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Temperature-dependent regulation of sugar metabolism in wild-type and low-invertase transgenic chipping potatoes during and after cooling for low-temperature storage

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ABSTRACT

Regulation of sugar metabolism in potato tubers stored at low temperatures has significant ramifications for producers and consumers of potato chips and French fries. Low-temperature storage reduces losses due to sprouting and disease but induces undesirable accumulation of the reducing sugars glucose and fructose. These sugars react with free amino acids during frying to produce dark-colored and bitter-tasting products that have elevated levels of acrylamide, a suspected human carcinogen. Elevated amounts of tuber glucose and fructose are produced when vacuolar acid invertase (VInv) hydrolyses sucrose derived from starch degradation. In this study, wild-type tubers and tubers in which *VInv* expression was reduced by RNA interference were used to study the temperature-dependent regulation of sugar accumulation and expression of carbohydrate metabolism genes during and after the cooling of tubers to 9 or 3 °C. It was found that transcriptional control of genes central to carbohydrate metabolism occurred in three overlapping phases. First, early in cooling, slight increases in *VInv* and *β-amylase* expression occurred. Second, at lower temperatures, large decreases in *AGPase* and *GBSS* expression were observed. Finally, when tubers reached 3–5 °C, large increases in *β-amylase* expression were observed, coinciding with large increases in sucrose in all genotypes and large increases in glucose and fructose in tubers with high *VInv* expression. Suppression of *VInv* expression did not alter the expression of *β-amylase*,*AGPase*, *GBSS*, or other genes, indicating that there was little or no feedback from reducing sugars to transcriptional control of carbohydrate metabolism. Invertase activity of WT tubers stored at 9 °C was comparable to that of WT tubers stored at 3 °C, but reducing sugars were markedly higher at the lower temperature. Taken together, these data support a model in which reducing sugar accumulation is controlled both by temperature-regulated changes in sucrose accumulation, resulting from increased starch degradation by β-amylase and decreased starch resynthesis by AGPase and GBSS, and by differences in VInv activity that persist throughout storage.

1. Introduction

Over half of the potato (*Solanum tuberosum* L.) tubers produced in the United States are processed into potato chips, French fries and other fried products (NASS-USDA, 2013). To meet industry quality standards, tubers must produce light-colored, flavorful products that satisfy consumer expectations for appearance and flavor. Product color and flavor depend largely on the reducing sugar contents of raw tubers. The major reducing sugars in potato tubers are glucose and fructose, and during frying these sugars react with free amino acids to produce pigments and flavor compounds in a non-enzymatic Maillard reaction (Maillard, 1912). Tubers with low amounts of reducing sugars produce light-colored products with desirable flavor (Denny and Thornton, 1941; Shallenberger et al., 1959). When reducing sugar contents are excessive, however, dark-colored, bitter-tasting products are produced; these also contain relatively high amounts of acrylamide (Mottram et al., 2002; Stadler et al., 2002). Dietary acrylamide is a suspected human carcinogen, and fried potato products have high acrylamide contents compared to many other foods (Hogervorst et al., 2010; Tareke et al., 2002). It has been suggested

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that decreasing tuber reducing sugar contents is one way to minimize potential adverse effects of dietary acrylamide (Amrein et al., 2003; Biedermann-Brem et al., 2003; FDA-USDHHS, 2013).

When potato tubers are cooled to temperatures below approximately 10 °C, they accumulate more sucrose, glucose and fructose than when stored at higher temperatures. This process of low temperature-induced sugar accumulation is called cold-induced sweetening (CIS) or low-temperature sweetening (Sowokinos, 2001). The pathway of starch catabolism to sucrose and reducing sugar formation has, for the most part, been elucidated, and many of the enzymes involved have been characterized (Sowokinos, 2001; Schreiber et al., 2014). During CIS, net sucrose synthesis increases (Isherwood, 1973), and vacuolar invertase (VInv) converts some of this sucrose into glucose and fructose. Accumulation of glucose and fructose during CIS is strongly linked to VInv activity (Matsuura-Endo et al., 2004; Pressey, 1970), and it has been shown that genetic manipulation to reduce *VInv* expression can prevent glucose and fructose buildup (Bhaskar et al., 2010; Chi et al., 2008; Wu et al., 2011; Ye et al., 2010; Zhu et al., 2014; Zrenner et al., 1996). Potato tubers destined for fry and chip processing are usually stored at 8–10 °C in order to minimize reducing sugar accumulation and maintain acceptable processing quality. However, tubers stored at these temperatures have much higher rates of disease and sprouting than those stored at 3–5 °C. It would be advantageous to the potato growing and processing community to develop potato cultivars that resist CIS when stored at low temperatures.

Potatoes for chip and fry processing in North America are stored using established protocols that include a preconditioning and wound-healing period in which tubers are stored at 11–15 °C for at least two weeks. This is often followed by application of a chemical sprout inhibitor and then by cooling at approximately 0.3 °C per day to the final holding temperature (Stark and Love, 2003). Extended preconditioning and slow cooling have been shown to decrease the severity of CIS and increase the processing quality of stored tubers (Burton, 1969; Pritchard and Adam, 1992; Stark and Love, 2003). The preconditioning period may be extended and rate of cooling may be slowed when tuber sugar contents are higher than benchmarks for a given variety (Sowokinos and Preston, 1988). Application of isopropyl *N*-(3-chlorophenyl) carbamate (CIPC), the most commonly used sprout inhibitor, may have effects on tuber sugars and metabolism that persist for weeks (Blenkinsop et al., 2002; Copp et al., 2000; Yang et al., 1999). In many cases, a transient increase in tuber sugar content follows CIPC application (Copp et al., 2000), and cooling to holding temperature does not proceed until sugar contents have returned to benchmark amounts for the variety in question.

Most research on CIS has been done using tubers that have been exposed to an abrupt temperature change, often one or two weeks after harvest (Bagnaresi et al., 2008; Cochrane et al., 1991; Deiting et al., 1998; Hill et al., 1996; Hopkins, 1924; Matsuura-Endo et al., 2004; Nielsen et al., 1997; Ohara-Takada et al., 2005; Zhang et al., 2014a,b). Thus, the storage conditions used in previous research are substantially different from those used in commercial practice. Tuber responses to rapid cold-shock treatments may be very different from those observed in commercial settings in response to gradual cooling following extended preconditioning and application of sprout inhibitor. One of the goals of the present study was to investigate temperature-dependent changes in expression of carbohydrate metabolism genes under storage conditions that are typical of those used in commercial storage of chip and fry processing potatoes.

'Atlantic' (Atl), 'Dakota Pearl' (DKP), and 'MegaChip' (MC) are three potato varieties developed for the production of potato chips

