

# Suppression of the Vacuolar Invertase Gene Prevents Cold-Induced Sweetening in Potato<sup>1,2</sup>[W][OA]

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Potato (*Solanum tuberosum*) is the third most important food crop in the world. Potato tubers must be stored at cold temperatures to prevent sprouting, minimize disease losses, and supply consumers and the processing industry with high-quality tubers throughout the year. Unfortunately, cold storage triggers an accumulation of reducing sugars in tubers. High-temperature processing of these tubers results in dark-colored, bitter-tasting products. Such products also have elevated amounts of acrylamide, a neurotoxin and potential carcinogen. We demonstrate that silencing the potato vacuolar acid invertase gene *VInv* prevents reducing sugar accumulation in cold-stored tubers. Potato chips processed from *VInv* silencing lines showed a 15-fold acrylamide reduction and were light in color even when tubers were stored at 4°C. Comparable, low levels of *VInv* gene expression were observed in cold-stored tubers from wild potato germplasm stocks that are resistant to cold-induced sweetening. Thus, both processing quality and acrylamide problems in potato can be controlled effectively by suppression of the *VInv* gene through biotechnology or targeted breeding.

The accumulation of reducing sugars in cold-stored potato (*Solanum tuberosum*) tubers, a phenomenon referred to as cold-induced sweetening (CIS; Dale and Bradshaw, 2003), is a persistent, costly problem for the potato processing industry. During potato chip and French fry production, reducing sugars (primarily Glc and Fru) in the tubers react with free amino acids in a nonenzymatic Maillard reaction (Shallenberger et al., 1959) to produce brown- to black-pigmented products that are not acceptable to consumers. In the United States, approximately 15% of potatoes are rejected at processing plants annually due to high levels of reducing sugars (Sowokinos, 2004). More problematically, the Maillard reaction also generates

acrylamide, a neurotoxin and a potential carcinogen (Mottram et al., 2002; Stadler et al., 2002). In 2002, high levels of acrylamide were reported in carbohydrate-rich foods processed at high temperatures. Potato chips had especially high acrylamide contents (Rosén and Hellenäs, 2002; Tareke et al., 2002), raising a worldwide food safety concern that has resulted in lawsuits against major potato and fast food companies (<http://ag.ca.gov/newsalerts/release.php?id=1207>). Reducing sugars and Asn are the two major substrates for acrylamide formation in processed potato products (Goekmen and Palazoglu, 2008). Developing methods to reduce acrylamide in fried potato products has become an urgent requirement for the potato processing industry. One effective way to decrease acrylamide content is to decrease the amount of reducing sugars in raw tubers (Matsuura-Endo et al., 2006; Muttucumaru et al., 2008).

The enzymes responsible for CIS have been identified, and most have been characterized at the biochemical and molecular levels (Sowokinos, 2001; Kumar et al., 2004). During CIS, a change occurs in carbon fluxes linking starch with Suc that causes the net rate of Suc synthesis to increase (Isherwood, 1973). This is accomplished through the combined activity of UDP-Glc pyrophosphorylase, Suc phosphate synthase, and Suc phosphate phosphatase (Sowokinos, 2001). Some of the Suc produced is transported into the vacuole, where it is hydrolyzed to Glc and Fru (Isla et al., 1998). This step is predominantly controlled by the vacuolar acid invertase (VINV), and there is a

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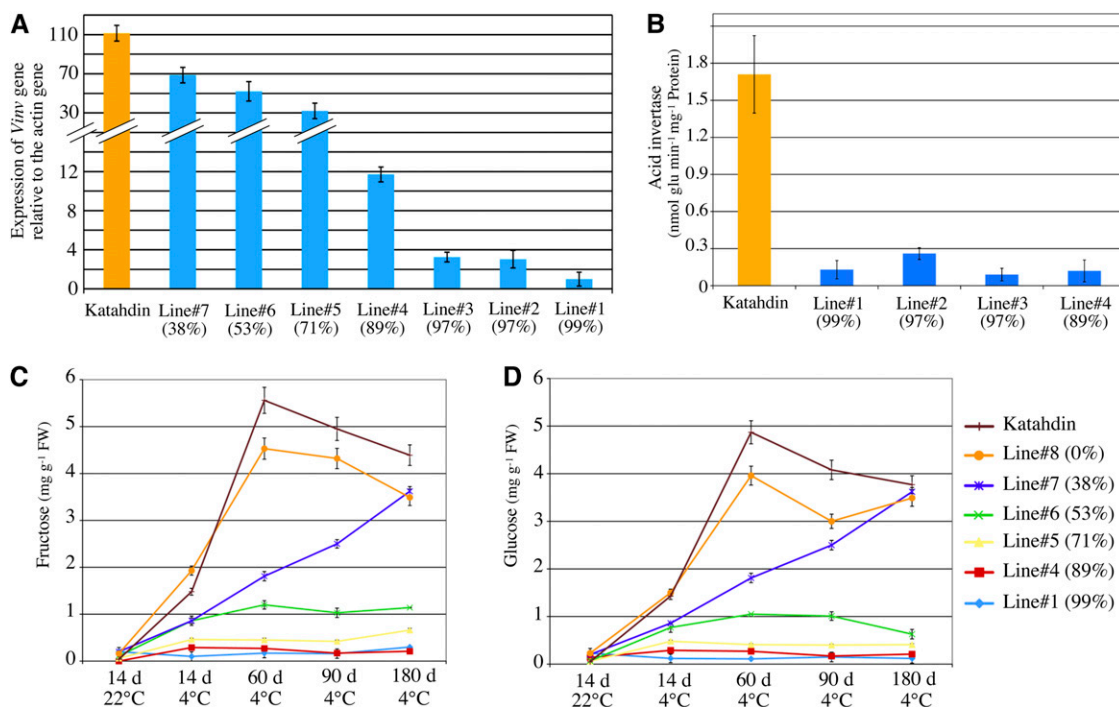
strong association between VINV activity and the accumulation of reducing sugars during cold storage (Matsuura-Endo et al., 2004).

