Developing Cold–Chipping Potato Varieties by Silencing the Vacuolar Invertase Gene

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ABSTRACT

Accumulation of reducing sugars during cold storage is a persistent and costly problem for the potato (*Solanum tuberosum* L.) processing industry. High temperature processing of potato tubers with elevated amounts of reducing sugars results in potato chips, fries, and other products that are unacceptable to consumers because of their bitter taste and unappealing dark color. More problematically, such products contain increased amounts of acrylamide, a neurotoxin and a potential carcinogen. We have demonstrated that silencing of the potato vacuolar acid invertase gene *VInv* can prevent reducing sugar accumulation in cold-stored tubers. Using this approach we developed *VInv* silencing lines using RNA interference (RNAi) from four potato cultivars grown currently for potato chip production in North America. Accumulation of reducing sugars during cold storage was reduced by ~93% or more in all RNAi lines that had >90% reduction of *VInv* transcript. Potato chips produced from these lines were light colored and significantly lower in acrylamide than controls. Changes in growth and tuber yield were not associated with *VInv* suppression using RNAi. We demonstrate that silencing of *VInv* is an effective approach to control the cold-induced sweetening problem in potato.

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Abbreviations: cDNA, complementary DNA; CIS, cold-induced sweetening; FW, fresh weight; HPLC, high performance liquid chromatography; mRNA, messenger RNA; PCR, polymerase chain reaction; qRT-PCR, quantitative real-time polymerase chain reaction; RNAi, RNA interference; RT, room temperature, UDP-glucose, uridine diphosphate glucose; UGPase, uridine diphosphate glucose pyrophosphorylase; VINV, vacuolar acid invertase enzyme; *VInv*, vacuolar acid invertase gene.

CULTIVATED POTATO (*Solanum tuberosum* L.) is grown commercially worldwide and is the world's number one nongrain food commodity (FAO, 2010). Global potato production is estimated at over 325 million t and is increasing rapidly in the developing nations including China and India (FAO, 2008). Potato tubers are used to produce starch for industrial purposes and food for human consumption. In the United States, over half of all potatoes are used by the potato processing industry to make French fries, potato chips, and similar items (NASS-USDA, 2009). Tubers used by the potato processing industry must meet stringent requirements for quality. One of the most important of these is the ability to produce light–colored products after frying, and this depends primarily on the content of the reducing sugars glucose and fructose in raw tubers. During high temperature processing, reducing sugars react with free amino acids in a nonenzymatic Maillard reaction to

produce dark-colored pigments (Kumar et al., 2004; Shallenberger et al., 1959). Acrylamide is a byproduct of this reaction that results from the reaction between reducing sugars and asparagine (Mottram et al., 2002; Rommens et al., 2008; Stadler et al., 2002). Acrylamide in food has emerged recently as a potential health risk, and decreasing the content of acrylamide in fried potato products is a priority with the potato industry (Foot et al., 2007).

Potato is a fresh vegetable crop that must be stored carefully to maintain high quality. As for many other vegetables, low temperature storage has many benefits for growers and consumers. These include decreased rates of respiration, prolonged sprout dormancy, and substantial reductions in spoilage caused by pathogens. Unfortunately, potato tubers respond to low temperatures by accumulating relatively high amounts of sucrose, part of which is converted into glucose and fructose by the vacuolar acid invertase enzyme (VINV). This process is referred to as cold-induced sweetening (CIS) (Dale and Bradshaw, 2003; Kumar et al., 2004; Matsuura-Endo et al., 2004; Sowokinos, 2001). Because of CIS, potato tubers destined for high temperature processing are stored at relatively warm temperatures, typically 9 to 11°C, even though this increases the likelihood of spoilage and the need for chemical sprout inhibitors. An alternative method is to store tubers at colder temperatures and then warm them before processing (Illeperuma et al., 1998). This is referred to as reconditioning and in many cases it can reverse the adverse effects of low temperature storage. Unfortunately, reconditioning large stores of potatoes takes several weeks and during this time tubers are at increased risk of spoilage and sprouting. Reconditioning is not favored by potato storage managers in the United States and is used primarily in attempts to recover useful product from tubers that have unexpectedly undergone CIS.

Most of the enzymes involved in the net conversion of starch to sucrose and reducing sugars during CIS have been characterized at the molecular and biochemical level (Geigenberger et al., 2004; Kumar et al., 2004; Sowokinos, 2001). Transgenic potato lines expressing antisense constructs or overexpressing sense constructs have been used to evaluate the contribution of many of these enzymes to tuber carbohydrate metabolism (Agarwal et al., 2003; Chen et al., 2008; Geigenberger et al., 2004; Greiner et al., 1999; Junker et al., 2005; Lorberth et al., 1998; Schwall et al., 2000; Zhang et al., 2008; Zrenner et al., 1996). These data, and data from potato clones with varying degrees of CIS, have been used to identify a short list of candidate enzymes thought to be most important in controlling CIS. Of these, cytosolic uridine diphosphate glucose pyrophosphorylase (UGPase) and VINV have received the most attention (Greiner et al., 1999; Gupta et al., 2008; Kumar et al., 2004; Matsuura-Endo et al., 2004; Sowokinos, 2001; Zrenner et al., 1996). Uridine diphosphate glucose pyrophosphorylase is responsible for the initial, rate-limiting step in the formation of sucrose. In this reversible reaction, glucose-1 phosphate and uridine triphosphate (UTP) form uridine diphosphate glucose (UDP-glucose) and pyrophosphate. Two additional cytosolic enzymes, sucrose phosphate synthase and sucrose phosphate phosphatase, produce cytosolic sucrose from UDP-glucose. Some of this sucrose is transported into the vacuole where VINV carries out an irreversible reaction to produce equal amounts of glucose and fructose. Increased transcription of UGPase genes and the vacuolar acid invertase gene (*VInv*) and increases in activity of the respective enzymes occur in tubers stored at temperatures of less than ~10°C (Bhaskar et al., 2010; Illeperuma et al., 1998; Matsuura-Endo et al., 2004; Zrenner et al., 1993).

