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Profiling of *StvacINV1*, *BAM1* and *INH2α* Expressions in Relation to Acid Invertase and β-amylase Activities During Development of Cold-Induced Sweetening in Indian Potato (*Solanum tuberosum* L.) Tubers

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Abstract

Cold-induced sweetening (CIS) characterized by reducing sugars (RS) accumulation during low temperature storage of potato (*Solanum tuberosum* L.) tubers remains a serious postharvest concern for the potato processing industry. Enzymes involved in carbohydrates metabolism and the genes modulating their activities are of paramount importance in the events associated with the development of CIS. Expression of vacuolar acid invertase gene *StvacINV1*, β-amylase gene *BAM1* and invertase inhibitor gene *INH2α* and their consequence on acid invertase and β-amylase activities with resulting RS accumulation were followed in one CIS-tolerant (Kufri Jyoti) and one CIS-susceptible (Kufri Badshah) Indian potato varieties stored in cold conditions. Differential gene expression analysis showed that during cold storage, expression of *StvacINV1* and *BAM1* increased at low temperature and their transcripts were more expressed in the CIS-tolerant variety than the CIS-sensitive. Besides, correlation between *BAM1* expression and β-amylase activity affirmed the hypothesis of several enzymes and pathways involved in starch degradation during cold storage of potato. Expression of invertase inhibitor gene *INH2α* however was higher in the CIS-tolerant variety than the CIS-sensitive. Correlating *StvacINV1* and *INH2α* expressions with RS content and acid invertase activity established that post-translational regulation of acid invertase by the invertase inhibitor protein could be an important component of resistance to CIS.

Keywords: Cold-induced sweetening, Potato, Expression profiling, Invertase, Amylase.

1. Introduction

Potato (*Solanum tuberosum*) being the third most important food crop and the most important non-grain food crop in the world, it serves not only as a staple food in many countries, but is also intensely demanded by processing industry. Potato tubers must be stored at cold temperatures to prevent sprouting, minimize disease-induced losses, and supply consumers and the processing industry with high quality tubers throughout the year. Unfortunately, cold storage triggers an accumulation of reducing sugars (RS) in tubers, a phenomenon known as cold-induced sweetening (CIS). Processing at high temperature of these tubers results in dark-colored, bitter-tasting products. Such products also have elevated amounts of acrylamide, a neurotoxin and potential carcinogen (Bhaskar et al. 2010).

The amount of sugars increases in cold stored potatoes due to the increased activities of carbohydrate degrading enzymes. Many enzymes along the pathway of carbohydrate metabolism in potato tubers have been investigated as possible control points for CIS. The activities of acid invertase (INV) (Matsuura-Endo et al. 2004; McKenzie et al. 2005) and β-amylase (BAM) (Nielsen et al. 1997; Karim et al. 2008) play a major role in accumulation of hexose sugars. Correlations between INV activity and the hexoses:sucrose ratio have been made in cultivars exhibiting varying resistance to CIS (Zrenner et al.1996; Matsuura-Endo et al. 2004; McKenzie et al. 2005). Furthermore, Brummell et al. (2011) suggested that endogenous potato invertase inhibitors, if present in particular cultivars could potentially be important in determining the extent of CIS.

The CIS process in potato tubers involves a network of genes and the final content of sugar in a potato tuber at any given point in time is influenced by several genes (Sowokinos 2001). Variability in resistance to CIS between cultivars is presumably due to a composite of differences in mRNA expression patterns and post-transcriptional events (Brummell et al. 2011). The cold-responsive genes encoding key enzymes involved in carbohydrate metabolic pathways according to previous research include α-amylase gene *Amy23*, β-amylase genes *BAM1* and *BAM9*, invertase gene *StvacINV1*, ADP-glucose pyrophosphorylase gene *sAGP*, starch phosphorylase gene *SP*, sucrose-phosphate-synthase gene *SPS*, sucrose synthase gene *SuSy* and UDP-glucose pyrophosphorylase gene *UGPase* (Zhang et al. 2013). Recent reports have established essential roles of other acid invertase genes *StInv1* (Zhang, 2007), *StvacINV1*