Genetic mapping studies have shown that CIS is associated with a large number of quantitative trait loci (QTLs; Menéndez et al., 2002; Li et al., 2008). The genetic complexity of the CIS trait is consistent with the involvement of numerous enzymes in the metabolic pathways linking starch synthesis and breakdown to sugar formation and utilization in plants (Nguyen-Quoc and Foyer, 2001; Sowokinos, 2001; Geigenberger et al., 2004; Nägele et al., 2010). Genes associated with any of these enzymes and their regulation could influence steady-state amounts of Glc and Fru. In several cases, molecular markers associated with genes involved in starch or sugar metabolism have been linked to QTLs for sugar content or chip color (Menéndez et al., 2002).

The potato breeding community has strived for many decades to develop “cold chippers,” cultivars that do not accumulate reducing sugars during cold storage (Mackay et al., 1990; Pereira et al., 1994; Thill and Peloquin, 1995; Xiong et al., 2002). Although slow

progress has been made over the past several decades toward developing cultivars that maintain relatively low amounts of reducing sugars during cold storage (Love et al., 1998; Hamernik et al., 2009), none of the cultivars in production can be stored at cold temperatures without undergoing CIS. In contrast to *S. tuberosum*, several wild *Solanum* species, including some accessions of *Solanum raphanifolium* ( $2n = 2x = 24$ ), show excellent potential as cold chippers (Hamernik, 1998; McCann et al., 2010). Potato chips processed from tubers of *S. raphanifolium* accessions stored for 3 months at 2°C showed acceptable color (Hamernik, 1998). This extreme cold-chipping phenotype was heritable when cold-chipping *S. raphanifolium* accessions were crossed with diploid potato clones (Hamernik, 1998; Hamernik et al., 2009).

Several attempts have been made to control CIS by manipulating VINV activity (Greiner et al., 1999; Agarwal et al., 2003) or by reducing the transcription of the *VInv* gene (Zrenner et al., 1996; Zhang et al., 2008). Ectopic expression in potato of an invertase inhibitor from tobacco (*Nicotiana tabacum*) cell walls reduced cold-induced accumulation of reducing



**Figure 1.** Silencing of the *VInv* gene and its impact on acid invertase activity and reducing sugar accumulation. A, Real-time PCR-based transcription analysis of the *VInv* gene in potato tubers after 14 d of storage at 4°C. Data are presented for seven representative RNAi lines with different degrees of *VInv* gene silencing. Numbers in parentheses indicate the extent of *VInv* gene silencing for each RNAi line as a percentage of Katahdin controls. Bars represent mean expression of the *VInv* gene in three to five independent tuber samples relative to expression of the potato *Actin97* gene. B, Maximal extractable acid invertase activity in three RNAi lines in which the *VInv* gene is silenced 97% to 99% (indicated in parentheses). Potato tubers were harvested from field-grown plants and stored at 4°C for 14 d. Acid invertase activity is shown as nmol Glc min<sup>-1</sup> mg<sup>-1</sup> protein. Data are means  $\pm$  SE of three to eight independent tuber samples. C and D, Patterns of tuber Fru (C) and Glc (D) accumulation among representative RNAi lines and controls stored for 14 d at 22°C or for 14 to 180 d at 4°C. Fru and Glc content are expressed as mg g<sup>-1</sup> fresh tuber weight (FW). Each data point represents the mean  $\pm$  SD of two to three independent tuber samples.