A long-term goal of the potato breeding community has been to develop cultivars that maintain acceptable amounts of reducing sugars when stored at cold temperatures. Slow, steady progress has been made over the past fifty years, but no cultivars are resistant to CIS (Douches et al., 1996; Love et al., 1998). Alternatively, molecular approaches have been used to further our understanding of CIS and to demonstrate proof of concept for CIS-mitigation strategies using biotechnology. We recently showed that suppressing *VInv* expression using RNA interference (RNAi) dramatically reduces the accumulation of reducing sugars in tubers of Katahdin, a potato cultivar released in 1938 that has no resistance to CIS. When *VInv* accumulation was reduced to ~3% of control expression, CIS was effectively prevented (Bhaskar et al., 2010). Many modern potato cultivars have a higher resistance to CIS than Katahdin, and potato breeders have known for many years that VINV plays an important role in the development of CIS (Matsuura-Endo et al., 2004; Pressey, 1969; Pressey and Shaw, 1966). It was unclear, therefore, if a similar approach using potato cultivars currently in production would lead to comparable, dramatic improvements in CIS resistance, and if so, how much of a reduction in *VInv* transcript would be required to effectively prevent CIS.

To determine if *VInv* suppression could be used to control CIS in modern potato germplasm, and to determine the extent that *VInv* expression would need to be reduced to effectively control CIS, we transformed four potato chipping cultivars with RNAi constructs that target *VInv* messenger RNA (mRNA). The cultivars selected, Atlantic, Snowden, Dakota Pearl, and MegaChip, are representative of chipping cultivars developed over the last four decades. Atlantic was released in 1976 and is currently the most widely grown public variety for chip production in the United States. Snowden, released in 1990, shares one parent with Atlantic but has greater resistance to CIS. Dakota Pearl and MegaChip (Groza et al., 2007), released in 1999 and 2007, respectively, are still more resistant to CIS than Atlantic and Snowden and the pedigrees for each of them are distinctly different from those of the other three cultivars. We demonstrate that *VInv* suppression and CIS prevention can be achieved in all four cultivars.

MATERIALS AND METHODS

Plant Materials

Potato varieties Dakota Pearl, Atlantic, MegaChip, and Snowden were used in this study. In-vitro clones of each variety were propagated and maintained in tissue culture under a 16 h 23°C/8 h 21°C light/dark regime on potato modified Murashige and Skoog (MS) medium (Phytotechnology Labs, Shawnee Mission, KS) containing 3% sucrose and 0.2% phytogel. *VInv*-RNAi lines resulting from successful transformations and control plants were grown at greenhouse facilities of University of Wisconsin-Madison. Plants were grown at 15 to 25°C with a light (irradiance 500 μmol m^{-2} s^{-1}) and dark cycle of 16 and 8 h, respectively. Plants were fertilized with 5 g Nutricote (13–13–13, Type 100; Sun Gro Horticulture, Bellevue, WA) at 20 and 60 d after planting. Plants were grown for 100 to 130 d and harvested at full vine senescence. Data were collected for fresh tuber weights at harvest computed as the total weight of all tubers harvested from each pot. Tubers were cured for 1 wk at room temperature (RT) (23°C) in the dark and subsequently moved to 4°C cold storage up for 4 mo.

Development and Characterization of Transgenic Plants

Transgenic potato lines in which the vacuolar acid invertase gene was silenced were achieved through RNAi. RNA interference construct InvBP1 (Bhaskar et al., 2010) was used in transformation of Atlantic, MegaChip, and Snowden; construct InvBP2 (Bhaskar et al., 2010) was used in transformation of Dakota Pearl. These two constructs were designed to cover different parts of *VInv* and were found to be equally effective in silencing (Bhaskar et al., 2010). An *Agrobacterium tumefaciens* strain GV3101 containing pHellsGate8-*VInv* plasmid InvBP1 or InvBP2 was used for potato transformation. The transformation method using stem internode explants was as described before (Bhaskar et al., 2008). Kanamycin antibiotic was used as a transgenic plant selection marker. The extent of *VInv* suppression in transformed plants was characterized initially by assessing the amount of *VInv* transcript in leaves as described by Bhaskar et al. (2008).

Measurement of *VInv* Transcription

The *VInv* expression in the transgenic lines was initially assessed by RNA blot analysis using RNA isolated from leaf tissue. Although the expression levels of *VInv* in leaf and tuber tissues were generally correlated in the transgenic lines (P.B. Bhaskar and J. Jiang, unpublished data, 2010), we selected 12 independent transgenic lines derived from each variety along with controls for precise quantification of *VInv* transcript amount in tubers. The 12 selected transgenic lines of each variety represent the four lines with the lowest transcript levels in leaves and eight lines with partial suppression of *VInv* based on Northern data. Quantitative real-time polymerase chain reaction (PCR) was performed on tuber samples that had been stored at 4°C for 30 d. Potato tuber RNA was isolated using Plant RNA isolation kit (Agilent, Wilmington, DE) according to the manufacturers' instructions. Total RNA samples were treated with TURBO DNA-free (Ambion, Austin, TX) to eliminate DNA contamination. First strand complementary DNA (cDNA) was synthesized from 2 μg deoxyribonuclease (DNase)-treated total RNA using Super Script III reverse transcriptase (Invitrogen, Carlsbad, CA). For quantitative real-time PCR, quadruplicate samples of each transgenic line as well as control were prepared using DYNAMO SYBR Green master mix (Finnzymes; New England Biolabs, Ipswich, MA) and run on a MJ Research Opticon 2 (Bio-Rad Laboratories, Hercules, CA). The quantitative real-time polymerase chain reaction (qRT-PCR) was performed for 40 cycles of heat denaturation at 94°C for 20 s, annealing at 55°C for 20 s, and extension at 72°C for 30 s after an initial heat denaturation at 95°C for 15 min. *Solanum tuberosum Actin-97* (TC164213) was used as a reference gene. The silencing percentage for *VInv* was calculated by the comparative Ct method (Livak and Schmittgen, 2001), where delta Ct of untransformed cultivar controls was defined as 100% transcript presence. Primers for *VInv* and *Actin-97* amplification were those used by Bhaskar (Bhaskar et al., 2010).

