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Amylase Analysis in Potato Starch Degradation During Cold Storage and Sprouting

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Abstract As the fourth most important food crop, potato plays a key role in food safety and economic development of the world. Harvested potato tubers can be stored for a long time, but sprouting and cold-induced sweetening (CIS) can seriously affect the quality of tubers during storage. One of the key pathways involved in CIS is starch degradation, in which both α-amylase and β-amylase play important roles. However, each amylase belongs to an extensive gene family and it is not clear which genes are the key regulators. In this study, we identified genes most likely regulating starch degradation. We first selected candidate genes from the public potato genome database and then investigated their expression patterns associated with reducing sugars and amylase activities. The results showed that the activity of α-amylase was mainly caused by *StAmy23* and the activity of β-amylase was mainly caused by *StBAM1* and *StBAM7*. In addition, α-amylase and β-amylase may play important roles in starch degradation of the tubers stored at low temperature and during sprouting, and the amylase activity may be regulated by the amylase inhibitor in cold-stored tubers.

Keywords Amylase genes · Amylase inhibitor · Cold-induced sweetening · Potato · Sprouting

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Introduction

Potato (*Solanum tuberosum* L.) is the fourth most important crop behind the cereal species rice (*Oryza sativa*), wheat (*Triticum aestivum*), and maize (*Zea mays*) and plays crucial roles in food security and economic development in the world. Tubers contain high-quality proteins and substantial amounts of vitamins, minerals, and trace elements. Therefore, there is a huge demand for potato tubers all year round. However, potato is grown in moderate climates, so continuous cultivation throughout the year is impossible, which makes tuber storage essential.

During storage, sprouting seriously affects potato quality since tubers need a large amount of water and nutrients to break bud dormancy. Previous studies have shown that bud dormancy can be regulated by changing hormones (Horvath et al. 2003; Rohde et al. 2007). The maintenance of tuber bud dormancy seems to involve abscisic acid (ABA) (Suttle 1995; Biemelt et al. 2000), whereas bud dormancy release possibly needs gibberellins (GA) and cytokinins (Suttle 2004; Hartmann et al. 2011). Starch degradation is also involved in the process of dormancy and sprouting, but the relationship between amylase activity and sprouting is not clear. Bailey et al. (1978) reported that a transient increase in α-amylase occurred at the time of sprouting. Biemelt et al. (2000) found an increase of both α-amylase and β-amylase in the sub-eye tissue after the onset of sprouting. However, Davies and Viola (1988) reported a decrease in total amylase activity during sprouting.

When potato tubers are stored under cold conditions post harvest, sprouting is inhibited but reducing sugars (RS) can accumulate, a process known as cold-induced sweetening (CIS). RS can cause a nonenzymatic Maillard reaction at a high frying temperature, which leads to unacceptable dark-colored products (Talburt et al. 1975). More concernedly, this reaction can also generate carcinogen acrylamide (Shepherd et al. 2010). The carbohydrate metabolic pathways of potato tubers have been extensively investigated, and some genes encoding the enzymes involved in the CIS pathway of potato tubers have been studied. In order to clarify the key pathways of CIS, the expression pattern of 188 genes from the cDNA microarray was analysed and results showed that amylolysis, sucrose decomposition, and glycolysis pathways play important roles in potato CIS (Chen et al. 2012).

Starch degradation occurs hydrolytically or phosphorolytically. The hydrolytic pathway involves enzymes like α-amylase and β-amylase, while the phosphorolytic pathway involves starch phosphorylase (Preiss 1982; Solomos and Mattoo 2005). It was found that the activity of starch-degrading enzymes was increased in the process of potato tuber CIS (Cottrell et al. 1993) although, previously, there were some arguments for a predominant pathway (Morrell and Rees 1986) and incompatible results between potato genotypes (Claassen et al. 1993; Hill et al. 1996). It has been shown that a potato RING finger gene *SbRFP1* plays an important role in inhibiting β-amylase, which consequently slows down starch degradation and the accumulation of reducing sugars in cold-stored tubers (Zhang et al. 2013). With the release of genome information, genes encoding enzymes related to starch degradation are now available. However, their functions in potato CIS are still unclear.

In this study, we selected genes encoding enzymes related to starch degradation and analysed their expression patterns. We found a few amylase genes that may play important roles in starch degradation and further discussed the regulation mechanisms of the amylase activity.