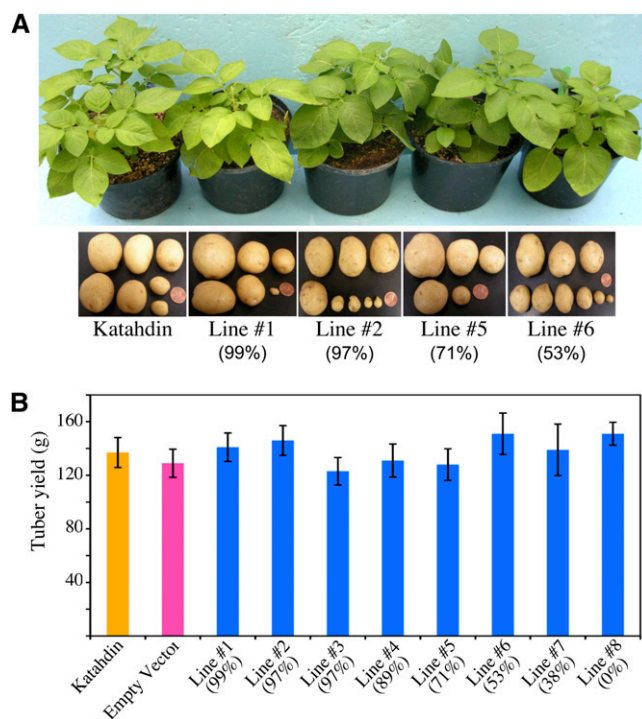
sugars by up to 75% (Greiner et al., 1999). Antisense inhibition of *VInv* in cv Desire decreased tuber invertase activity in cold-stored tubers by as much as 92%, but only a 43% reduction in tuber hexoses was observed (Zrenner et al., 1996). RNA interference (RNAi) suppression of *VInv* has been used to decrease *VInv* gene expression by up to 78% (Zhang et al., 2008). Nevertheless, CIS was not controlled effectively in these previous efforts, and one hypothesis to explain this is that VINV activity or *VInv* gene transcription was only partially suppressed in these experiments. Under this hypothesis, complete control of CIS would occur if sufficiently low rates of VINV enzyme activity were achieved. Alternatively, it can be hypothesized that cellular feedback loops employing alternative enzymes of hexose synthesis, such as Suc synthase, cytosolic neutral invertase, or apoplastic acid invertase, circumvent the biochemical block imposed by low rates of vacuolar invertase activity (Nguyen-Quoc and Foyer, 2001; Koch, 2004; Nägele et al., 2010). Under this hypothesis, accumulation of reducing sugars in cold-stored tubers would occur in the absence of *VInv* expression or enzyme activity. To directly test these competing hypotheses, we used RNAi to inhibit the accumulation of *VInv* transcript and to assess the relationship between *VInv* transcript amount and CIS in potato. We report that a nearly complete silencing of the *VInv* gene can effectively control CIS. We also observed low levels of *VInv* gene expression in CIS-resistant potato germplasm. Thus, we demonstrate that CIS and its associated acrylamide problem may be controlled by suppression of the *VInv* gene through biotechnology or targeted breeding.

## RESULTS

### Silencing of the *VInv* Gene Does Not Affect Potato Development

An RNAi-based approach was used to develop transgenic lines of the CIS-susceptible potato cv Katahdin with different levels of *VInv* gene transcription. A total of 150 RNAi lines were developed using three RNAi constructs that target different regions of the *VInv* gene. Gene silencing was achieved using all three constructs. Initial northern-blot hybridization analysis using RNA from leaf tissues revealed large variation in the amount of *VInv* gene transcript among different RNAi lines (Supplemental Fig. S1). A sensitive real-time quantitative PCR method was developed and used to quantify the *VInv* transcript in tubers. The amount of *VInv* gene transcript was reduced 90% to 99% in 23 lines, 10% to 90% in 69 lines, and 0% to 10% in 58 lines (Fig. 1A).

Multiple plants from 70 representative RNAi lines were grown in the greenhouse. Differences in plant and tuber growth were not observed between RNAi lines and controls (Fig. 2). Tuber fresh weights were not significantly different from controls across the full

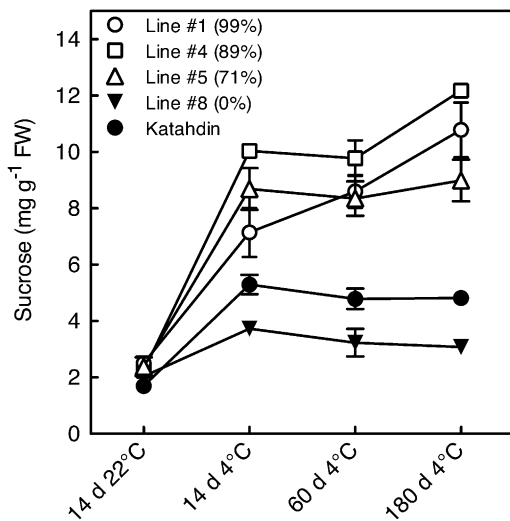


**Figure 2.** Phenotypes of representative RNAi lines and Katahdin controls. A, Plant and tuber phenotypes of four representative RNAi lines with different degrees of *VInv* gene silencing. Plant photographs were taken 50 d after tissue culture plantlets were transferred to greenhouses. The diameter of the coin is 18 mm. B, Tuber yield of representative RNAi lines and controls grown in greenhouses. The mean total yield (g) per plant ( $n = 2$ ) is shown. Error bars represent SD. Yield of the RNAi lines was compared with both Katahdin and a Katahdin line transformed with an empty vector. Fisher's LSD test as a comparison of mean total yields revealed no significant differences ( $\alpha = 0.05$ ).

range of RNAi suppression from 1% of wild-type transcript to 100% ( $P < 0.05$ ; Fig. 2). Three RNAi lines in which *VInv* transcription was highly suppressed were evaluated in a replicated field trial at the Hancock Agricultural Research Station, Wisconsin, in the 2009 growing season. These lines showed no growth abnormalities. The specific gravity of tubers, which is a measure of starch content, did not differ between the three RNAi lines and the controls (Supplemental Fig. S2). Tuber yield for one of the three lines (line 3), however, was slightly less than that of controls ( $P > 0.05$ ; Supplemental Fig. S2).

