h1 subgroup (Gelhaye et al. 2004) in potato.

Plant thioredoxins were reported to be involved in responses to various biotic and abiotic stresses (Kocsy et al. 2004). In our research, the transcripts of *SbTRXh1* and *SbTRXh1* protein accumulated in both WT and transgenic tubers stored at 4 °C, indicating that *SbTRXh1* is associated with the cold response of potato tubers (Fig. 4). This finding is in accordance with previous reports that



**Fig. 5** Sugar contents and correlations between sugar contents and transcripts of *SbTRXh1* of potato tubers in storage. **a–c** Contents of reducing sugar (RS), sucrose (Suc) and starch of potato tubers during the storage at 4 and 20 °C. The error bar indicates the SE of the three

replications. **d–f** Correlations between sugar contents and transcripts of *SbTRXh1* in RNAi-transgenic and WT potato E-potato-3 tubers stored at 4 and 20 °C, and between RS and Suc contents in all stored tubers. **\*\***Significant at the  $p < 0.01$  level

thioredoxins could be induced by cold treatment in maize (Kocsy et al. 2004) and rice (Xie et al. 2009).

During cold storage, the starch in potato tubers is converted into hexosephosphate, which can generate Suc 6-phosphate and then Suc (Halford et al. 2011). The accumulation of Suc leads to an increase of cytochlema concentration to cope with the cold. Furthermore, Suc can be degraded into glucose and fructose (Malone et al. 2006) resulting in CIS. In the present research, RS content was increased in the tubers stored at 4 °C (Fig. 5a). More importantly, this increase was significantly correlated to the expression of *SbTRXh1* (Fig. 5d), strongly suggesting that the *SbTRXh1* gene is involved in regulation of CIS in potato tubers.

Further analysis of present research showed a logarithmic relationship between transcripts and its protein content of the *SbTRXh1* gene (Fig. 4d), implying that the function of *SbTRXh1* in potato CIS may be mainly regulated at the transcriptional level. Protein content of PsTRXh1, an isoform thioredoxin h group in Pea (*Pisum sativum*) L. cv Lincoln, showed a positive correlation with transcripts of *PsTRXh1* in leaves and roots under oxidative stress (Traverso et al. 2007). In potato, protein content of CDSP 32, a drought-induced thioredoxin, was increased with the

accumulation of transcripts when plants were exposed to high light/low temperature (8 °C) or  $\gamma$ -irradiation (Broin et al. 2000). However, Broin and colleagues also found that treatment with 10  $\mu$ M methyl viologen led to a sharp increase of both transcripts and protein content of CDSP 32 at 6 h, but subsequently, the protein content continued to rise whereas the transcripts showed a consecutive drop. These results may suggest different regulation models of thioredoxin in response to varied stimulations.

Decreasing in RS accumulation in RNAi-transgenic tubers was accompanied by a declined Suc (Fig. 5a, b) and a stable starch content (Fig. 5c), demonstrating that the CIS of potato tubers might mainly result from Suc degradation. This relationship was further confirmed by analyzing all type of tubers in present research which still showed a significant relationship between RS and Suc (Fig. 5f) but not statistically significant between RS and starch. Therefore, it is reasonable to speculate that *SbTRXh1* may function in Suc–RS pathway to regulate potato CIS. It is known that thioredoxin functions by reducing disulfide bridge of target protein with a CXXC active site. Some enzymes involved in Suc–RS pathway have been identified to be potential target proteins of thioredoxin, such as ADP-glucose pyrophosphorylase and phosphoglucomutase

(Lindahl and Florencio 2003), UDP-glucose pyrophosphorylase (Wong et al. 2004; Alkhalifioui et al. 2007) and sucrose synthase 1 (Hägglund et al. 2008). There could be more target proteins not revealed so far that are worthy of further investigation to elucidate the mechanism by which *SbTRXh1* implements its function in the CIS process of potato tubers.

When stored at 4 °C after 15 days, there was no significant difference in RS content observed between OE-transgenic and WT tubers (Fig. 5a), and the Suc content in the former was slightly lower than that in the latter (Fig. 5b). This phenomenon might have resulted from a function potential of endogenous *SbTRXh1* gene and its homologues. Although little information is available for potato TRX h isoforms, their complex composition in other plant species have been reported, for example, there are 9 TRX h isoforms in rice (Nuruzzaman et al. 2008), 11 in *A. thaliana* (Meyer et al. 2008) and 12 in *Medicago truncatula* (Renard et al. 2011). Another possible explanation could be that *SbTRXh1* may not play a direct role in CIS regulation; looking into its target proteins could be necessary to reveal this speculation. Characterization of molecular modifications (e.g., accumulation of sucrose precursors, fructose and glucose) in RNAi-transgenic lines would be helpful in the investigation of target proteins of *SbTRXh1* in the regulation of potato CIS. In barley grain, over-expression of a TRX h with a signal peptide sequence for targeting to the protein body using an endosperm-specific B1-hordein promoter led to release of starch-hydrolyzing enzymes and reducing storage proteins (Cho et al. 1999; Wong et al. 2002). In our research, there was no significant difference in starch content found between OE-transgenic and WT tubers, suggesting that the functions of TRX h isoforms may be isoform-specific.