Assessment of Chip Color, Tuber Sugar Contents, and Chip Acrylamide Levels

Chipping experiments were performed on tubers that were stored at 4°C for either 30 d or 120 d without reconditioning. Tubers were washed, dried, cut lengthwise, and sliced into 1.5 mm thick chips for frying. Remaining tuber samples were frozen in liquid nitrogen for either quantification of *VInv* transcript or determination of tuber sugar profiles. Tuber slices were fried in vegetable oil for 2 min at 191°C or until the cessation of bubbles. Chip color scores were based on the Potato Chip Color Reference Standards (Snack Food Association, Arlington, VA) from 1 (light) to 10 (dark).

The amount of sucrose, glucose, and fructose was quantified in tubers using high performance liquid chromatography (HPLC) as described previously (Bethke et al., 2009). All tubers used in sugar measurements were subjected to cold storage for 30 d. For acrylamide analysis, chipping experiments were performed on tubers stored at 4°C for 30 d without reconditioning. Potato tubers were cut lengthwise to obtain slices and fried in vegetable oil for 2 min at 184°C or until the cessation of bubbles. Fried chips were left at RT (23°C) for 5 min to cool and thoroughly ground to a fine powder in liquid nitrogen. The powder samples were submitted to the Department of Bacteriology, University of Wisconsin-Madison, for acrylamide analysis. Samples for acrylamide were analyzed by a modified USEPA method described previously (Park et al., 2005).

For statistical analysis, a *t* test (two-sample unequal variance and two-tailed distribution) was used to determine whether the means of two groups were significant from each other. Linear regression analysis was conducted with R (R Development Core Team, 2010) and the data analysis package of Microsoft Excel (Microsoft Corporation, 2003).

RESULTS

Development of *VInv* Silencing Lines in Four Potato Chipping Varieties

Four potato varieties, Atlantic, Dakota Pearl, Snowden, and MegaChip, were selected for *VInv* silencing. A total of 44, 45, 71, and 86 independent transgenic lines were developed
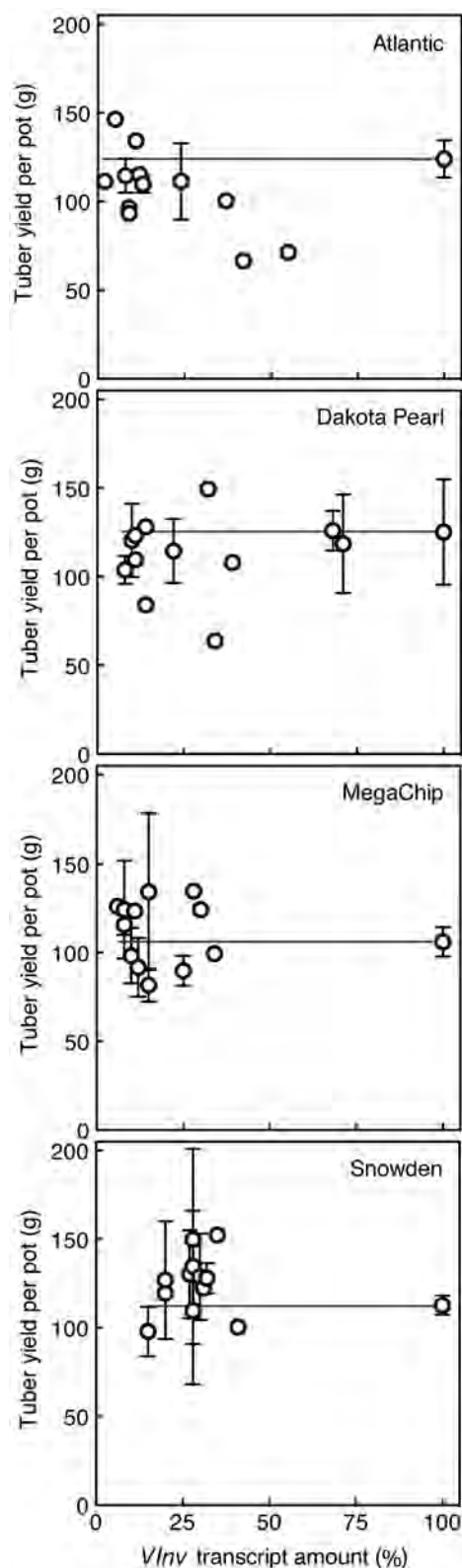
Figure 1. Tuber yield per pot of RNA interference (RNAi) lines and nontransformed controls. Error bars represent mean standard deviations of lines with two or more individuals. Horizontal lines are mean values for control cultivars (*n* = 3; 100% vacuolar acid invertase gene (*VInv*) transcript amount).

from these four varieties, respectively. The reduction of *VInv* expression in the transgenic lines was analyzed initially by

Northern blot hybridizations using RNA isolated from leaf tissue. From each variety we selected four RNAi lines that showed the lowest amounts of *VInv* transcript on Northern blots and eight additional lines in which *VInv* was silenced to varying degrees. These 48 lines were evaluated by real-time quantitative PCR to determine the extent that *VInv* expression was reduced in tubers that had been stored at 4°C for 30 d (Supplemental Table S1). There was good agreement between relative expression of *VInv* in leaves, measured by Northern blots, and expression in tubers as measured by qRT-PCR (data not shown). Although the 12 RNAi lines from each variety were selected using a common approach, different levels of *VInv* silencing were achieved in the four varieties. The 12 Atlantic RNAi lines showed a range of 45 to 98% *VInv* transcript reduction and five lines showed >90% reduction (Supplemental Table S1). In contrast, the 12 Snowden RNAi lines showed a narrow range of *VInv* transcript reduction (59–85%) and none of the lines had >90% reduction (Supplemental Table S1).