Methods and Materials

Genes Related to Starch Degradation

Genes encoding key enzymes in the process of starch degradation were tested in this study. Two α-amylase genes, seven β-amylase genes, five isoamylase genes, two starch phosphorylase genes, and two amylase inhibitor genes were selected from the Potato Genome Sequence Consortium (PGSC) database and the National Center of Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) database. Amino acid sequences of the β-amylase and isoamylase genes of *Arabidopsis thaliana* were selected from the NCBI database. The phylogenetic tree was constructed using CLUSTAL W (2.0) (Tompson et al. 1997) with neighbor-joining 1,000 bootstrap analysis. The tree obtained was viewed using TreeView software (version 1.6.6).

Plant Materials and Tuber Storage

CIS-tolerant potato genotypes (*Solanum berthaultii*, AC030-06, and AC142-01) and CIS-sensitive genotypes (AC035-01, AC041-03, and E3) were used in the present research (Liu et al. 2010). The plants were grown at 20–25 °C in diameter of 24 cm plastic pots in the greenhouse of Huazhong Agricultural University (Wuhan, China) with 16 h light per day, supplemented with mercury lamps. Leaves and stems were sampled at tuber formation stage about 45 days after planting. When mature tubers were harvested, a portion of them was kept at 20 °C in darkness. After about 75 days of storage, tubers without visible outgrowths were considered to be before sprouting and tubers with outgrowths longer than 0.5 cm were considered to be after sprouting. Tuber discs 1.5 cm in diameter around the buds were sampled from tubers both before and after sprouting. For the analysis of cold storage, tubers were kept at 20 °C for 10 days for the skin set. Then, they were divided into two groups: one group was stored at 4 °C in darkness as the cold treatment and the other at 20 °C for comparison. Tubers were sampled at 0, 5, 15, 30, and 60 days after storage, immediately peeled and frozen in liquid nitrogen, and then stored at −80 °C for further molecular and biochemical analyses.

RNA Isolation and Real-Time qRT-PCR

RNA isolation and reverse transcription were performed as described previously (Liu et al. 2010). The real-time reverse-transcription PCR (qRT-PCR) was performed to quantify the transcripts on the CFX96^{TM} real-time PCR system (Bio-Rad, USA). A total volume of 20 μL PCR contained 1 μL of cDNA, 0.6 μL primers (10 μmol L^{-1}) for specific gene, and 1× SsoAdvanced^{TM} SYBR® Green supermix (Bio-Rad, USA). The amplification program was 95 °C for 30 s, 40 cycles of 95 °C for 5 s, and 56 °C for 30 s. Relative quantification of each individual gene expression was calculated with the $2^{-\Delta CT}$ method as described in the manufacturer's introduction. *Ef1α* of potato (GenBank accession no. AB061263) was used as a control (Nicot et al. 2005). Primer Express v2. 0 was employed for primer design, and all genes' sequences blasted from the above-mentioned databases were referenced for primer design to affirm their specificity. Information and primers of the genes tested in this research are listed in Table 1.