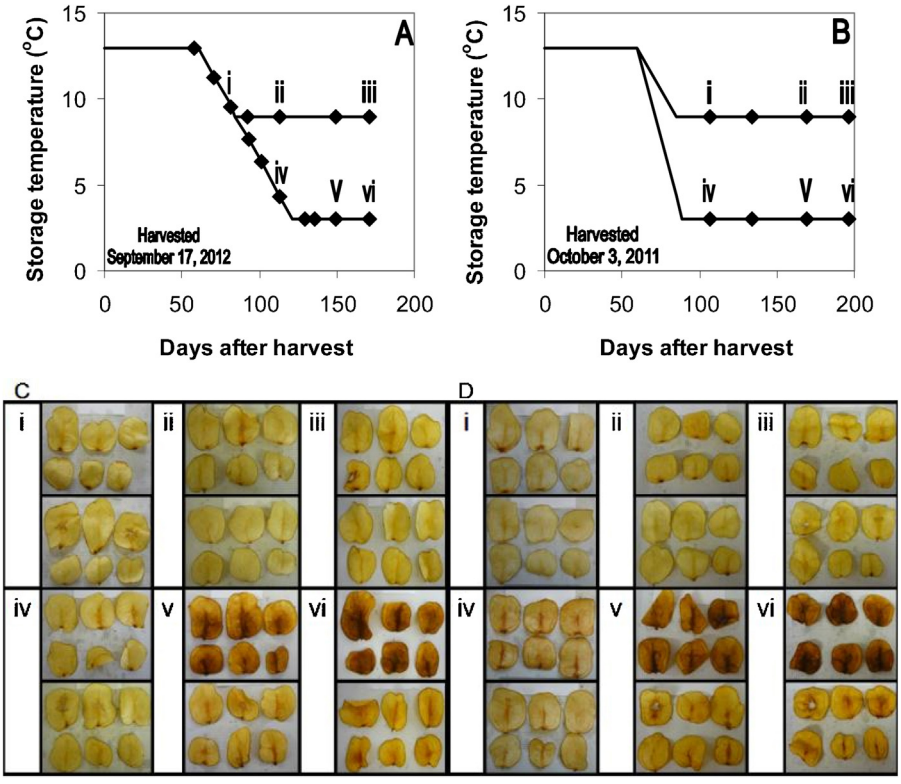

Fig. 1. Storage temperature profile for tubers harvested in 2012 (A) and 2011 (B). Sampling time points in both years are indicated by diamond symbols. Roman numerals in (A) and (B) indicate when photographs of chips in (C) and (D), respectively, were taken. Within each section of (C) and (D), the upper photo shows six representative wild-type MegaChip chips and the lower photo shows six representative RNAi MegaChip chips. MegaChip line 3 is shown in Ci, ii, iv, and v, as well as in Di-Dvi; line 11 is shown in Ciii and vi.

(Groza et al., 2007; Thompson et al., 2005; Webb et al., 1978). Atl tubers are typically processed into chips directly from the field and are rarely stored for more than a short time, as their chipping quality from storage is unpredictable. DKP tubers have resistance to CIS and excellent long-term storage capabilities. MC tubers can be stored successfully for several months and have moderate resistance to CIS. These three potato varieties were used for the research described here, along with two transgenic lines, having different levels of suppression of *VInv* expression, from each variety (Wu et al., 2011). Both transgenic lines of Atl and MC and one transgenic line of DKP had effective RNAi suppression of *VInv* expression compared to wild-type (WT) tubers. *VInv* suppression was relatively ineffective in line 11 of DKP. Tubers were harvested and cooled, at rates commonly used in commercial storages, to either 9 °C, a typical temperature for storage of chip processing potatoes, or 3 °C, a temperature at which all commercial potato varieties will accumulate substantial amounts of sugars as a result of CIS. Tissue samples were collected both during cooling and after tubers reached their final storage temperatures. Fried chip color, sugar contents, *VInv* expression, and transcript abundance of genes encoding several other enzymes involved in starch or sugar metabolism in stored potato tubers were evaluated. Differences between WT and RNAi tubers with respect to sugar accumulation and the timing and extent of temperature-dependent changes in gene expression were characterized and are of interest in light of ongoing efforts to commercialize low-VInv potato lines produced using RNAi (Ye et al., 2010) and genome editing (Clasen et al., 2015).

2. Materials and methods

2.1. Plant materials; growth and storage conditions

Tubers of wild-type Atl, DKP, and MC, as well as Atl RNAi lines 1 (2011 only), 3, and 8 (2012 only); DKP RNAi lines 3 and 11; and MC RNAi lines 3 and 11 (Wu et al., 2011) were planted at the University of Wisconsin Hancock Agricultural Research Station in Hancock, Wisconsin, during the 2011 and 2012 growing seasons. All required regulatory approvals for field growth of transgenic plants were obtained from the U.S. Department of Agriculture Animal and Plant Health Inspection Service prior to planting. Seed tubers were planted in a randomized complete block design with four (2011) or five (2012) blocks of 8-hill replicates of each RNAi line and WT control within each block. Standard cultivation and management practices were used during the growing season. Tubers were mechanically lifted and hand-harvested on October 3, 2011 and September 17, 2012 and were stored initially at 13 °C to promote skin set and formation of wound periderm. Tubers were treated with CIPC approximately one month after harvest, to prevent sprouting.

In 2012–13, cooling of storage lockers began on November 17, 2012. All tubers were cooled at a maximum rate of 0.06 °C per 8 h. The storage locker with a final set point temperature of 9 °C reached that temperature on December 10, 2012, and the locker with a final set point temperature of 3 °C reached that temperature on January 16, 2013. Six tubers per line per storage temperature were selected at random for biochemical, molecular, and chip color analyses 58 days after harvest (DAH), prior to the start of cooling; 71, 81, 93, 101, and 113 DAH, while tubers were being cooled to their final set point temperatures; 92, 113, 169, and 196 DAH, after tubers stored at 9 °C had reached that temperature; and 129, 136, 149, and 171 DAH, after tubers stored at 3 °C had reached that temperature (Fig. 1A) .

In 2011–12, cooling of storage lockers began on December 2, 2011. Tubers that were to have a final storage temperature of 9 °C were cooled at a maximum rate of 0.06 °C per 8 h, and tubers that were to have a final storage temperature of 3 °C were cooled at a maximum rate of 0.11 °C per 8 h. The storage locker with a final set point temperature of 9 °C reached that temperature on December 27, and the locker with a final set point temperature of 3 °C reached that temperature on December 31. Six tubers per line per storage temperature were selected at random for biochemical, molecular, and chip color analysis 107, 134, 169, and 196 DAH (Fig. 1B).

2.2. Chipping analysis

Tubers were cut in half from apical to basal end and three, 1-mm-thick slices were cut from one half of each tuber for frying. Slices were fried for 2 min 10 s in cottonseed oil at 188 °C. Fried chips were crushed for color analysis with a D25LT colorimeter (HunterLab, Reston, VA). Each sample consisted of 18 chips, three chips from each of six tubers per line per storage temperature. Chips with Hunter luminosity (L) values of 50 or higher may be acceptable to the potato chip processing industry (Marquez and Añon, 1986; Halford et al., 2012).

2.3. Sugar, protein, and mRNA extraction and analysis

At each sampling time, two tissue samples were collected from the center of each of six tubers per line per storage temperature, frozen in liquid nitrogen, and stored at −80 °C until use. Each sample was a cylinder of tuber tissue with a fresh weight of approximately 0.6 g. One tissue sample from each tuber was used for sucrose, glucose, and fructose quantification by high-performance liquid chromatography as described by Bethke et al. (2009). The other tissue sample was used for RNA and protein analysis. For each line at each sampling time and storage temperature, the six remaining tissue samples were grouped into three pairs, and each pair of samples was ground under liquid nitrogen using a freezer mill (model 6770, SPEX SamplePrep, Metuchen, NJ) to make three pooled tissue samples for each line at each sampling time and storage temperature. Ground samples were stored at −80 °C.

Protein extraction and desalting were carried out as described by Bhaskar et al. (2010), with about 0.5 g of pooled, ground tissue homogenized in 2 mL of extraction buffer. Protein concentrations in the extracts were determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Extracts were assayed for acid invertase activity as described by Bhaskar et al. (2010). All samples were assayed in triplicate, and activities were expressed as nmol glucose min^{-1} mg^{-1} protein. Both extracts that had been vortexed to inactivate endogenous invertase inhibitors and non-vortexed extracts were assayed.