The 12 selected RNAi lines for each variety together with nontransformed controls were grown in greenhouses. Plant growth (data not shown) and tubers (Supplemental Fig. S1) of the transgenic lines were comparable in appearance to the controls. Mean total tuber fresh weights of individual transgenic lines were measured and yield per pot for the transgenic lines was not different from that of the cultivar controls across the range of *VInv* suppression examined (Fig. 1). Best-fit linear regression lines through the data in any of the panels in Fig. 1 have slopes that are not statistically different from zero (data not shown).

Sugar Contents of the RNA Interference Lines

For each variety, five RNAi lines spanning a range of *VInv* suppression from the highest obtained for that variety to less than 70% suppression (Fig. 2) were selected for detailed characterization. Tuber sugar contents of these selected lines were quantified by HPLC. High amounts of reducing sugars were observed in tubers from nontransformed controls after 30 d at 4°C (Fig. 3). Nontransformed Dakota Pearl had the lowest reducing sugar content in tubers of the controls, but both glucose and fructose were still commercially unacceptable at ~2 mg g^{-1} fresh weight (FW) (Fig. 3). In contrast, tubers from the RNAi lines with >90% *VInv* transcript reduction had very low amounts of reducing sugars (0.04–0.13 mg g^{-1} FW) (Fig. 3). For example, RNAi line #1 of Atlantic (98% reduction of *VInv* expression) contained only 0.12 and 0.15 mg g^{-1} FW of fructose and glucose, respectively, which is a 50- and 33-fold reduction compared to the Atlantic control.

Tubers from RNAi lines of Atlantic, Dakota Pearl, and MegaChip with strongly reduced amounts of *VInv* transcript (>90%) had low to very low amounts of reducing sugars whereas tubers from lines with greater *VInv* transcript
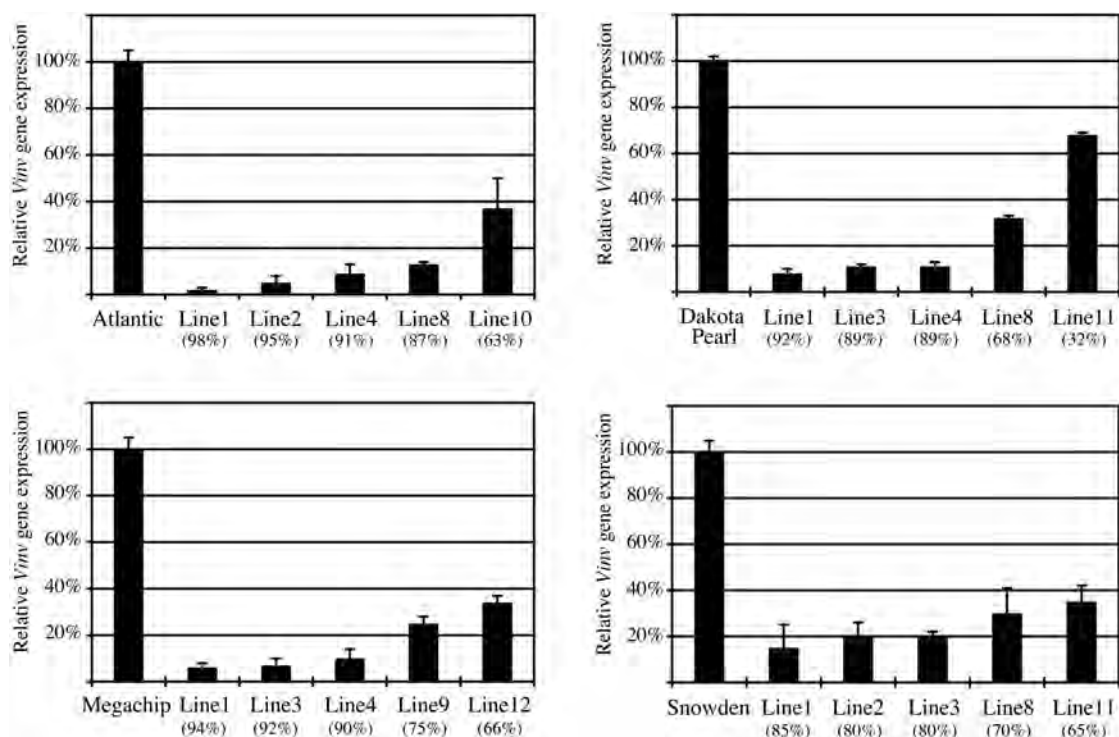
Figure 2. Quantification of vacuolar acid invertase gene (*VInv*) expression in five RNA interference (RNAi) lines derived from four potato varieties. Real-time polymerase chain reaction (PCR) was performed using complementary DNA (cDNA) prepared from tubers after 30 d of storage at 4°C. Numbers in parentheses indicate the extent of *VInv* silencing for the respective RNAi line as a percentage of nontransformed controls. Bars represent mean expression of *VInv* in two independent tuber samples from two plants relative to expression of the potato *Actin-97* gene.
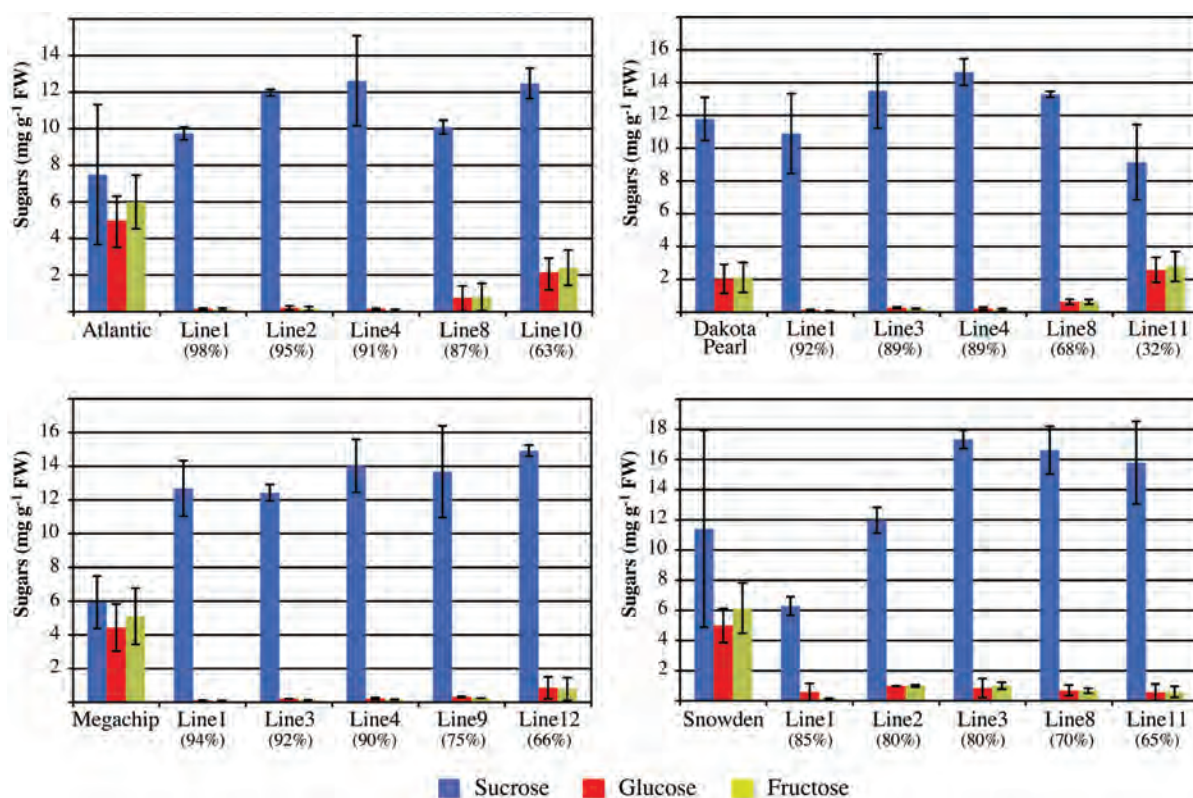