**Acknowledgments** This research was partially supported by grants from Teacher Foundation of Ministry of Education (20070504085), National Natural Science Foundation of China (30571181) and Earmarked Fund for Modern Agro-industry Technology Research System (CARS-10-P06).

## References

- Abderrakib Z, Samia A, Roland C (2008) Thioredoxin h system and wheat seed quality. *Cereal Chem* 85:799–807
- Alkhalifioui F, Renard M, Vensel WH, Wong J, Tanaka CK, Hurkman WJ, Buchanan BB, Montrichard F (2007) Thioredoxin-linked proteins are reduced during germination of seeds of *Medicago truncatula*. *Plant Physiol* 144:1559–1579
- Blenkinsop RW, Yada RY, Marangoni AG (2004) Metabolic control of low-temperature sweetening in potato tubers during postharvest storage. *Hortic Rev* 30:317–354
- Broin M, Cuine S, Peltier G, Rey P (2000) Involvement of CDSP 32, a drought-induced thioredoxin, in the response to oxidative stress in potato plants. *FEBS Lett* 467:245–248
- Cabrillac D, Cock JM, Dumas C, Gaude T (2001) The S-locus receptor kinase is inhibited by thioredoxins and activated by pollen coat proteins. *Nature* 410:220–223
- Cho MJ, Wong JH, Marx C, Jiang W, Lemaux PG, Buchanan BB (1999) Overexpression of thioredoxin h leads to enhanced activity of starch debranching enzyme (pullulanase) in barley grain. *Proc Natl Acad Sci USA* 96:14641–14646
- Gelhay E, Nicolas R, Jean-Pierre J (2004) The thioredoxin h system of higher plants. *Plant Physiol Biochem* 42:265–271
- Greiner S, Rausch T, Sonnewald U, Herbers K (1999) Ectopic expression of a tobacco invertase inhibitor homolog prevents cold-induced sweetening of potato tubers. *Nat Biotechnol* 17:708–711
- Guo H, Zhang H, Li Y, Ren J, Wang X, Niu H, Yin J (2011) Identification of changes in wheat (*Triticum aestivum* L.) seeds proteome in response to anti-trx s gene. *PLoS ONE* 6(7):e22255. doi:10.1371/journal.pone.0022255
- Hägglund P, Bunkenborg J, Maeda K, Svensson B (2008) Identification of thioredoxin protein disulfide targets using a quantitative proteomics approach based on isotope-coded affinity tags. *J Proteome Res* 7:5270–5276
- Halford NG, Curtis TY, Muttucumaru N, Postles J, Mottram DS (2011) Sugars in crop plants. *Ann Appl Biol* 158:1–25
- Kobrehel K, Yee BC, Buchanan BB (1991) Role of the NADP/thioredoxin system in the reduction of  $\alpha$ -amylase and trypsin inhibitor proteins. *J Biol Chem* 266:16135–16140
- Kocsy G, Kobrehel K, Szalai G, Duviau MP, Buzás Z, Galib G (2004) Abiotic stress-induced changes in glutathione and thioredoxin h levels in maize. *Environ Exp Bot* 52:101–112
- Li YC, Ren JP, Cho MJ, Zhou SM, Kim YB, Guo HX, Wong JH, Niu HB, Kim HK, Morigasaki S, Lemaux PG, Frick OL, Yin J, Buchanan BB (2009) The level of expression of thioredoxin is linked to fundamental properties and applications of wheat seeds. *Mol Plant* 2:430–441
- Lindahl M, Florencio FJ (2003) Thioredoxin-linked processes in cyanobacteria are as numerous as in chloroplasts, but targets are different. *Proc Natl Acad Sci USA* 100:16107–16112
- Liu X, Song BT, Zhang HL, Li XQ, Xie CH, Liu J (2010) Cloning and molecular characterization of putative invertase inhibitor genes and their possible contributions to cold-induced sweetening of potato tubers. *Mol Genet Genomics* 284:147–159
- Lozano RM, Wong JH, Yee BC, Peters A, Kobrehel K, Buchanan BB (1996) New evidence for a role for thioredoxin h in germination and seedling development. *Planta* 200:100–106
- Malone JG, Mittova V, Ratcliffe RG, Kruger NJ (2006) The response of carbohydrate metabolism in potato tubers to low temperature. *Plant Cell Physiol* 47:1309–1322
- Meyer Y, Siala W, Bashandy T, Riondet C, Vignols F, Reichheld JP (2008) Glutaredoxins and thioredoxins in plants. *Biochim Biophys Acta* 1783:589–600
- Muttucumaru N, Elmore JS, Curtis T, Mottram DS, Parry MAJ, Halford NG (2008) Reducing acrylamide precursors in raw materials derived from wheat and potato. *J Agric Food Chem* 56:6167–6172
- Ni XM, Tian ZD, Liu J, Song BT, Li JC, Shi XL, Xie CH (2010) StPUB17, a novel potato UND/PUB/ARM repeat type gene, is associated with late blight resistance and NaCl stress. *Plant Sci* 178:158–169
- Nuruzzaman M, Gupta M, Zhang C, Wang L, Xie W, Xiong L, Zhang Q, Lian X (2008) Sequence and expression analysis of the thioredoxin protein gene family in rice. *Mol Genet Genomics* 280:139–151
- Pinhero RG, Copp LJ, Amaya C, Marangoni AG, Yada RY (2007) Roles of alcohol dehydrogenase, lactate dehydrogenase and pyruvate decarboxylase in low-temperature sweetening in