(Liu et al. 2011; Ou et al. 2013), a novel RING finger gene *SbRFP1* (Zhang et al. 2013) and the vacuolar invertase inhibitor gene *INH2α* (McKenzie *et al.* 2013) in development of potato CIS. India ranks second in world potato production with 45.3 million metric tons, grown on an area of 1.9 million hectares (FAO 2013). Potato is one of the main commercial crop in 23 States of India and the majority of potato crop in the country is harvested during February-March, which coincides with a steep rise in temperature. Consequently, potatoes are stored in cold storage to provide round the year supply to markets and consumers (Marwaha et al. 2010). However, many events associated with CIS in Indian potato varieties still remain unknown, and the genetic basis for the variation in CIS sensitivity among different Indian genotypes has yet to be explained. Our recent study (Galani 2014) have found that K. Jyoti was CIS-tolerant and K. Badshah was CIS-susceptible. These presented ideal models for the initial characterization of CIS enzyme pathways in Indian germplasm. Therefore transcriptional analysis of *StvacINV1*, *BAM1* and *INH2α* in these two potato varieties, in comparison with the related carbohydrates splitting enzymes activities and RS accumulation was initiated.

2. Materials and Methods

2.1. Plant materials

Tubers of K. Jyoti and K. Badshah, two Indian potato (*Solanum tuberosum* L.) highly cultivated varieties were obtained from Main Vegetable Research Station, Anand Agricultural University, Anand. The tubers were preconditioned during 15 days at room temperature, then they were separately packed in small plastic net bags and stored in different conditions i.e. (i) at room temperature (25-32ºC), (ii) in the incubator (15ºC) and (iii) in cold storage (4ºC), with 80-95% relative humidity. Samples (3 tubers with similar size per replication) were collected for analysis at 0 and 60 days of storage. The tubers were thoroughly washed with tap water, the peal was removed and the flesh was cut into small dices of approximately 0.5 cm^3 and pooled. Approximately 10 g of pooled flesh was ground to fine powder in liquid nitrogen using a mortar and pestle. The powder was kept in deep-freezer at -70ºC until use.

2.2. Estimation of starch

The procedure of Kuan et al. (2011) was used with some modifications. Ten ml of distilled-deionised water (ddH_2O) was added to 0.1 g of potato powder and incubated in a water bath (80°C) with regular shaking for 30 min. The slurry was then centrifuged at 13,000 g for 10 min at 4°C. The residue was washed 8 times with 40 mL of ddH_2O for removing soluble

carbohydrates, the resultant precipitate was oven-dried at 80°C for 8 h, and 2 ml of ddH_2O was added and hydrolyzed at 100°C in 2 ml of 6 N HCl for 15 min. After cooling the glucose content was estimated by phenol sulphuric acid method as described by Dubois et al. (1959). The value of glucose content obtained from a standard curve was multiplied by a factor 0.9 to obtain the starch content (Sadasivam and Manickam 2007), expressed in percentage of fresh weight tuber (% fw).

2.3. Reducing sugars measurement

Sugars were extracted from 100 mg of the sample in 10 mL of 80% (v/v) methanol for 2 h on an orbital shaker. The solution was centrifuged at 13,000 g for 10 min and the methanol was evaporated from the supernatant by keeping it on a water bath at 80°C for 25 min. After cooling, extracted sugars were dissolved by adding 10 mL of distilled water in the tube and RS content was measured using the dinitrosalicylic acid (DNS) method (Miller 1959) and expressed in mg 100 g^{-1} fw.

2.4. Determination of enzymes activities

For extraction of carbohydrates degrading enzymes, 1g of potato powder was homogenized in 1.5 mL of ice-cold extraction buffer containing 50 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulphonic acid (HEPES)-KOH, pH 7.3, 15 mM $MgCl_2$, 2 mM ethylene diamine tetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 2 mM dithiothreitol (DTT), 10% (v/v) glycerol, and 2% (w/v) polyvinyl polypyrrolidone (PVP). The crude extract was centrifuged at 14,000 g for 20 min at 4°C and the supernatant was collected. Protein concentration of extracts was determined according to Lowry et al. (1951). The activity of β-amylase (BAM) was determined using the protocol developed by Kuan et al. (2011) and expressed as gram of maltose produced per min (one unit) per gram of fresh weight tuber (g Mal $min^{-1}g^{-1}$ fw or U g^{-1} fw). For acid invertase activity (INV) the procedure of Brummell et al. (2011) was used and the result was expressed as nmol of glucose formed per hour (one unit) per mg protein (nmol Glu $h^{-1}mg^{-1}$ or U mg^{-1} protein)