Table 1 The primer sequences of genes related to starch degradation

Gene name	Gene ID	Forward primer (5′–3′)	Reverse primer (5′–3′)
StAmy1	PGSC0003DMC400035807[a]	GGGATCAAATCGGTTGCTGA	CATCCGTCGTTCCTCCTTCA
StAmy23	PGSC0003DMC400017447[a]	GGCATACACAGCCGTTCATCT	ATCCGTCCCCAATCTTCACG
StBAM1	PGSC0003DMC400002800[a]	TGAGATGCGTGACCATGAGC	CAAGTGGAACTTGCGCTTCC
StBAM2	PGSC0003DMC400035625[a]	TTGTTCGAGCCCGAGAATTG	TCGAATCGCATTCTGGAAGC
StBAM3	PGSC0003DMC400021443[a]	AGGGATGTTGCTGCTTCACG	GTGAATCCCAGCCACTTTCC
StBAM4	PGSC0003DMC400045472[a]	CGGGCTATATGATTGGTCTGC	GCCACCACATTGATGGAATG
StBAM5	PGSC0003DMC400000368[a]	GAACACCCACCACAATGGTG	GTGTTCTGAAACCCGGAAGG
StBAM6	PGSC0003DMC400041754[a]	TTACAACCCAACGAACCAGGA	TGGAACCTGAAGCCCTGAACT
StBAM7	PGSC0003DMC400018848[a]	CCTTGATGGAAAGACTCCGG	AGAAACGCCCGTGATTGTG
StAI1	PGSC0003DMC400063289[a]	CCGATGATTGTGGGCATACTC	CCTTGATCTGCTTGCGTGC
SbAI	HS989783[b]	CCATCAGCAACGAATTCCG	AATGCAGCAATGAGGACCAAT
StISA1	PGSC0003DMC400035985[a]	CAGGCAAACCAGCACCATTT	GGATACTGGTTCGCGTCCAG
StISA2	PGSC0003DMC400001818[a]	AGGCGACTACTTCTGGGAAGG	GGACGTATCTCAGACCCACCC
StISA3	PGSC0003DMC400012889[a]	ATTGCTGAGCCATGGGATTG	TGATGTCATCGCGGTACTTCC
StISA4	PGSC0003DMC400046519[a]	CTTAACCACAGCGTTTCCCC	TACCCAGAGCAACCTCAGCA
StISA5	PGSC0003DMC400030763[a]	CAGAACCCAGATGCATTTCCA	CGAACGAATACCATCCACCC
Pho1	X52385[b]	CAGGAACCAGATGCTGCTCTT	CATAGCCCCATGCTGGGTAGT
Pho2	PGSC0003DMC400013565[a]	GATGCAGGGTTCTGGCATG	TCATTCCATTTTGAAGCCCAC

[a] Gene from the Potato Genome Sequence Consortium database

[b] Gene from the National Center of Biotechnology Information database

Assessment of Enzyme Activity and Sugar Contents

Activities of amylases were quantified as described by Li et al. (2005). The amount of reducing sugars was determined as previously described (Ou et al. 2013). The data were shown as means of three repeats. Each test was repeated three times. Correlation analysis of the data was conducted using the Microsoft Excel program (Microsoft Office XP, 2010).

Results

Selection of Candidate Genes Related to Starch Degradation

Genes related to starch degradation were selected from the PGSC database and the NCBI database, including two α-amylase genes, *StAmy1* (PGSC0003DMC400035807) and *StAmy23* (PGSC0003DMC400017447) (Rentzsch et al. 2012); seven β-amylase genes (*StBAM1*, *StBAM2*, *StBAM3*, *StBAM4*, *StBAM5*, *StBAM6*, and *StBAM7*); five isoamylase genes (*StISA1*, *StISA2*, *StISA3*, *StISA4*, and *StISA5*); two starch phosphorylase genes, *pho1* (Brisson et al. 1989) and *pho2* (Lorberth et al. 1998), which represent cytosolic and plastidic starch phosphorylase, respectively; and two putative amylase inhibitor genes, *StAI1* (PGSC0003DMC400063289) and *SbAI* from EST C20-3-E15 (GeneBank ID: HS989783) (Chen 2012). The β-amylase genes and isoamylase genes in potato were respectively clustered with the genes identified in *A. thaliana*. The cluster showed that *StBAM1*, *StBAM2*, *StBAM4*, *StBAM5*, *StBAM6*, and *StBAM7* were most identical to *AtBAM1*, *AtBAM3*, *AtBAM5*, *AtBAM7*, *AtBAM8*, and *AtBAM9*, respectively, while *StBAM3* had no homologs in *Arabidopsis* (Fig. 1a). *StISA1*, *StISA2*, and *StISA3* were most identical to *AtISA1*, *AtISA2*, and *AtISA3*, respectively, while *StISA4* and *StISA5* were grouped together without *Arabidopsis* homologs (Fig. 1b).

Expression Analysis of Genes Related to Starch Degradation

The transcripts of genes related to starch degradation in different potato organs of AC030-06 and E3 were estimated by qRT-PCR. Results showed that the transcripts of the α-amylase gene *StAmy23* were much higher than those of *StAmy1* in the leaves, stems, and tubers at different storage times. In addition, *StAmy23* had the highest expression in tubers before sprouting, and the expression decreased after sprouting. Moreover, the expression of *StAmy23* increased when the tubers were stored at 4 °C for 30 days (Fig. 2a, d). These data indicate that *StAmy23* may play roles in potato tubers during cold storage and sprouting.