### *VInv* Gene-Silencing Lines Showed Dramatically Reduced Acid Invertase Activity and No Accumulation of Reducing Sugars in Cold-Stored Potato Tubers

Acid invertase activity was measured in a number of RNAi lines and in Katahdin controls. Enzyme activity in tubers from the RNAi lines was dramatically reduced after 14 d of storage at 4°C. Several RNAi lines with greater than 97% reduction of *VInv* transcript showed extremely low acid invertase activity (less



**Figure 3.** Suc accumulation in tubers from four independent RNAi lines and in the Katahdin control. Plants were grown in greenhouses, and tubers were stored for 14 d at 22°C or for 14 to 180 d at 4°C. Suppression of the *VInv* gene results in a dramatic accumulation of Suc in cold-stored tubers. Numbers in parentheses indicate the extent of *VInv* gene silencing for the respective RNAi line as a percentage of Katahdin controls. FW, Tuber fresh weight.

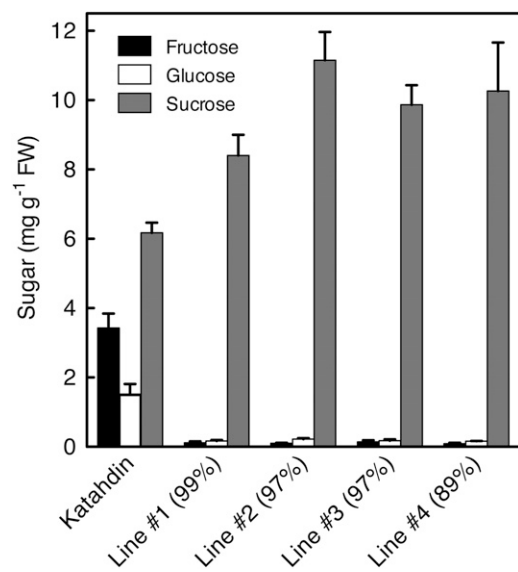
than 0.2 nmol Glc min<sup>-1</sup> mg<sup>-1</sup> protein; Fig. 1B). The amount of Suc, Glc, and Fru in tubers stored at room temperature (22°C) or at 4°C was quantified using HPLC. Katahdin and RNAi lines had low levels of reducing sugars when stored at room temperature (Fig. 1, C and D). The amounts of Fru and Glc in Katahdin tubers increased rapidly after 14 d of cold storage and peaked at 60 d (Fig. 1, C and D). Remarkably, tubers from RNAi lines with more than approximately 90% reduction of *VInv* transcript showed almost no increase in Fru and Glc content after storage at 4°C for 180 d (Fig. 1, C and D). Tubers from RNAi lines with intermediate levels of *VInv* silencing (approximately 38%–70% transcript reduction) showed relatively small increases in Fru and Glc (Fig. 1, C and D). Conversely, cold-stored tubers from the *VInv*-suppressed RNAi lines had higher amounts of Suc than controls, most likely because Suc was less effectively cleaved by invertase in the RNAi lines (Fig. 3). Very low amounts of Fru and Glc and significant accumulation of Suc were also observed in cold-stored tubers from field-grown RNAi lines (Fig. 4). Thus, the sugar profile data from multiple RNAi lines revealed a correlation between *VInv* gene expression and the accumulation of reducing sugars in cold-stored tubers.

#### Quality and Acrylamide Levels of Potato Chips Processed from the *VInv* Gene-Silencing Lines

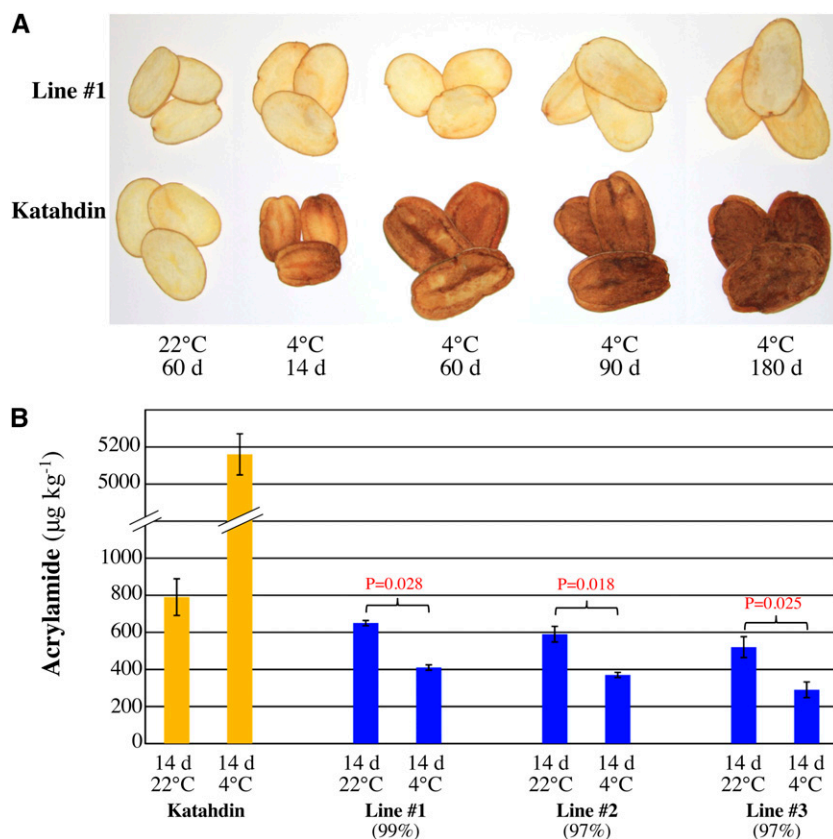
We performed potato chipping analysis of greenhouse-grown RNAi lines using tubers stored at room temperature or 4°C. Chips processed from room tem-