RNA was extracted from pooled, ground DKP and MC samples using the Agilent Plant RNA Isolation Mini Kit (Agilent Technologies, Inc., Santa Clara, CA) according to the manufacturer's instructions. Between 20 and 120 μL of extraction solution were used per milligram of tissue. RNA was extracted from pooled, ground Atl samples as follows: 100 mg of tuber tissue were homogenized in 750 μL extraction buffer (2% w/v cetyltrimethyl ammonium bromide, 2% w/v polyvinylpyrrolidone, 100 mM Tris–HCl pH 8.0, 25 mM EDTA, and 2 M NaCl in diethylpyrocarbonate-treated H_2O, with 2% v/v β-mercaptoethanol added immediately before use). The homogenate was centrifuged at 16,000 × *g* for 10 min at 4 °C and then extracted twice with an equal volume of 5:1 phenol:chloroform and once with an equal volume of chloroform. The upper aqueous layer was collected and precipitated with 0.5 volume of 7.5 M lithium chloride on ice for 1 h. This solution was centrifuged at 16,000 × *g* for 20 min at 4 °C; the supernatant was removed and the pellet washed twice with 70% ethanol. The pellet was then allowed to air-dry and was resuspended in 30 μL of nuclease-free water.

RNA integrity was checked visually by agarose gel electrophoresis, and purity was evaluated using a NanoDrop 1000 (NanoDrop

Products, Wilmington, DE) with absorbance values measured at 230, 260, and 280 nm. RNA was DNase-treated using a DNase Treatment Kit (Ambion, Austin, TX) according to the manufacturer's instructions. In 2011–12, reverse transcription was carried out on 500 ng of total RNA using the SuperScript III kit (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions. After reactions were completed, nuclease-free water was added to bring the volume to 100 μL. Two microliters of this mixture (containing 10 ng of total RNA) were used as template for each quantitative PCR. In 2012–13, reverse transcription was carried out on 600 ng of total RNA using M-MLV reverse transcriptase, RNase H minus (Promega Corporation, Madison, WI) according to the manufacturer's instructions, and the reactions were brought to a final volume of 80 μL, with 15 ng of total RNA used as template for each quantitative PCR. For each gene, only a single product was formed, and calculated PCR efficiencies were between 90% and 105%.

A Bio-Rad iCycler (Bio-Rad Laboratories, Hercules CA) was used for quantitative PCR of 2011–12 samples, and a Bio-Rad MyiQ thermocycler was used on 2012–13 samples, with Maxima SYBR Green/Fluorescein qPCR Master Mix used for all samples (Fermentas Inc., Glen Burnie, MD). The thermal profile consisted of one cycle of 95 °C for 10 min; 40 cycles of 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s; and a melt curve with temperatures from 55 to 95 °C. Fluorescence data were collected during the extension phase and the melt curve. For every gene in each sample, reactions were carried out in triplicate. Primer sequences and final concentrations are listed in Table S1.

2.4. Statistics

Statistical analyses were carried out, with Tukey's HSD or Student's *t*-test as appropriate, using JMP® Pro version 11.0.0 (SAS Institute, Cary, NC).

3. Results

3.1. Chip color evaluation

In 2012–13, L-values of WT chips were similar to those of RNAi chips and remained constant during cooling to 3 °C (Figs. 1Ci-iv and S1A-C). When WT tubers reached 3 °C, chips became much darker and there was a sudden and large decrease in L-values of all three varieties, and L-values continued to decrease with increased time at 3 °C (Fig. S1A-C). When RNAi tubers reached 3 °C, chips became slightly darker and there was a slight decrease in L-values, but these chips remained paler and their L-values higher than those of WT chips in all lines except DKP line 11, the line in which *VInv* silencing was ineffective. L-values of chips from WT tubers stored at 9 °C were equivalent to those from RNAi tubers stored at the same temperature. Chips from 2011 to 2012 tubers had similar patterns in chip color and L-values: in tubers stored at 3 °C, chips from all RNAi lines except DKP line 11 had higher L-values than WT chips, and values for Atl and MC WT chips decreased over time (Figs. 1D and S1D–F). Within each variety, all lines of MC and DKP had similar L-values when tubers were stored at 9 °C. L-values for RNAi lines of Atl stored at 9 °C, however, were often higher than those of WT.

3.2. Sugar contents of stored tubers

During cooling to 3 °C in 2012–13, MC tuber sucrose contents remained constant through 101 DAH, when storage temperature was 6 °C (Fig. 2A) . Tuber sucrose content was slightly higher ($p < 0.05$) 113 DAH, when the storage temperature reached 5 °C. Sucrose contents in MC tubers rose dramatically once the storage temperature reached 3 °C (Fig. 2A). The same pattern was seen in DKP and Atl tubers (Fig. 2B,C) and in all of the RNAi lines (Figs. 2 A–C). Glucose (Fig. 2D–F) and fructose (Fig. 2G–I) contents in all three varieties remained low and stable during cooling to at least 5 °C. Glucose and fructose contents in WT lines increased

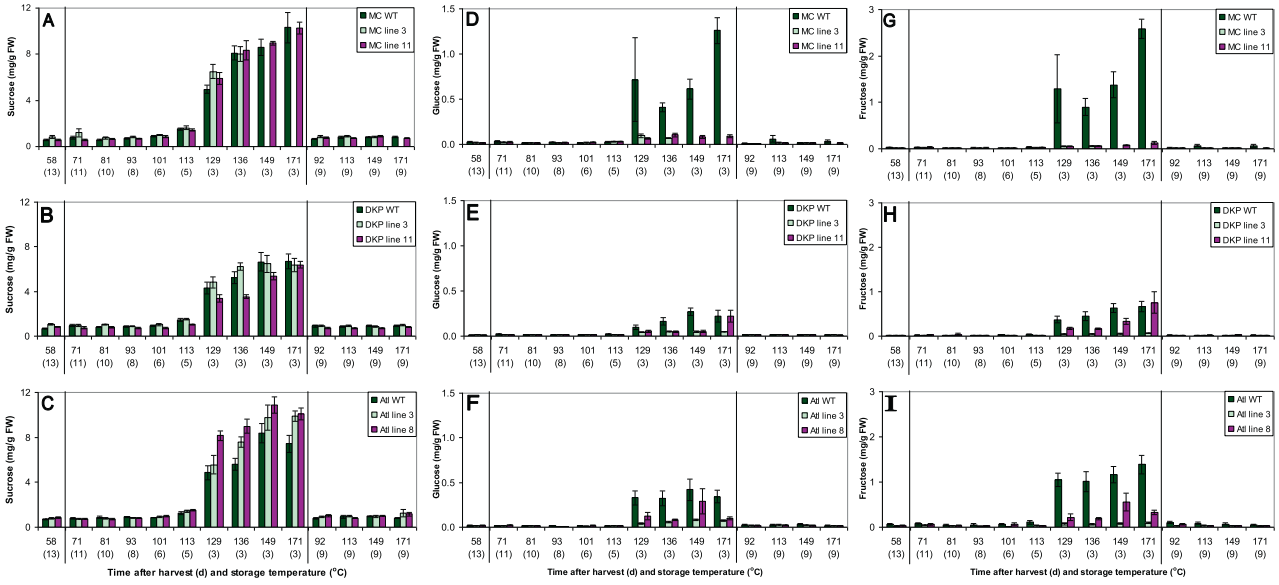

Fig. 2. Sucrose (A–C), glucose (D–F), and fructose (G–I) concentrations in MegaChip (A,D,G), Dakota Pearl (B,E,H), and Atlantic (C,F,I) tubers grown in 2012 and sampled in 2012–13. Tubers were cooled to a final storage temperature of 3 or 9 °C as indicated. Values from wild-type (dark green bars); MegaChip line 3, Dakota Pearl line 3, and Atlantic line 3 (light green bars); and MegaChip line 11, Dakota Pearl line 11, and Atlantic line 8 (purple bars) tubers are shown. Numbers in parentheses under the DAH indicate the storage temperature of the tubers at each sampling time. Bars represent mean concentrations in six independent samples ± standard error.

significantly once tubers reached 3 °C and either remained high, in Atl, or continued to increase, in DKP and MC, through 171 days after harvest. When tubers from RNAi lines reached 3 °C, glucose and fructose contents increased but in most cases remained below 0.1 mg g^{-1} fresh weight. The one exception to this was DKP line 11, which had glucose contents 171 DAH and fructose contents 129 DAH that were as high as those of WT DKP tubers (Fig. 2E,H).