For β-amylase, seven candidate genes were selected from the potato database (PGSC) and their expression was tested in AC030-06 and E3 tubers. Results showed that the expression of *StBAM1* and *StBAM7* was much higher than that of the rest of the candidate genes and increased sharply after cold storage, but there were no significant differences between the tubers before and after sprouting (Fig. 2b, e). The data indicated that the activity of β-amylase could be mainly caused by *StBAM1* and *StBAM7*. We also tested the expression of amylase inhibitors and found that the expression of *SbAI* was much higher than that of *StAI1* in all the organs tested (Fig. 2c, f), which indicated that the activity of amylase inhibitors was mainly caused by *SbAI*.

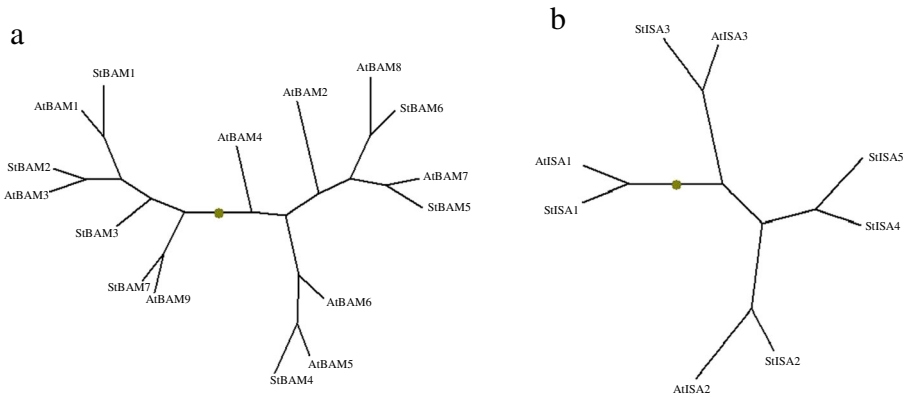

Fig. 1 Phylogenetic relationships of β-amylase genes (**a**) and isoamylase genes (**b**). Amino acid sequences of β-amylase genes are selected from Solanum tuberosum (*StBAM1*, PGSC0003DMC400002800; *StBAM2*, PGSC0003DMC400035625; *StBAM3*, PGSC0003DMC400021443; *StBAM4*, PGSC0003DMC400045472; *StBAM5*, PGSC0003DMC400000368; *StBAM6*, PGSC0003DMC400041754; *StBAM7*, PGSC0003DMC400018848) and Arabidopsis thaliana (*AtBAM1*, GenBank:AEE76832; *AtBAM2*, GenBank: AEE81889; *AtBAM3*, GenBank: AEE83848; *AtBAM4*, GenBank: AED96669; *AtBAM5*, GenBank: AEE83568; *AtBAM6*, GenBank: AEC08663; *AtBAM7*, GenBank: AEC10613; *AtBAM8*, GenBank: AED95229; *AtBAM9*, GenBank: AED92597). Amino acid sequences of the isoamylase genes are selected from Solanum tuberosum (*StISA1*, PGSC0003DMC400035985; *StISA2*, PGSC0003DMC400001818; *StISA3*, PGSC0003DMC400012889; *StISA4*, PGSC0003DMC400046519; *StISA5*, PGSC0003DMC400030763) and Arabidopsis thaliana (*AtISA1*, GenBank: AAQ56791; *AtISA2*, GenBank: AEE27557; *AtISA3*, GenBank: AEE82713)