perature-stored tubers from RNAi lines were light colored and similar to those from room temperature-stored Katahdin tubers (Fig. 5A; Supplemental Table S1). Potato chips from Katahdin tubers after 14 d of cold storage were significantly darker in color than those from room temperature-stored tubers, and the chip color became darker with increasing time in storage (Fig. 5A). Strikingly, potato chips made from cold-stored tubers of several RNAi lines with greater than 97% reduction of *VInv* transcript were light colored and showed subtle or no darkening even when tubers were stored at 4°C for up to 180 d (Fig. 5A; Supplemental Table S1). Similarly, potato chips were light colored when processed from tubers of three RNAi lines grown in the field and stored at 4°C for 60 d (Supplemental Fig. S3). We quantified the chip color using a colorimeter, and all scores were greater than 50 for the three field-grown RNAi lines (Supplemental Fig. S4), which is considered to be a commercially acceptable color score. Potato chip color was highly correlated with the extent of *VInv* gene silencing (Supplemental Fig. S5; Supplemental Table S1).

We analyzed the acrylamide levels of potato chips from three RNAi lines with greater than 97% reduction of *VInv* transcription. Potato chips were processed from greenhouse-grown tubers stored at room temperature or 4°C for 14 d. After 14 d at 4°C, the average acrylamide content of chips from Katahdin increased from 790 to 5,160 μg kg<sup>-1</sup>. Remarkably, the acrylamide contents in the three RNAi lines did not increase but significantly decreased ( $P < 0.05$ ) after the tubers were stored at 4°C for 14 d (Fig. 5B). A similar reduction in acrylamide content after cold storage of tubers was



**Figure 4.** Fru, Glc, and Suc concentrations in tubers from field-grown plants. Tubers were stored for 14 d at 4°C before samples were taken for measurements. The sugar concentrations are expressed as mg g<sup>-1</sup> tuber fresh weight (FW).



**Figure 5.** Potato chip color and acrylamide content in chips derived from representative RNAi lines and Katahdin. A, Potato chips processed from RNAi line 1, which had a 99% reduction of the *VInv* transcript, and from Katahdin. Tubers were harvested from greenhouse-grown plants and stored at 22°C for 60 d or at 4°C for 14 to 180 d. B, Acrylamide content in potato chips produced from three greenhouse-grown RNAi lines and Katahdin controls. Potato chips were processed from tubers after 14 d of storage at either 22°C or 4°C. Acrylamide contents are shown as  $\mu\text{g kg}^{-1}$  and represent means  $\pm$  SD of two independent measurements. Chips processed from tubers stored at 4°C for all three RNAi lines had significantly ( $P < 0.05$ , two-tailed  $t$  test, unequal variance) lower acrylamide compared with the chips processed from tubers stored at 22°C.

observed in chips processed from field-grown tubers for two of the three RNAi lines (Supplemental Fig. S6).

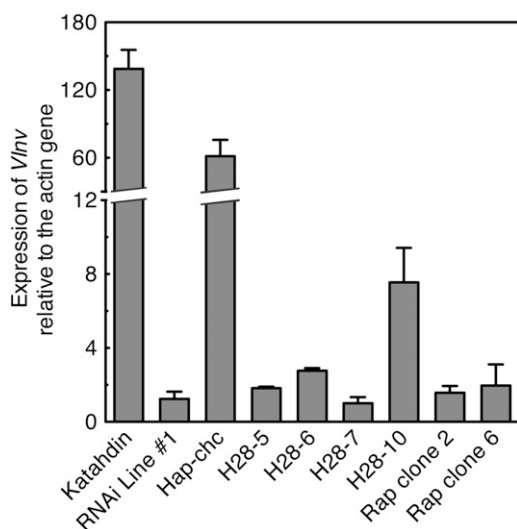
#### Cold-Chipping Germplasm with Low Levels of *VInv* Gene Expression

We examined *VInv* gene expression in cold-stored tubers from two genotypes of *S. raphanifolium* and in progeny from interspecies crosses between *S. raphanifolium* and a diploid potato (Hap-chc) derived from *S. tuberosum* and *Solanum chacoense*. Low amounts of *VInv* transcript were observed in both *S. raphanifolium* clones but not in the Hap-chc female parent (Fig. 6). Strikingly, quantitative PCR-based analysis revealed that *VInv* gene expression in some progeny of Hap-chc and *S. raphanifolium* was comparable to that in Katahdin RNAi lines with greater than 97% reduction of *VInv* transcript (Fig. 6).