2.5. RNA Extraction and cDNA synthesis

RNA was isolated from potato tubers using CTAB phase extraction and precipitation with LiCl. Approximately 100 mg of fine powder was transferred to 2 mL micro-centrifuge tube and 600 µL of 65°C-preheated CTAB extraction buffer (100 mM Tris-HCl pH 8.0, 2 M NaCl, 2% (w/v) CTAB, 2% (w/v) PVP, 25 mM EDTA and 2% β-mercaptoethanol) was added. Tubes were

vortexed for 10 s and 600 µL of phenol: chloroform: isoamylalcohol (25:24:1) was added, mixed and incubated for 10 min in water bath at 55°C. Afterward, the tubes were allowed to cool at room temperature for 5 min and the mixtures were centrifuged at 4°C for 10 min at 12,000 g. The upper phase was collected and a volume of 700 µL chloroform : isoamylalcohol (24:1) was added to each tube, gently mixed for 1 min, centrifuged at 4°C for 10 min at 12,000 g and 600 µL of upper aqueous layer were transferred to a clean 1.5 mL micro-centrifuge tube. The RNA was then precipitated by adding 600 µL of cold 4 M LiCl solution and the tubes left overnight at -20°C. To pellet the RNA, the tubes were centrifuged at 10,000 g for 10 min at 4°C. The supernatant was decanted and the RNA pellet was washed with 600 µL cold 2 M LiCl solution and the tubes were centrifuged at 10,000 g for 10 min at 4°C. The pellet was again washed with 600 µL of 70% ethanol, then ethanol was carefully aspirated and the RNA pellets were dried by leaving the tubes open for 15 min at room temperature. The RNA pellet was re-suspended in 50 µL Diethylpyrocarbonate (DEPC)-treated water and stored at -20°C. The quantity and quality of the RNA were assessed spectrophotometrically using NanoDrop (Thermo Scientific) and on 1% agarose gel stained with 0.5 µg mL^{-1} ethidium bromide. Residual genomic DNA was eliminated from RNA samples (10 µg) by treatment with DNase I treatment (TaKaRa) according to the manufacturer's instructions. Synthesis of cDNA was carried out from 1 µg RNA sample by using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific).

2.6. Quantitative Real Time PCR

Relative expressions of 3 genes *viz*., invertase inhibitor *INH2α* (McKenzie et al. 2013), vacuolar invertase *StvacINV1* and β-amylase *BAM1* (Zhang et al. 2013) were assessed. The PCR reactions were carried out into 200 µL PCR tubes using SsoFast™ EvaGreen® Supermix kit (Bio-Rad Laboratories) in a final reaction volume of 15 µL containing 7.5 µL SsoFast™ EvaGreen® Supermix, 3 µL of 25-fold diluted cDNA template, 0.4 µL 10 µM of each forward and reverse primer and 3.7 µL nuclease free water. The qPCR reaction was performed on Bio-Rad MiniOpticon™ Real-Time PCR System. The following amplification program was used for *INH2α* gene: 10 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 60 s at 63 °C and 90 s at 72 °C. For *StvacINV1* and *BAM 1* genes the program was: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 60 s. Fluorescence signals were measured in each cycle at the end of the extension step (*INH2α*) or annealing-extension step (*StvacINV1* and *BAM 1*). Results were normalized to a potato gene *ef1α* (AB061263) (Nicot et al. 2005) and relative expression was calculated with $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) using

the software Bio-Rad CFX Manager Version 3.1. To validate the specificity of the primers, 10 µL of PCR reactions were mixed with 1 µL of gel loading dye and electrophoresed on 3% agarose gels.

2.7. Statistics Analysis

The results are presented as mean ± S.Em of three biological replicates used for each measurement. Pearson's correlation at 1% and 5% levels of significance was performed between the parameters by using the SPSS Statistics 17.0.3 software.