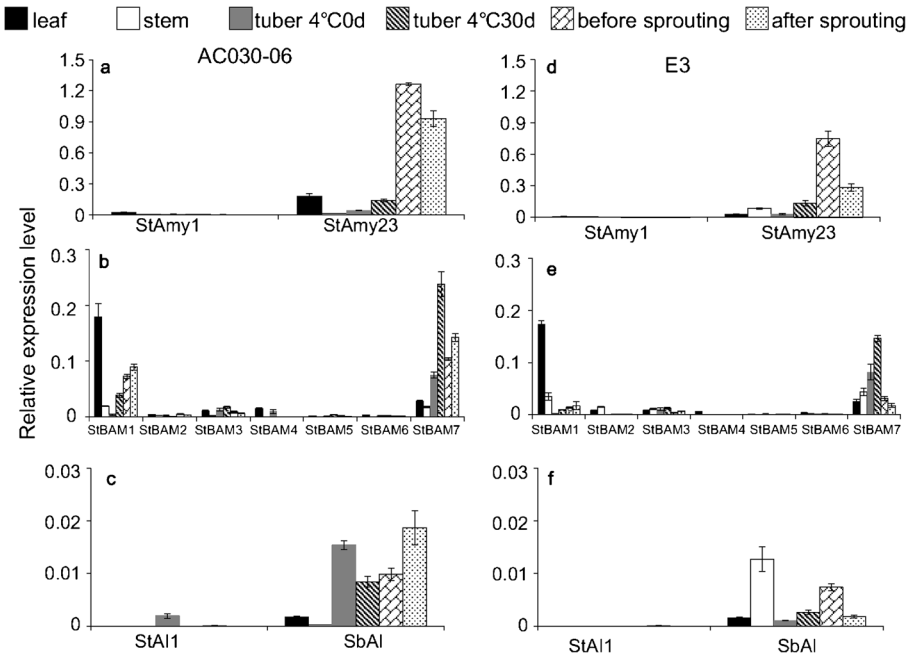

Fig. 2 Relative expression levels of α-amylase genes, β-amylase genes, and amylase inhibitor genes in different organs of AC030-06 (**a**–**c**) and E3 (**d**–**f**), respectively

Five isoamylase genes were selected from the PGSC database, and their expression was tested. The results showed that two genes, *StISA4* and *StISA5*, had much lower expression compared with the other three genes, *StISA1*, *StISA2*, and *StISA3*, while the expression patterns of these three genes in tubers during storage were inconsistent between AC030-06 and E3 (Fig. 3a, c). For *pho1* and *pho2*, the expression levels were similar in AC030-06 tubers before and after cold storage (Fig. 3b). In E3, the expression of *pho1* decreased after tubers were stored at 4 °C for 30 days and increased during sprouting, while the expression of *pho2* decreased during both cold storage and sprouting (Fig. 3d). These results indicate that isoamylase and starch phosphorylase genes may not play important roles in potato tubers during cold storage and sprouting.

Analysis of *StAmy23*, *StBAM1*, *StBAM7*, and *SbAI* in Potato Tubers During Cold Storage

According to the above gene expression analysis, *StAmy23*, *StBAM1*, *StBAM7*, and *SbAI* were considered as important genes regulating tuber starch degradation (Fig. 2). In order to analyze their functions in cold-stored tubers, six potato genotypes which have a well-defined resistance to potato CIS were employed, including CIS-resistant genotypes *S. berthaultii*, AC030-06, and AC142-01 and CIS-sensitive genotypes AC035-01, AC041-03, and E3 (Liu et al. 2010). The expression of *StAmy23*, *StBAM1*, *StBAM7*, and *SbAI* in the cold-stored tubers was tested by qRT-PCR with a potato housekeeping gene *ef1α* (GenBank accession no. AB061263) as an internal control. The results showed that the expression of *StAmy23* in *S. berthaultii* and AC030-06 was higher than in the other four genotypes (Fig. 4a). The expression of *StBAM1* in tubers stored at 4 °C for 5 days was the highest in all tested genotypes and decreased afterwards (Fig. 4b). The expression of *StBAM7* in tubers was increased after cold storage for 30 or 60 days (Fig. 4c). However, the expression levels of the three amylase genes in CIS-resistant tubers showed no significant differences compared to the CIS-sensitive tubers (Fig. 4a–c). The expression of amylase inhibitor gene *SbAI* in