#### DISCUSSION

CIS has long been perceived as a complex trait, and this idea has been reinforced by QTL mapping (Menéndez et al., 2002; Li et al., 2008). Individual QTLs, including those linked to vacuolar invertase, explained less than 16% of the phenotypic variance for tuber sugar content or chip color after storage at 4°C (Menéndez et al., 2002; Li et al., 2008). The relatively weak resistance to CIS of the parental lines used for

these genetic mapping studies may explain why vacuolar acid invertase did not contribute more to the observed traits. Thus, although the QTL data indicated that the *VInv* gene plays an important role in CIS, they did not clarify the potential impact of the *VInv* gene in CIS. Likewise, previous transformation using a tobacco cell wall invertase inhibitor gene or an antisense *VInv* gene produced potato lines with partial suppression of cold-induced hexose accumulation (Zrenner et al., 1996; Greiner et al., 1999). Zrenner et al. (1996) concluded that invertases do not control the total amount of soluble sugars in cold-stored potato tubers but are involved in the regulation of the ratio of hexose to Suc. The failure to effectively control CIS in these instances was likely the result of residual enzyme activity. Support for this supposition comes from data on enzymes of starch synthesis in potato. Reducing the activity of Suc synthase, UDP-Glc pyrophosphorylase, phosphoglucomutase, ADP-Glc pyrophosphorylase, or starch synthase by transformation with antisense constructs had little or no effect on starch synthesis unless activities dropped to approximately 20% or less of the wild type (Geigenberger et al., 2004). Our results from multiple RNAi lines revealed a clear correlation between *VInv* gene expression and the accumulation of reducing sugars in cold-stored tubers. A near 100% silencing of the *VInv* gene resulted in the complete suppression of reducing sugar accumulation (Fig. 1). The influence of genotype on the relationship between invertase gene expression and the accumulation of



**Figure 6.** Real-time PCR-based quantification of *VInv* gene transcription in potato tubers after 14 d of storage at 4°C. Hap-chc is a diploid clone derived from a cross between a diploid cultivated potato clone and the wild potato relative *S. chacoense*. It has good agronomic traits but accumulates high levels of reducing sugars during cold storage. Clones H28-5, H28-6, H28-7, and H28-10 are full-sibs derived by crossing the Hap-chc clone with bulked pollen from *S. raphanifolium* (Rap) accession 310998. Rap clone 2 and Rap clone 6 and the four H28 clones produced chips with acceptable color after tubers were stored for 3 months at 4°C.

reducing sugars is an area of investigation that warrants additional research.

Reducing sugars are required for acrylamide formation, and it was expected that the acrylamide content of potato chips would decrease if reducing sugar content in raw tubers decreased. The data in Figures 5 and 6 confirmed this. Surprisingly, acrylamide content in chips from RNAi lines with greater than 97% *VInv* suppression was often less when tubers were stored for 14 d at 4°C than at 22°C (Fig. 5; Supplemental Fig. S6). A plausible explanation for this is that low-temperature storage results in a decrease in the amount of Glc and Fru present in the cytoplasm and cell wall space. RNAi suppression of *VInv* effectively prevents Glc and Fru accumulation in the vacuole, resulting in a net decrease in tuber reducing sugar content and acrylamide production after 14 d at 4°C. This suggestion is consistent with chip color (Supplemental Figs. S3 and S5) and reducing sugar (Fig. 1, C and D) data.

Silencing the acid invertase gene in other species has resulted in large, undesirable changes in plant growth. In tomato (*Solanum lycopersicum*), antisense silencing of the acid invertase gene resulted in a 30% reduction in fruit size (Klann et al., 1996). In carrot (*Daucus carota*) and muskmelon (*Cucumis melo*), silencing of the acid invertase gene significantly altered plant and root/fruit development (Tang et al., 1999; Yu et al., 2008). In contrast, constitutive silencing of the *VInv* gene in several lines of potato, or inhibiting invertase activity by ectopic expression of an invertase inhibitor

(Greiner et al., 1999), did not alter plant development and did not reduce yields. These stark differences are likely to reflect differences in how Suc is broken down and the roles that Glc and Fru play in organ development. Potato tubers import Suc symplastically, and virtually all Suc is metabolized by the cytosolic enzyme Suc synthase to produce UDP-Glc and Fru (ap Rees and Morrell, 1990). Fru is phosphorylated by fructokinase and as hexose-phosphate enters into the starch synthesis and respiratory pathways. Vacuolar invertase does not have a documented role in developing tubers and in tubers stored at temperatures greater than approximately 10°C. Furthermore, Glc and Fru do not accumulate to high concentrations in the cytosol or vacuole of developing potato tubers. Instead, organic acids and potassium ions rather than sugars are the dominant contributors to the osmotic potential of potato tubers (Bethke et al., 2009). This situation is distinctly different from that in developing tomato fruit, where invertase activity and hexose accumulation function in the hydrolysis of Suc and turgor-driven fruit growth, respectively (Klann et al., 1996). In carrot taproots and in developing muskmelon fruit, vacuolar acid invertase activity is thought to have a similar role in promoting growth by producing hexoses that decrease osmotic potentials and promote water uptake (Tang et al., 1999; Yu et al., 2008). The severe consequences for growth and development when vacuolar invertase activity was reduced in these species are likely to have occurred because invertase activity contributes to organ growth and development over an extended period of time.