3. Results and Discussion

3.1. Carbohydrates dynamics and enzymes activities

The RS content in both the potato varieties increased during storage at 4ºC whereas no increase in RS was observed in tubers stored at 15 ºC. Also, during storage at the 3 temperatures, higher amount of RS was recorded in the CIS-sensitive variety as compared to the CIS-tolerant. Several authors have reported an increase of RS in cold stored potato tubers (Bhardwaj et al. 2011; Kumar, 2011; Chen et al. 2012; Kumar et al. 2012; Ou et al. 2013).

Starch content of the tubers increased during storage, with higher values obtained at 15ºC in both the varieties. Karim et al. (2008) have rather observed a continuous decrease in the amount of starch in potatoes stored at 0-2ºC for 8 weeks. Thus the increase in starch content observed in this study can be explained by starch and sugar metabolism balance oriented towards starch synthesis (Kim and Lee 1993).

Acid invertase activity augmented during storage and highest values were recorded at 4ºC in both the varieties. Moreover, at 4ºC the activity of the enzyme was highest in the CIS-susceptible variety K. Badshah. Our results are in agreement with the findings of Cheng et al. (2004), McKenzie et al. (2005), Karim et al. (2008) and Liu et al. (2011).

During storage, an increase of BAM activity was detected with higher values observed in the CIS-susceptible variety. Similar increases of BAM activity in potato tubers stored in cold temperatures were also observed by Nielsen et al. (1997) and Karim et al. (2008).

3.2. Gene expression analysis

At initial day, the increase of *StvacINV1* transcript expression was as high as 15.52-fold in K. Badshah as compared to K. Jyoti. However, at 60 days of storage at room temperature and 15ºC

both, no clear distinct expression differences could be observed between the two varieties as their relative expressions were all in the range of 3-fold. At 4ºC, the transcript expression was 6.48-fold higher in K. Badshah and 7.79-fold higher in K. Jyoti. Overall, at 60 days storage *StvacINV1* expression was higher in the CIS-tolerant variety than the CIS-sensitive (Table 1). Higher induction of *StvacINV1* transcript at low temperature affirms the results of Liu et al. (2011), Chen et al. (2012) and Ou et al. (2013). Moreover, Ou et al. (2013) also reported that the difference of *StvacINV1* expression in CIS-resistant and CIS-sensitive genotypes stored at stored at 4°C and 20°C was not clearly distinct.

The transcript of *BAM1* was highest at initial day (5.28-fold expression) in K. Badshah as compared to K. Jyoti. At 60 days storage, its expression increased with decrease of storage temperature in K. Badshah while in K. Jyoti the highest increase was obtained at 15ºC. But in general, the expression of *BAM 1* was higher in the CIS-sensitive variety than the CIS-tolerant (Table 1). Chen et al. (2012) also observed an increased expression of β-amylase genes at low temperature.

Expression of *INH2α* at initial day was as high as 27.36-fold and 24.27-fold increase in the CIS-sensitive and the CIS-tolerant varieties, respectively. At 60 days, the expression increased with decrease of storage temperature in K. Badshah while in K. Jyoti the highest increase of expression was recorded at 15ºC. Moreover, *INH2α* expression was higher in the CIS-tolerant variety than the CIS-sensitive (Table 1).

3.3. Correlation analysis

Correlation coefficients between transcript expression and other sugar metabolism parameters each for 8 observations are summarized in Table 2. A very strong correlation was found between INV and RS (R^2=0.874). The RS content in a potato tuber is synergistically controlled by several carbohydrate metabolism-related enzymes (Sowokinos 2001) and our results support that INV activity is crucial for the process of CIS (Ou et al. 2013). A surprising moderate positive relationship (R^2=0.593) was found between BAM and starch. However, a strong relationship was obtained between BAM and RS (R^2=0.689) which was comparable to the value (R^2=0.861) obtained by Zhang et al. (2013). This can be due to the fact that together with BAM, other enzymes such as starch phosphorylases (Zeeman et al. 2004; Rommens et al. 2006; Kamrani et al. 2011) and glucan water dikinase (Mikkelsen et al. 2005; Edner et al. 2007) are involved in the process of starch degradation in potato tubers during cold storage.