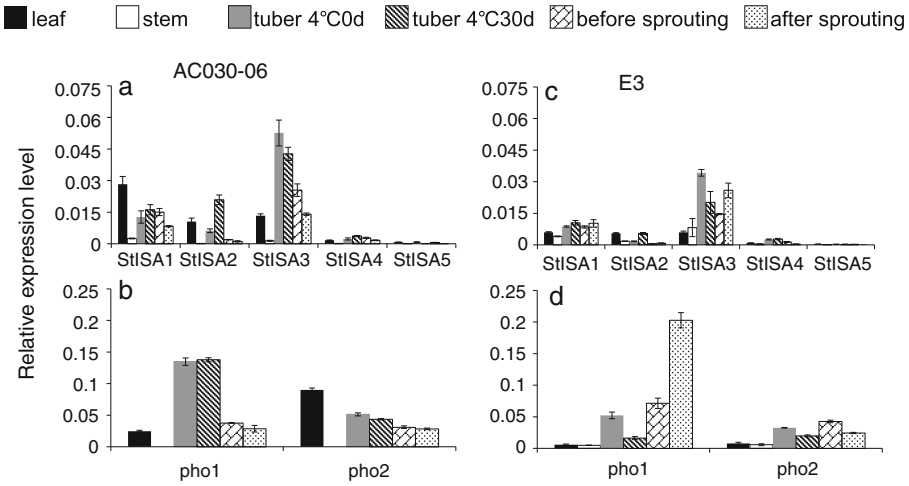

Fig. 3 Relative expression levels of the isoamylase genes and starch phosphorylase genes in different organs of AC030-06 (**a**, **b**) and E3 (**c**, **d**), respectively

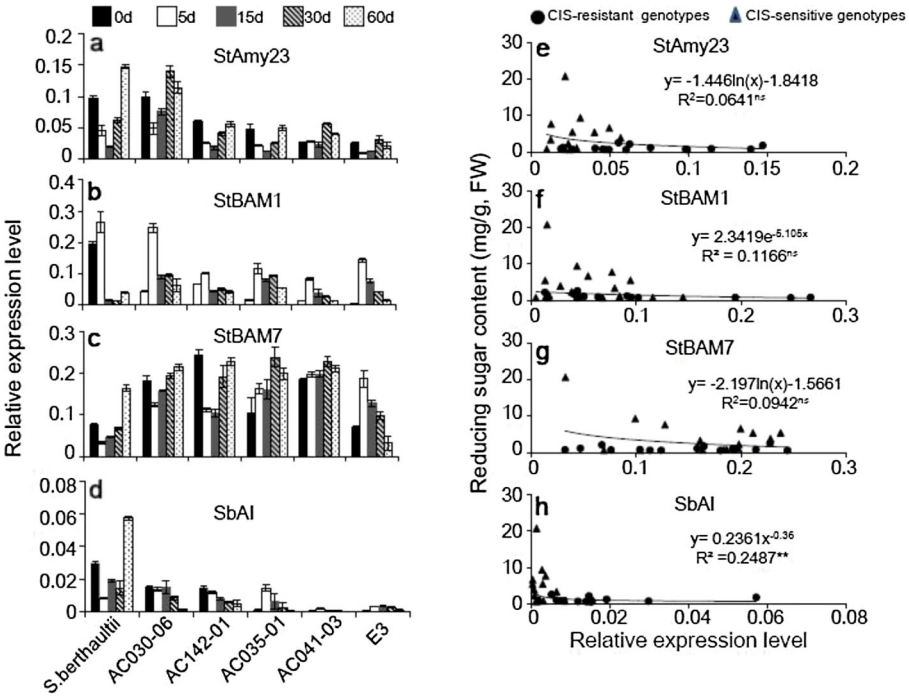

Fig. 4 The relationship of *StAmy23*, *StBAM1*, *StBAM7*, *SbAI*, and potato CIS (a–d). Expression patterns of *StAmy23*, *StBAM1*, *StBAM7*, and *SbAI* in six potato tubers during 4 °C storage, respectively. **e**–**h** Correlation of the reducing sugar content and the relative expression of *StAmy23*, *StBAM1*, *StBAM7*, and *SbAI*, respectively. $^{**}P<0.01$, significant level; *ns* no significance

CIS-resistant tubers was much higher than that in CIS-sensitive tubers (Fig. 4d). To look into the relationship between amylase genes and CIS, the RS content (Liu et al. 2010) was plotted against the expression level of *StAmy23*, *StBAM1*, *StBAM7*, and *SbAI* in the cold-stored tubers of six different genotypes. A significant negative correlation between the RS content and the expression of *SbAI* was established (Fig. 4h), while no significant correlations between the RS content and the expression of *StAmy23*, *StBAM1*, and *StBAM7* were observed (Fig. 4e–g). These results led to a conclusion that the roles of amylases in potato CIS may be regulated by the amylase inhibitor *SbAI*.