The data presented here suggest that the potato *VInv* gene may be adapted for a specific role in response to cold stress or that enzyme activities that compensate for VINV activity in leaves or other organs are lacking in tubers. Tubers from the highly suppressed RNAi lines do not appear different from Katahdin controls, which suggests that constitutive silencing of the *VInv* gene does not have a major impact on the expression of other essential potato genes at the relatively warm temperatures experienced during plant growth. This suggestion is consistent with previous data showing that overexpression of a yeast invertase gene in potato vacuoles increased invertase activity by up to 280-fold and decreased tuber Suc contents by more than 75% yet had a minor influence on plant growth and tuber metabolites (Junker et al., 2006). We performed whole transcriptome sequencing of total RNA (RNA-Seq) isolated from room temperature-stored tubers of Katahdin and RNAi line 1 (99% reduction of *VInv* transcript) to gain information on potential side effects associated with *VInv* silencing. More than 97% of the 17,936 potato genes with a minimum expression level of 1.6 FPKM (fragments per kilobase of exon model per million fragments mapped; median expression value across the experiment) in both Katahdin and RNAi line 1 had similar levels of expression in the two lines (difference of less than 2-fold), with only four genes differing in expression by 5- to 6-fold. We

identified 55 genes associated with different invertase families, invertase inhibitors, or starch-sugar metabolic pathways, with 41 genes showing a minimum expression level of 1.6 FPKM in both samples (Supplemental Table S2). Of these, only two genes, one for cytoplasmic phosphoglucosyltransferase and one for starch branching enzyme II, had a difference in expression in the two lines of more than 2-fold, 2.22- and 2.26-fold, respectively. In previous research where other genes in the potato starch and sugar metabolic pathways have been targeted for silencing to control CIS (Lorberth et al., 1998; Chen et al., 2008), major effects on starch quantity or quality were observed, thus limiting their practical application.

The narrow genetic base of cultivated potato is well known, as are the limitations this lack of diversity places on cultivar improvement through conventional breeding (Plaisted and Hoopes, 1989). One way to improve existing traits or incorporate novel traits is to introduce germplasm from wild *Solanum* species into cultivated potato (Plaisted and Hoopes, 1989). Tubers of some wild potato relatives, including lines of *S. raphanifolium*, *S. chacoense*, and *Solanum okadae*, have been shown to produce light-colored fried chips after low-temperature storage (Hamernik et al., 2009; McCann et al., 2010). The differences between members of these species and cultivated *S. tuberosum* that resulted in light-colored chips were not known. The data in Figure 6 suggest that low *VInv* expression is likely to contribute to this difference in two lines of *S. raphanifolium*. Significantly, this trait could be transferred to progeny by traditional breeding (Fig. 6). These data demonstrate that exploiting natural variation in *VInv* expression observed in wild species is a viable alternative to biotechnology for generating cultivars with low invertase activity and resistance to CIS.

## CONCLUSION

Both RNAi-based silencing and transcription analysis of cold-chipping germplasm showed that the *VInv* gene plays the key role in CIS. Suppression of this gene can be used to effectively solve both processing quality and acrylamide problems in potato. Although silencing of Asn synthetase genes provides an alternative approach to reduce the acrylamide content in processed potato products (Rommens et al., 2008), this approach does not address the CIS problem. The existence of heritable, natural variation in *VInv* gene expression provides an avenue for future breeding efforts to produce CIS-resistant cultivars through the development and application of DNA/RNA markers associated with *VInv* expression.

## MATERIALS AND METHODS

### Development of RNAi Constructs for *VInv* Gene Silencing

Total RNA was extracted from leaf tissue of potato (*Solanum tuberosum* 'Katahdin') using the Trizol reagent (Invitrogen) and was treated with TURBO

DNA-free (Ambion/Applied Biosystems) to remove DNA. First-strand cDNA was synthesized using 2  $\mu$ g of total RNA, oligo(dT) primer, and SuperScript reverse transcriptase (Invitrogen). A 2,351-bp full-length *VInv* cDNA was amplified by PCR using primers 5'-GCACGAGTATGGCCACCCAGTA-3' (forward) and 5'-GGCGAAATCACGTGCTCTAA-3' (reverse), corresponding to the previously described full-length vacuolar invertase mRNA sequence (TC163068; *S. tuberosum* Gene Index database [http://compbio.dfci.harvard.edu/cgi-bin/tgi/gireport.pl?gudb=potato]). Three different sequences, 506, 495, and 508 bp in length, were selected for RNAi construct design. All cDNA fragments were amplified from Katahdin using Platinum Taq DNA polymerase (Invitrogen) with 35 cycles of heat denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min after an initial heat denaturation at 95°C for 40 s. The 506-bp cDNA fragment (1,845–2,351 bp; construct InvBP3) was amplified using primers 5'-CACCACAGGGGCT-AGCGTGACTGC-3' (forward) and 5'-CGCGAAATCACGTGCTCTAAAG-3' (reverse). The 495-bp cDNA fragment (673–1,168 bp; construct InvBP2) was amplified using primers 5'-CACCCTGGGTCAAGTACAAAGGC-3' (forward) and 5'-CTCTTCGTTGTTTCTTCGGGTCA-3' (reverse). The 508-bp cDNA fragment (1,310–1,818 bp; construct InvBP1) was amplified using primers 5'-CACCGAAAGCTTAAGAGGCGGTGATCC-3' (forward) and 5'-CTGCTCCATCACTGCTTTGTTT-3' (reverse). The amplified PCR products were purified using the QIAquick PCR Purification Kit (Qiagen), gel verified, and cloned into pENTR/D directional TOPO cloning vector (Invitrogen). The *VInv* cDNA fragments were then transferred into the pHellsGate8 vector using the LR Clonase recombination method (Hellmich et al., 2002). Sequences in the recombinant pHellsGate8-*VInv* plasmids were confirmed by restriction digestion (*Xba*I and *Xba*I) and sequencing of inserts to ensure that the *VInv* sequences recombined in sense and antisense orientations.