Sucrose contents of MC, Atl, and DKP tubers stored at 9 °C were virtually unchanged between 58 and 171 DAH (Fig. 2A–C). Glucose and fructose contents in MC tubers at 9 °C were well below those in tubers at 3 °C and were higher 113 and 171 DAH than at 58 DAH in WT but not in RNAi lines (Fig. 2D,G). Changes in reducing sugars in Atl tubers during cooling and storage at 9 °C (Fig. 2F,I) were similar to those seen in MC tubers. Reducing sugar contents in DKP tubers during storage at 9 °C were the same as those at the end of preconditioning, and sugar contents in DKP RNAi tubers stored at 9 °C were the same as those in WT tubers (Fig. 2E,H).

In 2011–12, patterns of changes in sugar contents were similar to those seen in 2012–13. Sucrose contents were much greater in tubers stored at 3 °C than at 9 °C (Fig. S2A–C), with the exception of a sharp decrease observed in all Atl lines between 134 and 169 DAH at 3 °C. Glucose and fructose contents were significantly ($p < 0.05$)

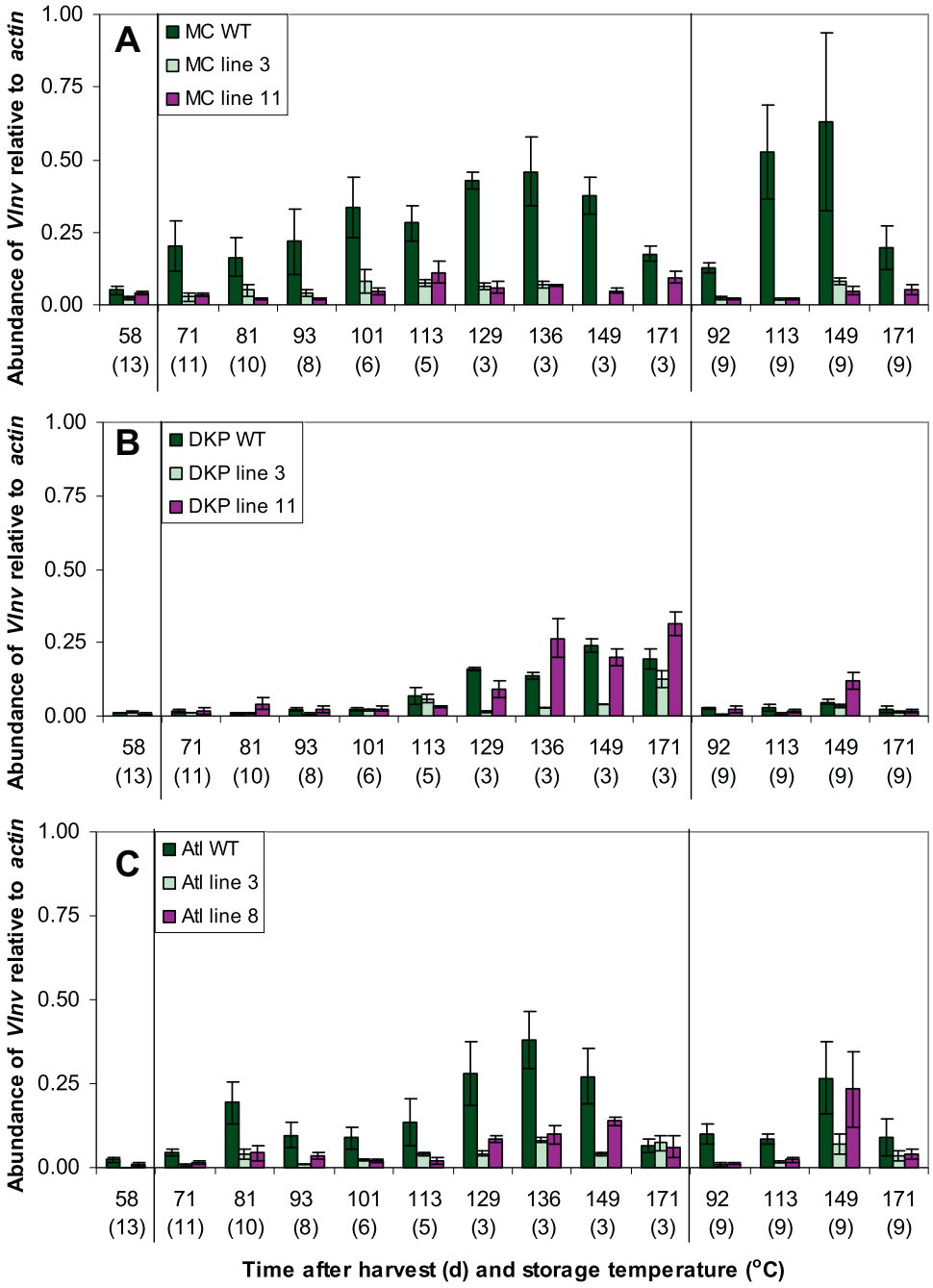

Fig. 3. Amount of *Vinv* transcript relative to *actin* in MegaChip (A), Dakota Pearl (B), and Atlantic (C) tubers grown in 2012 and sampled in 2012–13. Tubers were cooled to a final storage temperature of 3 or 9 °C. Values from wild-type (dark green bars); MegaChip line 3, Dakota Pearl line 3, and Atlantic line 3 (light green bars); and MegaChip line 11, Dakota Pearl line 11, and Atlantic line 8 (purple bars) tubers are shown. Numbers in parentheses under the DAH indicate the storage temperature of the tubers at each sampling time. Bars represent mean expression in three independent samples ± standard error.

Table 1
Largest difference in *VInv* mRNA accumulation between WT Atlantic (Atl), Dakota Pearl (DKP) and MegaChip (MC) and RNAi tubers of each variety during and after cooling to 3 °C. Numbers indicate the extent of *VInv* silencing for each line as a percentage of WT expression. Values were calculated using mean expression from three samples from each WT and RNAi line, with each sample containing tissue pooled from two tubers.

	VInv silencing (% of WT expression)	Time (DAH) and temperature (°C) when maximum difference was observed
Atl line 1	81	134 DAH, 3 °C
Atl line 8	85	113 DAH, 5 °C
DKP line 3	89	169 DAH, 3 °C
DKP line 11	58	134 DAH, 3 °C
MC line 3	92	107 DAH, 3 °C
MC line 11	90	107 DAH, 3 °C

lower in RNAi than in WT tubers of all varieties stored at 3 °C, with the exception of DKP line 11, and reducing sugar contents were lower in RNAi than in WT Atl stored at 9 °C (Fig. S2D–I).

3.3. VInv expression and activity in stored tubers

VInv expression in WT tubers of MC and Atl harvested in 2012 increased during the first two weeks of cooling, and expression was three- and eight-fold higher, respectively, when storage temperature reached 10 °C than at the end of the 13 °C-preconditioning period (Fig. 3A,C) . The amount of *VInv* mRNA in these varieties increased gradually as storage temperature decreased from 10 °C to 3 °C and reached a peak 129–149 DAH, 2 weeks after the final storage temperature was reached. Differences in *VInv* transcript amount between WT and RNAi tubers were greatest around this time (Table 1). *VInv* expression then declined, and by 171 DAH, *VInv* mRNA had returned to the levels seen early in cooling. Accumulation of *VInv* mRNA in WT DKP tubers was delayed relative to MC and Atl, and the greatest *VInv* mRNA accumulation was observed 149–171 DAH (Fig. 3B). *VInv* mRNA accumulation in MC RNAi lines 3 and 11, DKP RNAi line 3, and Atl RNAi lines 3 and 8 was significantly ($p < 0.05$) less than that in WT tubers of the same varieties after the tubers reached 3 °C. DKP RNAi line 11 *VInv* levels were comparable to or greater than those seen in WT tubers at almost all sampling times. Tubers cooled to 9 °C almost invariably had less *VInv* mRNA than those at 3 °C.