Figure 3. Sucrose, glucose, and fructose concentrations in tubers from selected RNA interference (RNAi) lines and nontransformed controls. Sugars levels were monitored among tubers stored at 4°C for 30 d. The sugar concentrations are expressed as mg g^{-1} of tuber fresh weight (FW). Data are means ±SD of two samples.
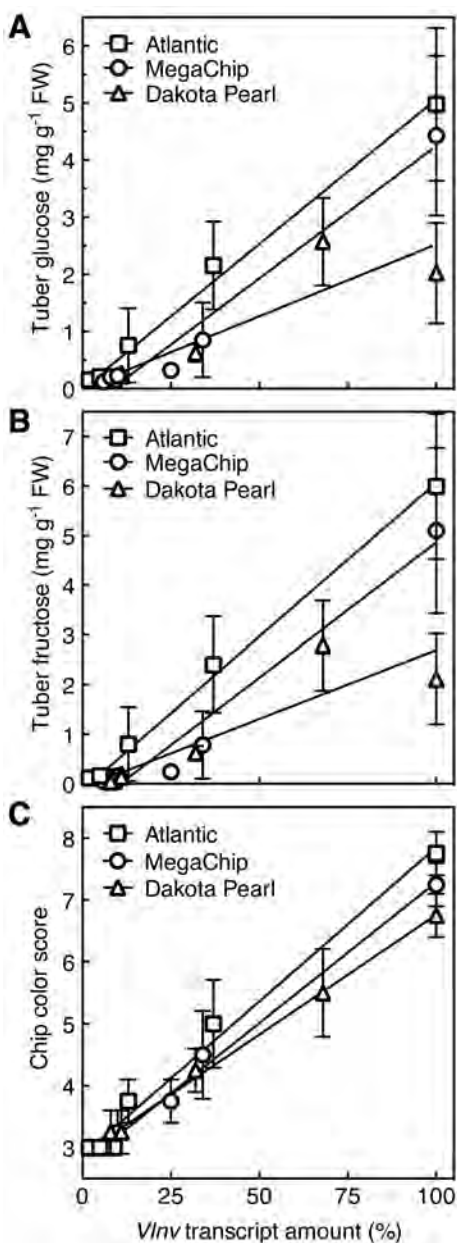
Figure 4. Correlation of the amounts of reducing sugars and chip color from cold stored potato tubers (30 d at 4°C) and the amount of vacuolar acid invertase gene (*VInv*) transcript in the corresponding RNA interference (RNAi) lines. The *x*-axis values represent *VInv* expression in the RNAi lines as a percentage of the nontransformed control of the same potato variety. Lines indicate linear regression between the transcription of *VInv* and glucose accumulation (A), fructose accumulation (B), and chip color (C). The slopes of all three linear regressions were significant with $p < 0.01$. FW, fresh weight.

accumulation had higher amounts of reducing sugars. For example, RNAi line #10 of Atlantic (63% reduction) had a decrease of 2.3-fold in fructose and 2.5-fold in glucose and RNAi line #8 of Dakota Pearl (68% reduction) had a decrease of 3.4-fold in fructose and 3.3-fold in glucose compared with controls. RNA interference line #11 of Dakota Pearl (32% reduction) accumulated almost the same amount of reducing sugars compared to the control (Fig. 3).

A linear regression analysis among RNAi lines of Atlantic, Dakota Pearl, and MegaChip showed that glucose and fructose in cold-stored tubers were correlated with the extent of *VInv* transcript reduction (Fig. 4A and 4B).

The 12 RNAi lines developed from Snowden showed a range of 59 to 85% reduction of *VInv* expression (Supplemental Table S1), which was narrower than that for the RNAi lines from the other three varieties. Interestingly, the tubers from each of the five Snowden RNAi lines examined in detail (with 65–85% reduction of *VInv* expression) showed a similar level of reducing sugar accumulation in the range of 6- to 15-fold reduction compared to Snowden controls (Fig. 3).

All RNAi lines developed from the four potato varieties showed high levels of sucrose accumulation in cold-stored tubers (Fig. 3). Thus, all lines responded to cold temperatures by accumulating sucrose. Cold-stored tubers from the RNAi lines of MegaChip accumulated more sucrose than cultivar controls ($p = 0.011$ with one-way ANOVA), with tuber sucrose in RNAi lines approximately twice that in controls. Sucrose contents in RNAi lines of the other three varieties were not statistically different from controls.