The *StvacINV1* expression did not correlated with RS content (R^2=0.093), neither with INV activity (R^2=-0.152) whereas the association between RS content and INV was very strong (R^2=0.874). Similar values were reported by Ou *et al*. (2013). These observations suggest that other factors playing roles in post-translational regulation of INV activity could be involved in RS accumulation (Liu et al. 2013; Ou et al. 2013; McKenzie et al. 2013).

The correlation between *BAM1* expression and BAM activity was moderate (R^2=0.523) while it showed no relationship with starch content (R^2=0.067), thereby supporting the opinion of several enzymes and pathways involved in starch degradation during cold storage of potato (Zeeman et al. 2004; Mikkelsen et al. 2005; Rommens et al. 2006; Edner et al. 2007; Kamrani et al. 2011).

The expression of *INH2α* showed a weak negative relationship with RS content (R^2=-0.450) but a strong negative relationship with INV activity (R^2=-0.789) suggesting that *INH2α* expression is related both with low INV activity and with tolerance to CIS. The results affirm with the previous report that post-translational regulation of acid invertase by the vacuolar invertase inhibitor is an important component of resistance to CIS (Rausch and Greiner 2004; Liu et al. 2013; Ou et al. 2013; McKenzie et al. 2013). Invertase inhibitors proteins may act through posttranslational modification of INV activity by protein–protein interactions (Rausch and Greiner 2004). From these results, it can be concluded that sucrose cleavage by INV is causal for RS accumulation in cold-stored tubers and INV activity is most probably modulated by post-translational mechanisms involving invertase inhibitor protein. This is the first report on gene expression related to CIS in Indian potato varieties. This work paves the way to biotechnologists and breeders for developing Indian CIS-resistant cultivars.

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Table 1: Relative gene expressions, enzymes activities and carbohydrates changes in potato tubers stored at different temperatures

Varieties	K. Badshah				K. Jyoti			
Treatments	**0 day, RT**	**60 days, RT**	**60 days, 15ºC**	**60 days, 4ºC**	**0 day, RT**	**60 days, RT**	**60 days, 15ºC**	**60 days, 4ºC**
StvacINV1	15.52±5.87	3.07±1.01	3.09±0.53	6.48±3.76	1.00±0.28	3.08±0.28	3.85±0.16	7.79±1.56
BAM1	5.28±1.42	2.12±0.53	4.15±0.87	5.00±2.66	1.00±0.25	1.72±1.13	2.45±0.59	2.02±0.62
INH2α	27.36±17.82	2.21±1.36	1.60±0.51	1.00±0.79	24.27±7.63	3.06±1.46	7.56±4.43	4.12±3.67
RS (mg 100 g^{-1} fw)	79.39±0.92	170.19±0.88	160.81±1.23	901.17±1.51	105.39±1.82	156.58±0.99	100.51±1.22	412.45±2.03
Starch (% fw)	4.56±0.04	16.72±0.06	25.82±0.07	20.81±0.05	7.08±0.05	17.17±0.08	20.75±0.07	18.7±0.05
INV (U mg^{-1} protein)	0.03±0.01	1.08±0.03	1.08±0.01	2.84±0.02	0.07±0.01	1.35±0.02	1.03±0.01	1.33±0.03
BAM (U g^{-1} fw)	1.10±0.01	1.85±0.02	1.84±0.02	2.53±0.04	0.88±0.01	1.32±0.01	1.32±0.03	1.04±0.01

Values are means ± S.Em. of 3 replicates. RT=Room Temperature

Table 2: Correlation coefficients between transcript expressions and other sugar metabolism parameters

	StvacINV1	***INH2α***	**INV**	**RS**	***BAM1***	**BAM**
INH2α	0.430					
INV	-0.152*	-0.789				
RS	0.093**	-0.450	0.874			
BAM1	0.675	-0.049	0.262	0.386		
BAM	-0.125*	-0.643**	0.804	0.689	0.523	
Starch	-0.433	-0.913	0.687	0.358	0.067**	0.593

*, ** mean correlation is significant at 5% and 1% level, respectively