VInv expression in tubers harvested in 2011 and cooled to 3 and 9 °C was consistent with that seen in 2012–13 tubers: all RNAi lines except DKP 11 had significantly less ($p < 0.05$) *VInv* mRNA accumulation than WT at 107 and 134 DAH (Fig. S3). Later in storage, the *VInv* expression levels in WT and RNAi tubers at 3 °C were not statistically different ($p < 0.05$), although mean values were consistently lower in RNAi lines than in WT, with the exception of DKP 11.

As expected, extracted VInv activity of MC was higher in WT than in RNAi tubers in 2012–13 (Fig. 4) and of MC and Atl in 2011–12 (Fig. S4). In contrast, VInv activities were usually similar in DKP WT and RNAi lines (Fig. S4). Invertase activity in tubers stored at 9 °C was, in general, not significantly higher than that in tubers stored at 3 °C, though activities after tubers had reached their final temperatures were usually higher than those during the first forty days of cooling.

3.4. Expression of genes involved in sugar metabolism

In 2012–13, expression of *β-amylase* was strongly induced in tubers cooled to 3 °C (Fig. 5). *β-amylase* expression was low in all genotypes when tubers were at 13 °C and remained low through 101 DAH, when tubers were at 6 °C, with the exception of the 81 DAH sampling in Atl tubers. At 113 DAH, when tubers had reached 5 °C, *β-amylase* expression increased significantly ($p < 0.05$) in Atl and DKP tubers; within one week of reaching 3 °C, the same occurred in MC tubers. In all WT and RNAi lines stored at 3 °C, *β-amylase* expression remained high through 171 DAH. *β-amylase* expression was lower in tubers stored at 9 °C than in those stored at 3 °C (Fig. 5).

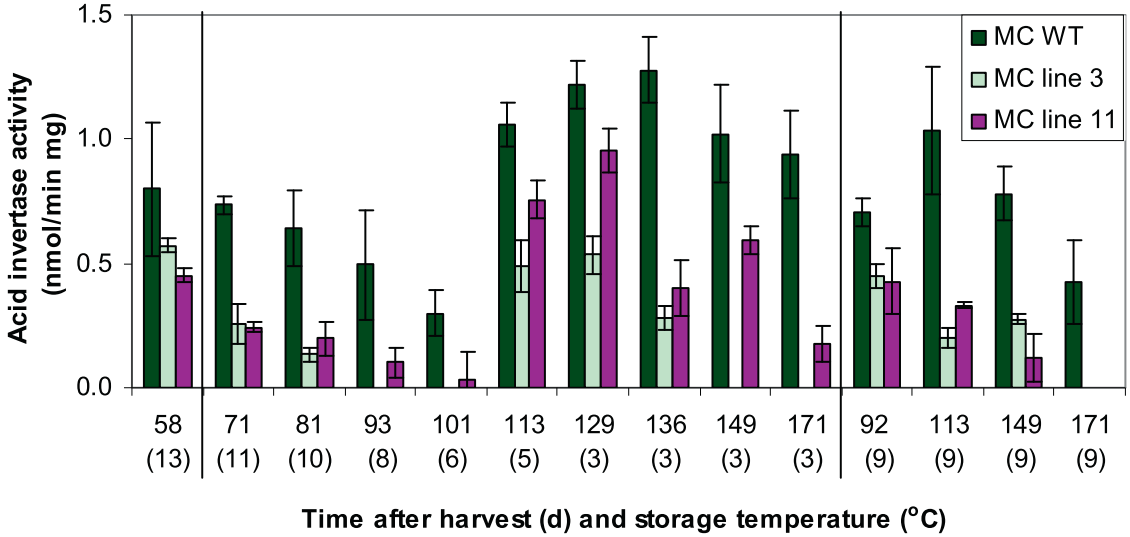

Fig. 4. Extractable acid invertase activity in MegaChip tubers grown in 2012 and sampled in 2012–13. Tubers were cooled to a final storage temperature of 3 or 9 °C. Values from wild-type (dark green bars), line 3 (light green bars), and line 11 (purple bars) tubers are shown. Numbers in parentheses under the DAH indicate the storage temperature of the tubers at each sampling time. Bars represent mean activities in three independent samples ± standard error.