Processing Quality of the RNA Interference Lines

We performed potato chip analysis of tubers from the same RNAi lines used for sugar profiling. Potato chips were processed from tubers stored at RT or at 4°C for 30 or 120 d. Chips processed from cold-stored tubers of nontransformed controls for all four varieties were dark colored (Fig. 5), with mean chip color scores in the range of 6.8 ±0.4 to 7.8 ±0.4 (Table 1). In contrast, potato chips processed from the RNAi lines with >90% *VInv* transcript reduction were light colored after 4°C storage at either 30 (Fig. 5) or 120 d (Supplemental Fig. S2). For example, chips from tubers of three RNAi lines of Atlantic with 91, 95, and 98% reduction in *VInv*, respectively, maintained a chip color score of 3.0 after the tubers were stored for 30 and 120 d, respectively (Table 1).

Chips processed from cold stored-tubers from RNAi lines with 60 to 80% gene silencing had medium levels of color, which were lighter than those from controls but darker than those of highly silenced RNAi lines (Table 1). A simple linear regression analysis among RNAi lines of Atlantic, MegaChip, and Dakota Pearl showed that the mean chip score values were correlated with the levels of *VInv* expression (Fig. 4C). The R^2 values for Atlantic, MegaChip, and Dakota Pearl were 0.982, 0.993, and 0.997, respectively ($p < 0.05$).

The chips processed from each of the five Snowden RNAi lines had similar color values, ranging from 3.75 to 4.75 at 30 d and from 4.0 to 4.75 at 120 d of cold storage (Table 1). A statistically significant correlation between the chip color values and the levels of *VInv* transcription
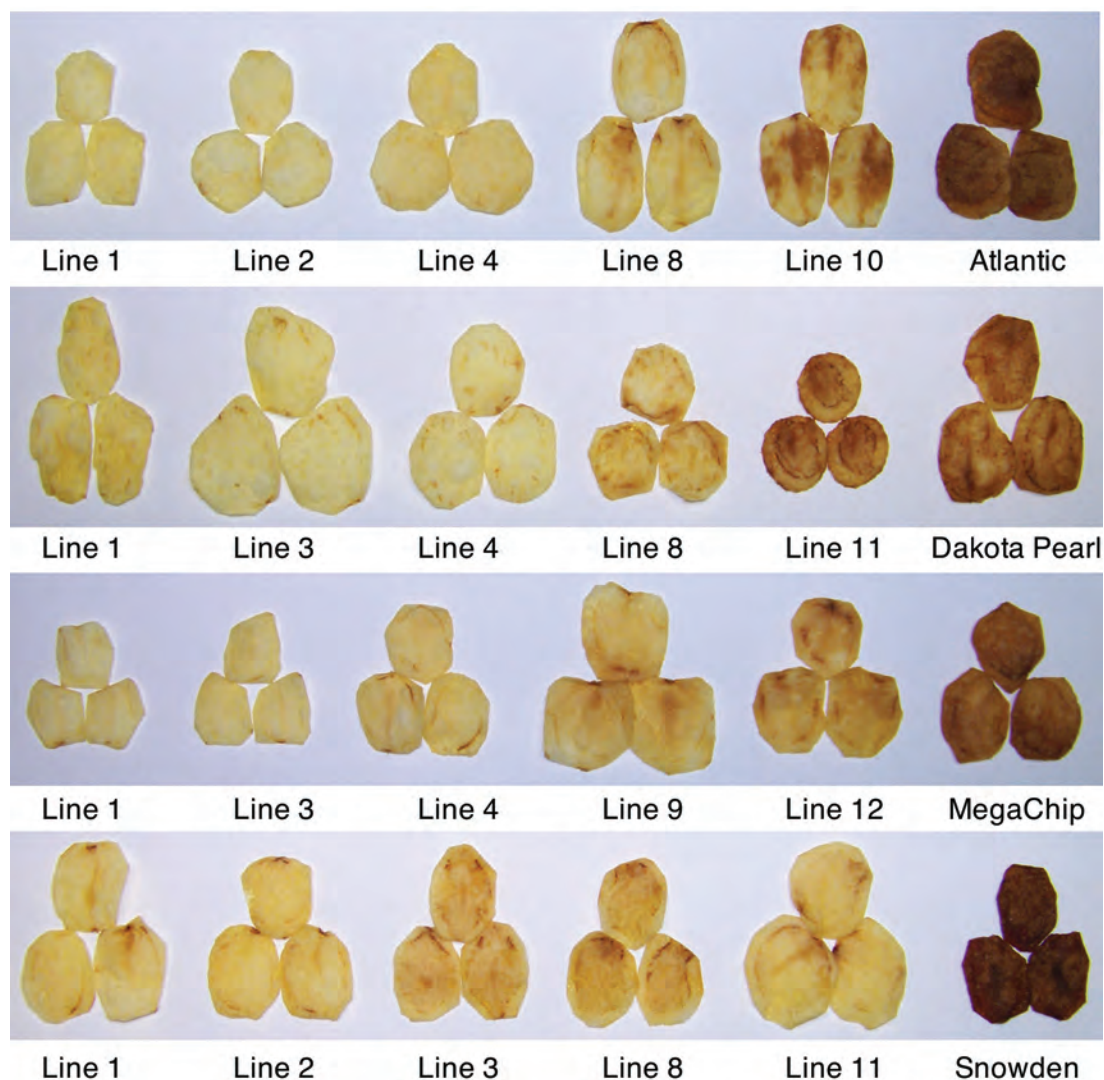
Figure 5. Potato chips processed from five highly silenced and partially silenced RNA interference (RNAi) lines and nontransformed controls. Tubers were stored at 4°C for 30 d before processing.

was not observed among the Snowden RNAi lines, and this is consistent with the reducing sugar data.