- tolerant and susceptible varieties of potato (*Solanum tuberosum*). *Physiol Plant* 130:230–239
- Renard M, Alkhalfioui F, Schmitt-Keichinger C, Ritzenthaler C, Montrichard F (2011) Identification and characterization of thioredoxin h isoforms differentially expressed in germinating seeds of the model legume *Medicago truncatula*. *Plant Physiol* 155:1113–1126
- Shepherd LVT, Bradshaw JE, Dale MFB, McNicol JW, Pont SDA, Mottram DS, Davies HV (2010) Variation in acrylamide producing potential in potato: segregation of the trait in a breeding population. *Food Chem* 123:568–573
- Si HJ, Xie CH, Liu J (2003) An efficient protocol for *Agrobacterium*-mediated transformation with microtuber and the introduction of an antisense class patatin gene into potato. *Acta Agron Sin* 29:801–805
- Song BT, Xie CH, Liu J (2005) Expression of potato sAGP gene and its effects on contents of starch and reducing sugar of transgenic potato tubers. *Sci Agric Sin* 38:1439–1446
- Sun L, Ren H, Liu R, Li B, Wu T, Sun F, Liu H, Wang X, Dong H (2010) An h-type thioredoxin functions in tobacco defense responses to two species of viruses and an abiotic oxidative stress. *Mol Plant Microbe Interact* 23:1470–1485
- Thill CA, Peloquin SJ (1995) A breeding method for accelerated development of cold chipping clones in potato. *Euphytica* 84:73–80
- Traverso JA, Vignols F, Cazalis R, Pulido A, Sahrawy M, Cejudo FJ, Meyer Y, Chueca A (2007) PsTRXh1 and PsTRXh2 are both pea h-type thioredoxins with antagonistic behavior in redox imbalances. *Plant Physiol* 143:300–311
- Von AA (2007) Subcellular localization of GUS- and GFP-tagged proteins in onion epidermal cells. *Cold Spring Harb Protoc* 3:pdb.prot4689
- Wismer WV, Worthing WM, Yada RY, Marangoni AG (1998) Membrane lipid dynamics and lipid peroxidation in the early stages of low-temperature sweetening in tubers of *Solanum tuberosum*. *Physiol Plant* 102:396–410
- Wong JH, Kim YB, Ren PH, Cai N, Cho MJ, Hedden P, Lemaux PG, Buchanan BB (2002) Transgenic barley grain overexpressing thioredoxin shows evidence that the starchy endosperm communicates with the embryo and the aleurone. *Proc Natl Acad Sci USA* 99:16325–16330
- Wong JH, Cai N, Balmer Y, Tanaka CK, Vensel WH, Hurkman WJ, Buchanan BB (2004) Thioredoxin targets of developing wheat seeds identified by complementary proteomic approaches. *Phytochemistry* 65:1629–1640
- Xie GS, Kato H, Sasaki K, Imai R (2009) A cold-induced thioredoxin h of rice, OsTrx23, negatively regulates kinase activities of OsMPK3 and OsMPK6 in vitro. *FEBS Lett* 583:2734–2738
- Yano H, Wong JH, Cho MJ, Buchanan BB (2001) Redox changes accompanying the degradation of seed storage proteins in germinating rice. *Plant Cell Physiol* 42:879–883