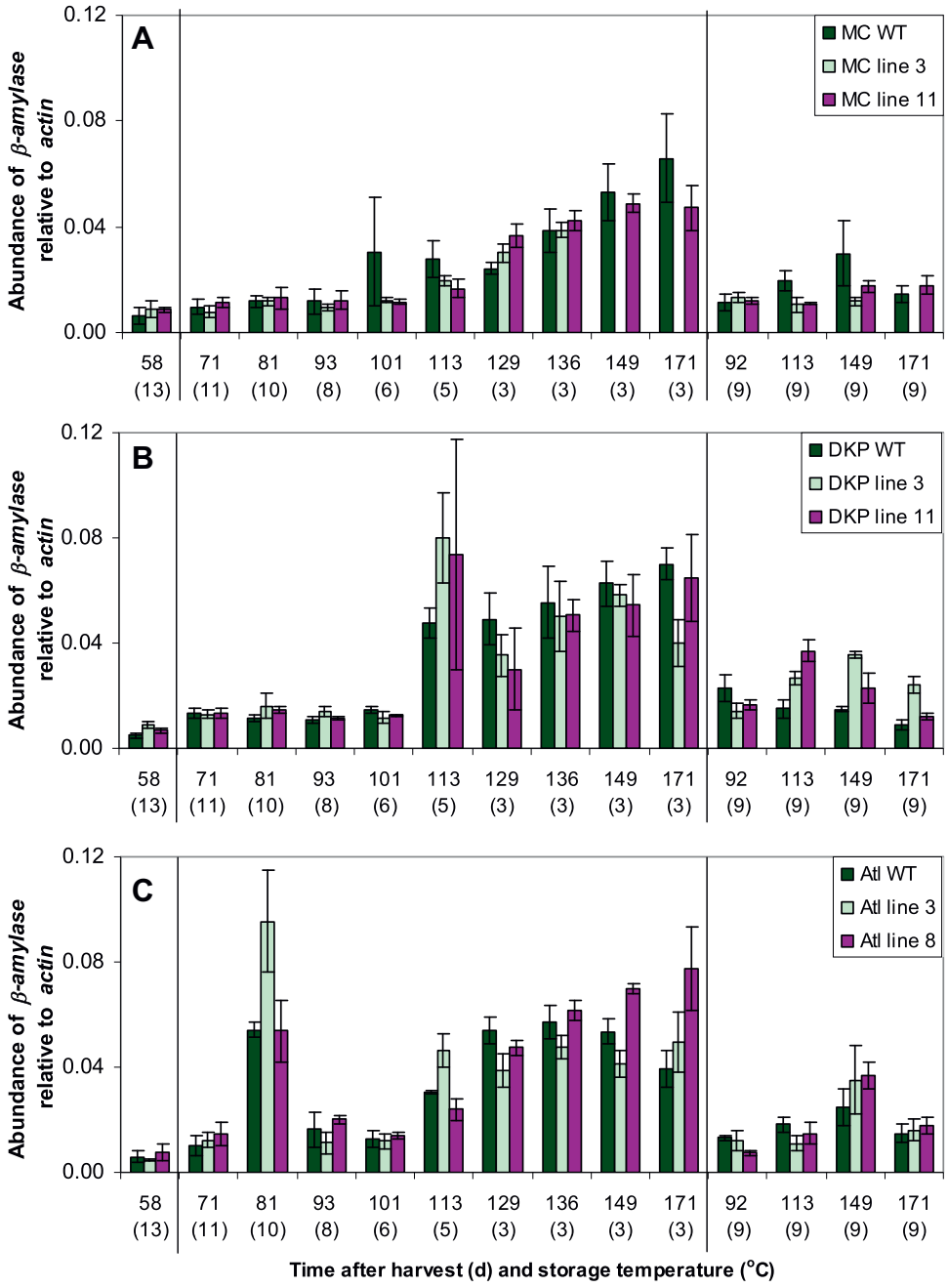

Fig. 5. Expression of *β-amylase* relative to *actin* in MegaChip (A), Dakota Pearl (B), and Atlantic (C) tubers grown in 2012 and sampled in 2012–13. Tubers were cooled to a final storage temperature of 3 or 9 °C. Values from wild-type (dark green bars); MegaChip line 3, Dakota Pearl line 3, and Atlantic line 3 (light green bars); and MegaChip line 11, Dakota Pearl line 11, and Atlantic line 8 (purple bars) tubers are shown. Numbers in parentheses under the DAH indicate the storage temperature of the tubers at each sampling time. Bars represent mean expression in three independent samples ± standard error.

Expression of the genes encoding the ADP-glucose pyrophosphorylase small subunit (*AGPase*) and granule-bound starch synthase (*GBSS*) decreased as tubers were cooled. In MC tubers, expression of both genes decreased gradually during cooling until tubers reached 5 °C and then decreased significantly ($p < 0.05$) with further cooling to 3 °C (Fig. 6A,D) . Expression remained low through 171 DAH in tubers stored at 3 °C, increasing slightly at the end of that time. Likewise, expression of both genes in DKP tubers decreased gradually during cooling, reaching its lowest point when tubers were at 5–6 °C and then increasing modestly at later times in storage (Fig. 6B,E). In Atl tubers, expression of both genes dropped significantly within 71 DAH, during the first ten days of cooling to 3 °C, and remained low until 171 DAH, when *AGPase* expression increased slightly (Fig. 6C,F). In Atl tubers stored at 9 °C, *AGPase* and *GBSS* expression remained low through 171 DAH, though expression levels observed at 9 °C were comparable to the highest level observed at 3 °C. Lower expression of these genes in tubers stored at 3 °C than in those stored at 9 °C was also seen in 2011–12 tubers (Fig. S5). In both years, expression in RNAi lines were similar to those seen in WT tubers; the few differences that were observed did not follow an apparent pattern.

Statistically significant differences in UDP-glucose pyrophosphorylase (*UGPase*) and sucrose phosphate synthase (*SPS*) expression in WT and RNAi MC tubers were not observed in response to

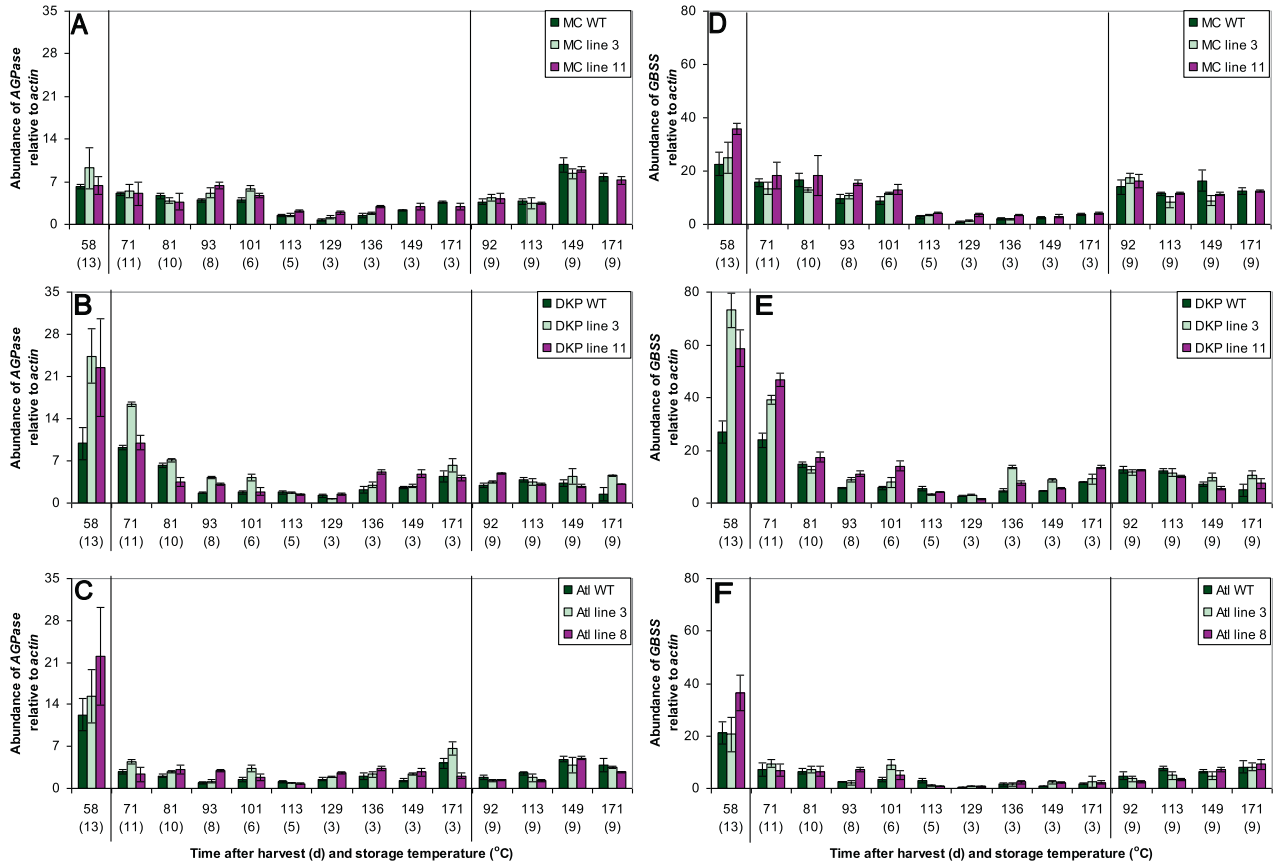

Fig. 6. Expression of *AGPase* (A–C) and *GBSS* (D–F) relative to *actin* in MegaChip (A,D), Dakota Pearl (B,E), and Atlantic (C,F) tubers grown in 2012 and sampled in 2012–13. Tubers were cooled to a final storage temperature of 3 or 9 °C. Values from wild-type (dark green bars); MegaChip line 3, Dakota Pearl line 3, and Atlantic line 3 (light green bars); and MegaChip line 11, Dakota Pearl line 11, and Atlantic line 8 (purple bars) tubers are shown. Numbers in parentheses under the DAH indicate the storage temperature of the tubers at each sampling time. Bars represent mean expression in three independent samples ± standard error.

cooling, final temperature, or time in storage (Fig. 7A,B). *SuSy* expression did vary slightly with changing time and temperature, but most differences were not statistically significant (Fig. 7C). Expression of invertase inhibitor (*INH*) genes in MC tubers sampled in 2012–13 was slightly lower after tubers reached 3 °C than during cooling or when tubers were at 9 °C (Fig. 7D). *SPS*, *SuSy*, and *INH* expression were similar between storage temperatures in MC, DKP and Atl tubers sampled in 2012 (data not shown).