The activity of amylase in the six potato genotype tubers stored at 4 and 20 °C was analysed. In tubers stored at 20 °C, the amylase activity was at a low level in all six genotypes (Fig. 5b). When tubers were stored at 4 °C, the activity was increased sharply in E3 (from 0.12 to 0.69 stored for 30 days) and was increased in AC041-03 tubers stored for 60 days, but it was low in the other four genotypes (Fig. 5a). The amylase activity was not consistent with the expression of *StAmy23*, *StBAM1*, and *StBAM7* (Fig. 4a–c), so they may be regulated by regulators such as amylase inhibitors.

Analysis of Amylase Activity in Potato Tubers During Sprouting

In order to identify the contribution of amylases to potato tubers during sprouting, reducing sugar amounts and amylase activities were measured. The RS amounts in tubers were decreased by about 67.3 and 46.2% after sprouting in E3 and AC030-06,

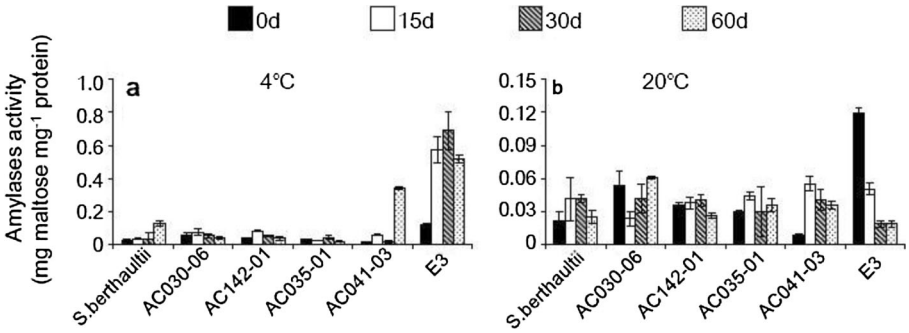

Fig. 5 The activity of amylases in six genotype tubers during storage. **a** Amylase activity after tubers were stored at 4 °C for 0, 15, 30, and 60 days. **b** Amylase activity after tubers were stored at 20 °C for 0, 15, 30, and 60 days. *Columns* represent mean values ±SD (n=3)

respectively (Fig. 6a). The amylase activity in tubers was decreased by about 77.7% after sprouting in E3, while it was slightly increased in AC030-06 (Fig. 6b).

Discussion

Starch degradation is an important process in plants, especially in starch-excess organs like potato tubers. It is also important for potato tubers stored after harvest. However,

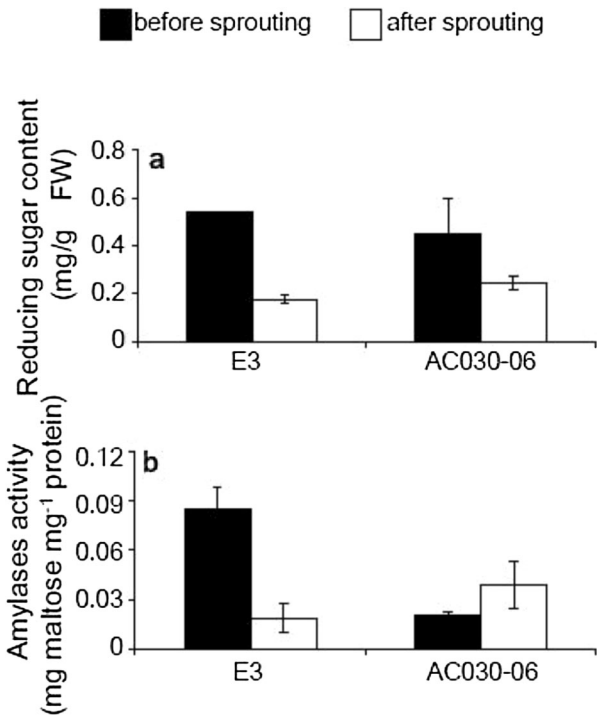

Fig. 6 The reducing sugar content (**a**) and amylase activity (**b**) in potato tubers of E3 and AC030-06 before and after sprouting. *Columns* represent mean values ±SD (n=3)

genes related to starch degradation in potato CIS remain to be clarified. In this study, we selected candidate genes involved in starch degradation according to the available potato information and tested their expression. Our data indicate that *StAmy23*, *StBAM1*, and *StBAM7* may play roles in tubers stored at low temperature and in the process of sprouting, and their activity may be regulated by amylase inhibitor gene *SbAI*.