Reducing sugars and asparagine are the two most important substrates for acrylamide formation in processed potato products. We measured the amount of acrylamide in potato chips from cold stored tubers of selected RNAi lines of Atlantic. Potato chips processed from nontransformed Atlantic tubers stored at 4°C for 30 d contained acrylamide at 3880 µg kg^{-1}. In contrast, the acrylamide contents in chips from RNAi line #1 (98% reduction of *VInv* transcript), #2 (95% reduction), and #10 (63% reduction) were 630, 440, and 1570 µg kg^{-1}, respectively (Fig. 6).

DISCUSSION

The data presented here support three key findings. First, that suppression of *VInv* using RNAi can dramatically increase the resistance of modern chipping potato varieties to CIS (Table 1; Fig. 3, 4, and 5; Supplemental Fig. S2). Second, that the approach is generally applicable since

large improvements in cold-chipping performance were observed in each of the cultivars used for transformations (Supplemental Table S1; Fig. 2, 3, 4, and 5; Supplemental Fig. S2). Third, for Atlantic, Dakota Pearl, and MegaChip, suppressing *VInv* expression by 90% was sufficient to nearly eliminate CIS with regard to reducing sugar accumulation (Fig. 3). These dramatic improvements in tuber reducing sugars and chip color were not accompanied by obvious changes in tuber yield (Fig. 1) or tuber shape (Supplemental Fig. S1) of transplants grown in the greenhouse.

The data presented in Fig. 3 can be used to gain novel insights into the relationship between tuber sucrose content and VINV activity. MegaChip had a mean tuber sucrose content of 6 mg g^{-1} FW after 30 d at 4°C compared with Dakota Pearl where tuber sucrose was approximately 12 mg g^{-1} FW. Mean sucrose in RNAi lines of MegaChip increased relative to that in controls, with the amount of increase indicating the extent that wild-type VINV activity functioned to limit tuber sucrose accumulation (Fig. 3). For Dakota

Table 1. Potato chip color scores from selected RNA interference (RNAi) lines and nontransformed controls.†

Potato lines	VInv gene silencing‡	4°C for 30 d§	4°C for 120 d§
Atlantic (Atl)	0%	7.75 ±0.35	7.75 ±0.35
Atl RNAi Line 1	98%	3.00 ±0.00	3.00 ±0.00
Atl RNAi Line 2	95%	3.00 ±0.00	3.00 ±0.00
Atl RNAi Line 4	91%	3.00 ±0.00	3.00 ±0.00
Atl RNAi Line 8	87%	3.75 ±0.35	3.75 ±0.35
Atl RNAi Line 10	63%	5.00 ±0.71	5.00 ±0.71
Dakota Pearl (DP)	0%	6.75 ±0.35	7.50 ±0.71
DP RNAi Line 1	92%	3.25 ±0.35	3.25 ±0.35
DP RNAi Line 3	89%	3.25 ±0.35	3.50 ±0.71
DP RNAi Line 4	89%	3.25 ±0.35	3.25 ±0.35
DP RNAi Line 8	68%	4.25 ±0.35	4.50 ±0.71
DP RNAi Line 11	32%	5.50 ±0.71	5.75 ±0.35
MegaChip (Meg)	0%	7.25 ±0.35	7.75 ±0.35
Meg RNAi Line 1	94%	3.00 ±0.00	3.00 ±0.00
Meg RNAi Line 3	92%	3.00 ±0.00	3.00 ±0.00
Meg RNAi Line 4	90%	3.25 ±0.35	3.25 ±0.35
Meg RNAi Line 9	75%	3.75 ±0.35	4.00 ±0.00
Meg RNAi Line 12	66%	4.50 ±0.71	4.50 ±0.71
Snowden (Sno)	0%	7.75 ±0.35	7.75 ±0.35
Sno RNAi Line 1	85%	3.75 ±0.35	4.00 ±0.00
Sno RNAi Line 2	80%	4.00 ±0.00	4.00 ±0.00
Sno RNAi Line 3	80%	4.75 ±0.35	4.75 ±0.35
Sno RNAi Line 8	70%	4.75 ±0.35	4.75 ±0.35
Sno RNAi Line 11	65%	4.00 ±0.00	4.25 ±0.35

†Potato chipping scores were collected from a minimum of 10 chips from two tubers of each line. Chipping color scores were given based on Potato Chip Color Reference Standards (Snack Food Association, Arlington, VA) from 1 (light) to 10 (dark). Chips were given a half score if their color was indistinguishable between 2 of the 10 color indicators. Values represent the mean ±SD.

‡Vacuolar acid invertase gene, VInv. Numbers indicate the extent of VInv silencing for the respective RNAi line as a percentage of control.

§The quality of potato chips is considered as good (chip score ≤ 4.5), medium (5–6), or poor (≥7.5).

Pearl, however, RNAi lines had tuber sucrose contents that were not statistically different from controls (Fig. 3). It seems likely, therefore, that VINV activity is low in this cultivar relative to the rate of sucrose production and that decreasing the activity of VINV through RNAi suppression was not sufficient to substantially change tuber sucrose content. These data indicate that breeding for low tuber sucrose content is likely to select for genotypes that have high rates of VINV activity relative to rates of sucrose production.

VInv suppression using RNAi decreased tuber glucose and fructose in all lines examined in detail with the exception of Line 11 for Dakota Pearl. This exception is likely the result of the relatively large percentage (68%) of wild-type mRNA present in tubers of that line (Fig. 3). For the other lines, RNAi suppression was very effective in decreasing tuber reducing sugar contents. Cultivars with higher amounts of tuber reducing sugars in controls, however, required a greater degree of suppression to achieve comparable amounts of glucose or fructose (Fig. 4A and 4B). This is easily seen when comparing tubers of Atlantic, a cultivar that accumulated ~11 mg reducing sugar g⁻¹ FW, with Dakota Pearl, whose tubers had ~4 mg reducing

sugars g⁻¹ FW. Line 8 of Dakota Pearl, with 32% of control VInv transcript amount, had lower reducing sugars than Line 8 of Atlantic even though VInv expression was reduced to 13% of controls in Atlantic Line 8. The large decrease in reducing sugar content of Dakota Pearl RNAi lines illustrates the power of the VInv-silencing approach. Dakota Pearl was developed to be a cold-chipping variety and was able to maintain acceptable glucose (0.4 mg g⁻¹ FW) in breeding trials for 7 mo at 5.5°C (Thompson et al., 2005). Yet even in this cultivar, reducing sugar contents were dramatically reduced by VInv suppression in tubers stored at 4°C for 30 d (Fig. 3).