4. Discussion

In the experiments described here, tubers of nine genotypes were stored under conditions representative of commercial storage, with extended preconditioning and slow cooling. Acceptable tuber processing quality was maintained by all genotypes when stored at 9 °C, a typical storage temperature for chipping potatoes. Tubers stored at this temperature had low and stable amounts of sucrose, glucose, and fructose, and they produced light-colored chips for more than five months after harvest (Figs. 1, 2, S1, S2). Processing quality of WT MC, DKP and Atl became unacceptable in 3 °C storage (Fig. 1). This was expected, given that no commercial chipping potato variety maintains acceptable reducing sugar contents and chip color at this temperature. Tubers that had strongly reduced *VInv* expression as a result of RNAi had lower reducing sugar concentrations than WT tubers and retained better processing quality for more than five months after harvest when stored at 3 °C (Fig. 1, S1).

Multiple pathways of molecular synthesis and degradation interact to establish the concentrations of sucrose and reducing sugars present in potato tubers. The data presented in Figs. 3–6 and S3–S5 support a model in which temperature-dependent transcriptional regulation of genes central to carbohydrate metabolism occurs in three overlapping phases during cooling and low-temperature storage. The first phase, which occurred as temperatures decreased from 13 to 10 °C, resulted in a marked increase in *VInv* transcript accumulation in WT MC and Atl tubers and a small increase in *β-amylase* expression in all nine genotypes. These changes in transcript abundance were not sufficient to promote chip color darkening or an increase in tuber sucrose or reducing sugar contents when tubers were stored at 9 °C. This initial accumulation of *VInv*, but not *β-amylase*, mRNA was delayed in WT DKP and all RNAi lines; in these lines, an increase in *VInv* mRNA was not apparent until storage temperatures were lower. For DKP, *VInv* mRNA abundance at approximately 6 °C was much lower than that observed in MC and Atl at temperatures of approximately 10 °C. This decreased sensitivity of DKP to temperature with regard to *VInv* mRNA accumulation may contribute to its ability to maintain acceptable chip color at temperatures as low as 5 °C (Thompson et al., 2005).

In the second phase, from the onset of cooling until the 3 °C set point was reached, amounts of *AGPase* and *GBSS* mRNA decreased substantially in all nine genotypes.

The third phase began when tubers reached approximately 3–5 °C; this phase was characterized by a rapid, large accumulation

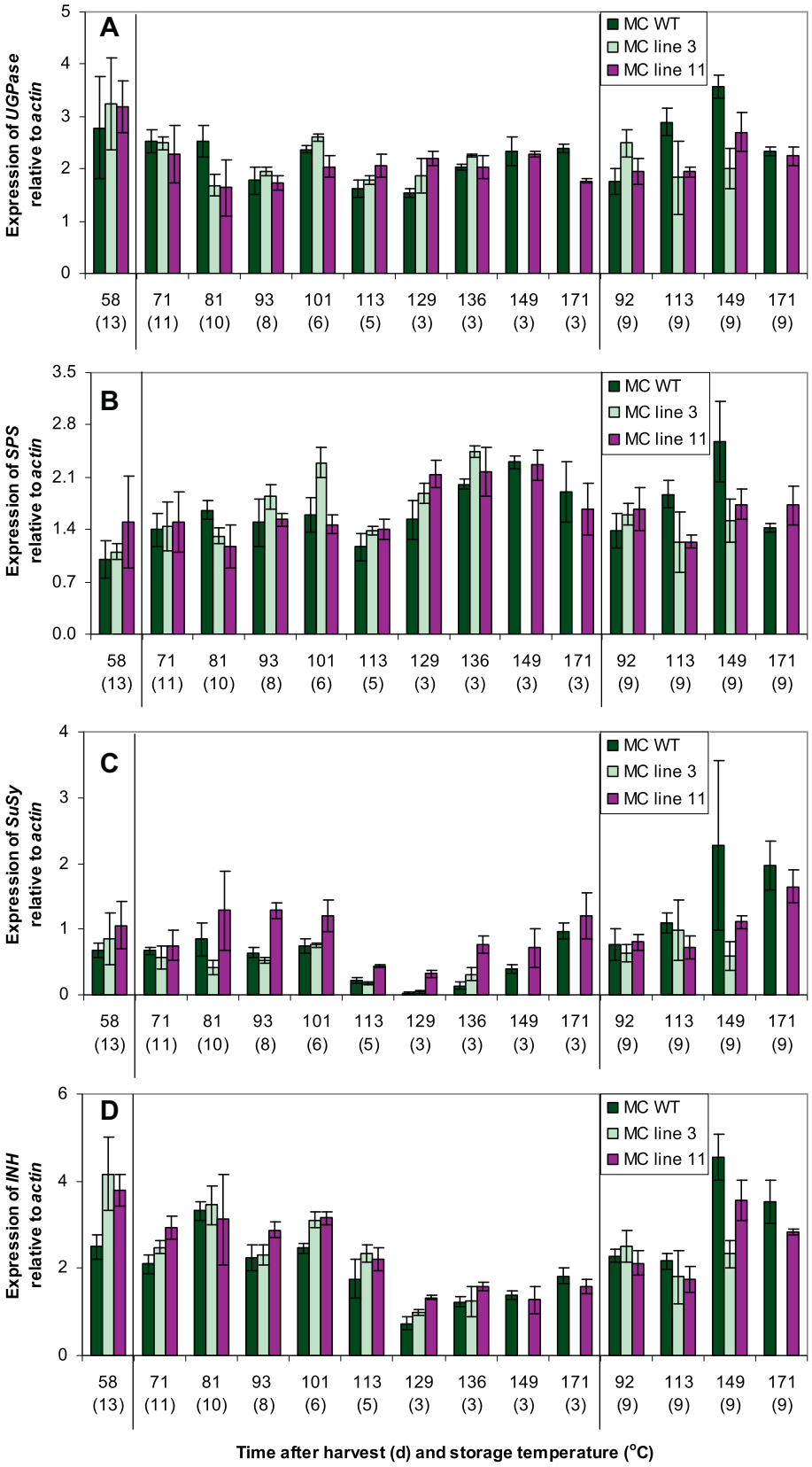

Fig. 7. Expression of *UGPase* (A), *SPS* (B), *SuSy* (C), and *INH* (D) in MegaChip tubers grown in 2012 and sampled in 2012–13. Tubers were cooled to a final storage temperature of 3 or 9 °C. Values from wild-type (dark green bars), line 3 (light green bars), and line 11 (purple bars) tubers are shown. Numbers in parentheses under the DAH indicate the storage temperature of the tubers at each sampling time. Bars represent mean expression in three independent samples ± standard error.

of *β-amylase* mRNA in all genotypes, further increases in *VInv* expression in all WT lines to reach their maximum levels shortly after tubers reached 3 °C, and gradual increases in *AGPase* and *GBSS* mRNA abundance. Changes in *β-amylase* mRNA accumulation during this phase coincided with comparably large, rapid increases in tuber sucrose contents in all genotypes; large, rapid increases in glucose and fructose in WT lines; and a corresponding darkening of fried chips in those lines. During this time period (>120 DAH in these experiments), tubers stored at 9 °C exhibited smaller increases in *β-amylase* mRNA; inconsistent changes in *VInv*, *AGPase*, and *GBSS* mRNA; no change in tuber sucrose; and little change in tuber reducing sugars and chip color. Given the negligible, if any, transcriptional control observed for *UGPase*, *SPS*, and *SuSy* during and after cooling, as well as the relatively small differences in VInv activity between 9 °C and 3 °C storage, it is reasonable to conclude that the large increase in sucrose observed as tubers were cooled to 5 °C and below and stored at 3 °C was driven by increased availability of substrate for sucrose synthesis as a result of increased rates of net starch breakdown. This supposition is consistent with numerous reports that concluded that cooling to low temperatures increases tuber sucrose content at the expense of tuber starch content (Müller-Thurgau, 1882; Appleman, 1912; Wright et al., 1936; Arreguin-Lozano and Bonner, 1949). Wright et al. (1936), for example, showed that Russet Burbank tubers stored for 124 days at 2.2 °C had 0.82% more sucrose, 1.22% more reducing sugars and 3.9% less starch on a fresh weight basis than tubers stored for 124 days at 10 °C. The data presented here suggest that an important part of this process is transcriptional control of *AGPase* and *GBSS*, which encode enzymes involved in starch synthesis, and *β-amylase,* which encodes a key enzyme in starch breakdown. The suggestion that availability of substrate would increase the rate of sucrose synthesis is supported by data showing that SPS protein is not rate limiting for sucrose synthesis, but rather that synthesis of sucrose 6-phosphate by SPS is strongly substrate limited (Hill et al., 1996).