Many enzymes were involved in the process of starch degradation, such as α-amylase, β-amylase, starch phosphorylase, and so on, but they may play different roles. When potato tubers were stored at 4 °C for 2 weeks, α-amylase activity and reducing sugar content increased sharply and showed significant deviation between different cultivars (Cottrell et al. 1993). We found that the *StAmy23* silencing tubers showed a significant reduction in the accumulation of reducing sugars when stored at 4 °C for 15 days (data not shown). β-Amylase genes were considered to play key roles in the process of starch degradation, and different members may be expressed in different cellular compartments and play distinct roles (Valerio et al. 2011; Fulton et al. 2008). Antisense plants of a chloroplast-targeted β-amylase gene, *PCT-BMY1* (*StBAM2* in this paper), showed a starch-excess phenotype in leaves but not in cold-storage tubers (Scheidig et al. 2002). In the present research, two β-amylase genes, *StBAM1* and *StBAM7*, out of the seven candidates showed higher expression levels, and their expression in tubers stored at 4 °C for 30 days was much higher than that in tubers stored at 4 °C for 0 day (Fig. 2b, e). This indicates that *StBAM1* and *StBAM7* may play roles in potato tubers during cold storage. In contrast, the starch phosphorylase and isoamylase may not be important in potato CIS according to our results (Fig. 3) although it was suggested that the increase in phosphorylase activity acts as a triggering event in potato CIS (Claassen et al. 1993).

The expression of *StAmy23*, *StBAM1*, and *StBAM7* in CIS-resistant potato genotypes is higher than that in the CIS-sensitive potato genotype (Fig. 4a–c). But, their amylase activity is low during cold storage (Fig. 5a). SbAI is a putative amylase inhibitor, and here, we show that its expression is higher in cold-stored tubers of CIS-resistant genotypes than that of CIS-sensitive ones (Fig. 4d). It demonstrates that the amylase activity may be regulated by *SbAI*. However, the regulation mechanism of SbAI and the roles of *StAmy23*, *StBAM1*, *StBAM7*, and *SbAI* in potato starch degradation are still not clear. As we know, different amylase genes may be located in different cellular compartments, plastidic or cytosolic, and their location may affect their functions. *StAmy23* and *StBAM1* were located in cytosolic (data not shown), and the localization of *StBAM7* and *SbAI* needs further investigation. In this study, we found that the amylase activity was only higher in one of the three CIS-sensitive genotypes (Fig. 5a), implying that there could be more pathways involved in regulating the potato CIS, such as sucrose cleavage. Invertase is the key enzyme in turning sucrose to reducing sugars, and suppression of acid invertase by silencing of *StvacINV1* resulted in a strong decrease in RS accumulation in cold-stored tubers (Bhaskar et al. 2010; Liu et al. 2010).

Starch degradation can provide energy for potato to tuber dormancy breaking. In this study, we found that the expression of *StAmy23* (Fig. 2a, d) and reducing sugar (Fig. 6a) contents in both AC030-06 and E3 tubers was significantly lower after sprouting. It suggested that the α-amylase gene *StAmy23* may play important roles in sprouting. In addition, the *StAmy23* RNAi-transgenic tubers sprouted later than the

wild-type control (data not published). The amylase activity of AC03-06 tubers was slightly higher after sprouting (Fig. 6b) accompanied by a lower reducing sugar content in the present research (Fig. 6a). The reason could be that other pathways may also affect the accumulation of reducing sugars in tubers except for starch degradation (Malone et al. 2006).

In conclusion, our data indicate that several key regulators are involved in potato starch degradation. The activity of α-amylase is mainly caused by *StAmy23*, and StAmy23 plays an important role in the process of potato CIS and tuber sprouting. The activity of β-amylase is mainly caused by *StBAM1* and *StBAM7*, and they may play roles only in potato CIS. Our data also indicate that the activity of amylases may be significantly regulated by the amylase inhibitor.

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