The chip color data in Fig. 5 and Supplemental Fig. S2 are consistent with the tuber sugar content data in Fig. 3. Suppression of VInv through RNAi produced lighter colored chips in all lines examined compared to cultivar controls. Chips were made from only two tubers per line at 30 d and at 120 d of cold storage because only a few tubers were available for each line. Invariably, RNAi lines had lower sugars and lighter chips than controls. The data as a whole, therefore, very strongly support the conclusion that suppression of VInv expression through RNAi markedly improved the resistance of potato tubers to CIS.

Acrylamide content in potato chips is expected to be high when reducing sugar contents are high and much lower when reducing sugar contents are low (Foot et al., 2007). This was observed for cultivar Atlantic and three RNAi lines derived from it (Fig. 6). Chip acrylamide contents decreased to ~500 μg kg⁻¹ in highly suppressed RNAi Lines 1 and 2 compared to almost 4000 μg kg⁻¹ in controls. This decrease, however, was not as large as the decrease in reducing sugar content. A plausible explanation for this is that at very low reducing sugar contents, factors in addition to glucose and fructose determine the final acrylamide content. One possible factor is asparagine content, and it has been demonstrated that decreasing tuber asparagine in Ranger Russet, a cultivar used for French fry production, decreased acrylamide content of fries (Rommens et al., 2008). The data in Fig. 6 indicate that significant reductions in chip acrylamide content are likely in lines with suppressed VInv. Differences in tuber growth conditions between greenhouses and commercial production fields, however, are likely to have a substantial impact on the absolute amount of chip acrylamide content since growth conditions and management practices influence both tuber reducing sugar contents and tuber asparagine.

"Resistance to CIS" is an important trait targeted by potato breeding programs in countries where a large portion of the potato crop is processed into French fries and potato chips. Cold-induced sweetening resistance has long been perceived as a complex trait that is associated with a large number of quantitative trait loci (QTLs) (Li et al., 2008; Menendez et al., 2002). Although progress toward developing cultivars that maintain relatively low amounts of reducing sugars during cold storage has been made over the past several decades (Hamernik et al., 2009; Love et al., 1998), the CIS

issue remains for all potato cultivars in the United States. One approach for increasing the resistance of cultivated potato to CIS is to introgress CIS resistant germplasm from wild species relatives into *S. tuberosum*. Several wild *Solanum* species show excellent resistance to CIS (Hamernik, 1998; McCann et al., 2010) and CIS resistant stocks have been developed from several *Solanum* species. For example, *Solanum berthaultii* Hawkes was used to produce the breeding clone S440, which has been used as a parental line by most potato breeding programs in the United States. Many new potato cultivars or breeding lines, including White Pearl (Groza et al., 2006), have been developed from S440. Progeny of S440 often have small tuber size and relatively low yields (Groza et al., 2006), however, and this decreases their appeal to commercial growers. Thus, a more precise, targeted breeding approach based on molecular markers associated with CIS resistance is likely to be required for more effective introgression of CIS resistance from wild species germplasm (Bhaskar et al., 2010).

Genetic engineering of specific traits is an ideal methodology for improving the potato crop, especially when changing the expression of a single gene results in a significant improvement in the desired trait. Potato is an autotetraploid and has a highly heterogeneous genome. This has made genetic analysis of many important agronomic traits a daunting task and impedes efforts to increase tuber quality through conventional breeding. Furthermore, the potato processing industry has optimized field management and processing facility practices for the growth requirements and physical characteristics of standard cultivars. To the extent possible it is preferable to replace these standard cultivars with others that have specific improvements in targeted quality traits but are similar in all other respects. Thus, improvement of specific traits in popular processing cultivars using genetic engineering could be a highly effective, rapid, and economical approach for potato improvement. The potential of this approach has been well demonstrated by genetic engineering for several important potato traits including resistance to potato late blight (Halterman et al., 2008; Kuhl et al., 2007) and high-amylose starch (Schwall et al., 2000). Genetically modified low amylose potatoes were approved for commercial release in Europe in 2010 (Ryffel, 2010). We demonstrate that silencing of *VInv* in four potato cultivars resulted in transgenic lines that minimize the CIS problem. Greenhouse evaluation of the RNAi lines from these four cultivars as well as field evaluation of RNAi lines of Katahdin (Bhaskar et al., 2010) have indicated so far that silencing of *VInv* does not have negative impacts on plant growth and yield. Large-scale field trials will be required to validate these initial observations, but as discussed in (Bhaskar et al., 2010), there is no evidence for an essential role of *VInv* in potato tuber development or growth. A function in response to a specific stress or growth condition, however, cannot be excluded.
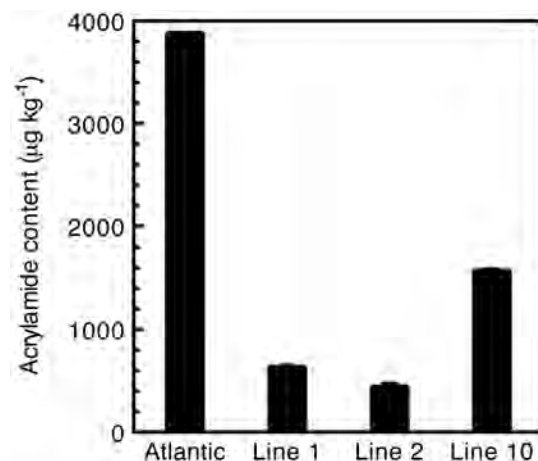

Figure 6. Acrylamide content in potato chips produced from three Atlantic RNA interference (RNAi) lines and Atlantic control. Acrylamide analysis was performed on chips processed from tubers stored at 4°C for 30 d. Vacuolar acid invertase gene (*VInv*) expression for lines 1, 2, and 10 was 2, 5, and 37% of Atlantic controls, respectively.

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