VInv expression and activity were significantly reduced in five RNAi lines relative to WT, yet the reducing sugar contents and chip colors of those RNAi lines were similar to those of their respective WT lines when tubers of MC and DKP were stored at 9 °C. Reducing sugar contents for WT Atl, however, were greater than those in Atl RNAi tubers stored at 9 °C. This leads to the working hypothesis that differences in invertase activity have little effect on chip color and tuber reducing sugar contents as long as tuber sucrose contents remain below a threshold concentration that is unique for each genotype. If this hypothesis is correct, then tuber sucrose contents for MC and DKP, but not for Atl, were below their respective thresholds.

Cooling to temperatures below 3–5 °C resulted in a dramatic increase in sucrose concentration, and it is reasonable to postulate that this large change in tuber sucrose increased the substrate available to VInv, caused large increases in tuber reducing sugars, and degraded chip color. This model for the synthesis of large amounts of reducing sugars at 3–5 °C does not require VInv activity to increase substantially above that which occurred during cooling to more moderate temperatures, and the data in Figs. 4 and S4 show that extracted VInv activities were comparable in tubers stored long-term at 9 °C and 3 °C. Deiting et al. (1998) found a similar pattern of invertase activity in tubers of the fresh market cultivar 'Desirée' preconditioned for four weeks and then cooled to various temperatures between 3 and 7 °C. VInv activities did not differ among storage temperatures, but reducing sugar contents did differ. Though VInv was active and reducing sugar contents increased during cold storage, sucrose concentrations also increased at 3 °C. Hence, net metabolic flux of carbon from starch to sucrose increased to a greater degree than did the rate of sucrose hydrolysis by invertase. Others have also observed coincident increases in VInv activity and tuber sucrose content during storage of fry and chip processing potatoes (Zhou and Solomos, 1998; McKenzie et al., 2005; Zommick et al., 2014). The data from McKenzie et al. (2005) highlight this concept of metabolic poise. Of ten chipping potato genotypes that had increased VInv activity between two weeks and five months of storage, three had increased tuber sucrose, four had decreased tuber sucrose, and three had no appreciable change in sucrose.

Lines in which *VInv* expression was effectively reduced had strongly reduced contents of glucose and fructose and substantially lighter chip color than WT lines. However, temperature- and time-dependent changes in expression of *β-amylase*, *AGPase*, *GBSS*, *UGPase, SPS* and *INH* did not differ between WT and RNAi lines of the same variety. This observation indicates that there is little feedback from reducing sugars to transcriptional control of carbohydrate metabolism.

Changes in *VInv* expression in WT Atl, DKP, and MC tubers over time were also consistent with those previously observed in Katahdin (Wiberley-Bradford et al., 2014). Late in storage, *VInv* mRNA levels decreased or remained constant in all four of these varieties. The latter observation is in stark contrast to patterns seen in WT Russet Burbank tubers, in which *VInv* expression increased 6- to 15-fold between 1 and 5 months after harvest (Zhu et al., 2014). Thus, it seems that *VInv* expression follows a similar pattern in different varieties of round potatoes used for chip processing, but that pattern is different from that seen in at least one potato variety used for French fry processing. With further research, it may be possible to define these patterns of *VInv* expression as a general difference between these two market classes.

Several of the changes in gene expression observed here are consistent with previous research. Large increases in β-amylase activity have often been observed in potato tubers and have been shown to result from storage at temperatures of ≤5 °C (Deiting et al., 1998), rather than from storage at 7–10 °C or extended time in storage (Bagnaresi et al., 2008; Cochrane et al., 1991; Deiting et al., 1998; Hill et al., 1996; Nielsen et al., 1997; Zhang et al., 2014a). Increased *β-amylase* expression has been shown to increase starch degradation. For example, decreased expression of an endogenous amylase inhibitor in diploid potato increased α- and β- amylase activity and increased the rate of starch degradation (Zhang et al., 2014a). Conversely, overexpression of an amylase inhibitor from *Solanum berthaultii* in cultivated potato decreased β-amylase activity and starch degradation (Zhang et al., 2014b). Furthermore, overexpression of *AGPase* has been found to increase starch and decrease sucrose, glucose, and fructose contents in stored tubers (Stark et al., 1996). Combined with increased *β-amylase* expression and activity, decreases in AGPase and GBSS activities that paralleled the observed decreases in expression would slow the rate of net starch synthesis, increasing hexose flux to sucrose synthesis and leading to the increased sucrose contents seen in tubers stored at 3 °C. This agrees with the observation that MC and DKP tubers stored at 9 °C had greater *AGPase* and *GBSS* expression than those stored at 3 °C and also had lower sucrose contents. Further investigations of β-amylase and AGPase activities in cooling tubers are underway.

Responses of the other studied genes to changes in storage temperature generally did not agree with observations from the literature. *UGPase* expression did not increase in cold-stored tubers in this study, though it did in two previous studies (Bagnaresi et al., 2008; Chen et al., 2008). *SPS* and *SuSy* expression did not change significantly in the current study, though increases in their expression or activity were previously observed after transfer to low temperatures (Bagnaresi et al., 2008; Deiting et al., 1998; Pressey, 1970). *INH* expression did not increase after tubers reached low temperature, though Brummell et al. (2011) reported an increase in activity in cold-stored tubers. These discrepancies

could be due to differences between varieties, differences in the length of the preconditioning period, or differences in the cooling regimens to which the tubers were subjected. In the studies cited, tubers were transferred directly from higher to lower storage temperatures, while in the current study tuber storage temperatures were lowered gradually, as is standard in commercial potato storage. Plant organs often respond differently to acute stresses than to chronic stress; the expression of the genes in question may differ in their responses to rapid or slow temperature changes. Such differences may be one reason that slow cooling is used in industry for chip and fry processing varieties. A comparison of tuber responses to rapid versus slow cooling would be an interesting area of future research.

5. Conclusions

Taken together, the data presented here give insights into a more nuanced view of cold-induced sweetening that encompasses effects of storage temperature and time in storage. The extent of reducing sugar accumulation is controlled by a combination of temperature-specific changes in sucrose accumulation and by the activity of VInv. For tubers of the storage varieties MC and DKP held at 9 °C, increased *VInv* expression due to cooling and decreased VInv activity due to RNAi had little effect on reducing sugar contents and chip color when tuber sucrose contents were unchanged from those observed at the end of preconditioning. Large increases in sucrose abundance at temperatures at and below 5 °C likely resulted from increased starch degradation by β-amylase and decreased starch resynthesis by AGPase and GBSS. This shifted net carbon flow away from starch synthesis and toward sucrose synthesis, and it increased the substrate available to VInv late in low-temperature storage, with the end result that sucrose hydrolysis by invertase led to large increases in tuber reducing sugars and a degradation of chip color. Suppression of *VInv* expression did not alter the expression of *β-amylase*, *AGPase*, *GBSS*, or other genes, indicating that there was little or no feedback from reducing sugars to transcriptional control of carbohydrate metabolism.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.postharvbio.2015.12.020.

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