### Development and Characterization of Transgenic Plants

The *Agrobacterium tumefaciens* strain GV3101 (pMP90) was transformed with pHellsGate8-*VInv* plasmids by the freeze-and-thaw method (Sambrook and Russell, 2001), and positive clones were selected on yeast extract peptone medium agar plates containing gentamycin (30 mg mL<sup>-1</sup>) and spectinomycin (50 mg mL<sup>-1</sup>). Single colonies were selected and grown in liquid yeast extract peptone medium at 28°C to an optical density of 0.6 to 0.8 before infecting potatoes. Stem internode explants from 5- to 6-week-old in vitro Katahdin plants were used in potato transformation (Bhaskar et al., 2008). The transgenic Katahdin plants obtained from the three RNAi constructs were first screened for the presence of the kanamycin resistance selection marker. PCR was performed on genomic DNA isolated from the transgenic plants using the kanamycin marker-specific primers 5'-CCAACGCTATGCTCTGATAG-3' (forward) and 5'-TTTGTCAGACCGACCTGTC-3' (reverse). PCR was performed for 40 cycles of heat denaturation at 95°C for 20 s, annealing at 53°C for 30 s, and extension at 72°C for 1 min after an initial heat denaturation at 95°C for 1 min. The PCR mix (25  $\mu$ L) consisted of 1 $\times$  PCR buffer (200 mM Tris-HCl, pH 8.4, and 500 mM KCl), 0.1 mM deoxyribonucleotide triphosphates, 0.2  $\mu$ M primers, 1.5 mM MgCl<sub>2</sub>, 1 unit of Platinum Taq polymerase (Invitrogen), and 1.5 ng of genomic DNA.

### Confirmation of *VInv* Gene Silencing in Transgenic Plants

#### Northern-Blot Hybridization Analysis

Approximately 15  $\mu$ g of total RNA isolated from leaf tissue was resolved on 1% denaturing agarose gels run at 200 V for 45 min using 1 $\times$  MSE buffer (0.2 M MOPS, pH 8, 50 mM sodium acetate, and 5 mM EDTA, pH 8) and then transferred to a Hybond+ nylon membrane (Amersham Biosciences). A 506-bp *VInv* cDNA sequence amplified using primers 5'-ACAGGGGCTA-GCGTGACTGC-3' (forward) and 5'-CGCGAAATCACGTGCTCTAAAG-3' (reverse) was used as a probe. The probe was radioactively labeled with 3,000 Ci mmol<sup>-1</sup> [<sup>32</sup>P]dATP (Amersham Biosciences) using the Strip-EZ DNA Kit (Ambion/Applied Biosystems) following the manufacturer's instructions. The membrane was prewashed in 65°C Church buffer (7% SDS, 0.5 M Na<sub>2</sub>HPO<sub>4</sub>, and 1 mM EDTA, pH 7.2) for a minimum of 1 h. The radioactive probes were denatured and then hybridized to the membrane overnight at 65°C. Membranes were washed twice in 2 $\times$  SSC and 0.1% SDS for 15 min and twice in 0.2 $\times$  SSC and 0.1% SDS for 15 min. Signals were detected using a Cyclone phosphor imager (Packard) and/or exposed to x-ray film (Kodak Biomax film; Sigma-Aldrich).

## Quantitative Real-Time PCR

Potato leaf RNA and tuber RNA were isolated using the RNeasy Plant Mini Kit (Qiagen) and Plant RNA Isolation Kit (Agilent), respectively, following the manufacturers' instructions, and were treated with TURBO DNA-free. SuperScript III reverse transcriptase (Invitrogen) was used to generate the first-strand cDNA. Controls without reverse transcriptase were tested for all cDNA reactions. cDNA was diluted 1:5 in distilled water and used for quantitative real-time PCR along with the DYNAMO SYBR Green master mix (Finnzymes, New England Biolabs) and 10 nM primers. Quantitative real-time PCR was performed in triplicate for each sample on MJ Research Opticon 2 (Bio-Rad Laboratories) using primers 5'-AAACGGGTGGACACATCAT-3' (forward) and 5'-AACCAATCCACAATCCAA-3' (reverse) for *Vlnv* transcript quantification (amplicon size, 159 bp) or 5'-GATGGCAGACGGAGAGGA-3' (forward) and 5'-GAGGACAGGATGCTCCTC-3' (reverse) for reference gene (*S. tuberosum Actin97*; TC164213) transcript quantification (amplicon size, 319 bp). The following protocol was performed for all quantitative real-time PCR experiments: 15 min at 95°C, 40 cycles of 20 s at 94°C, 20 s at the corresponding annealing temperature, and 30 s at 72°C, followed by a plate read. Then a melting curve was performed: 50°C to 95°C with 0.2°C steps, hold for 2 s, followed by a final extension step of 10 min at 72°C. Annealing temperatures were 55°C for *Vlnv* and 55°C for *Actin97*. PCR efficiencies for both genes were greater than 95%. Relative expression was calculated using Gene Expression Macro software version 1.1 (Bio-Rad Laboratories). The silencing percentage for the *Vlnv* gene was calculated by the comparative cycle threshold method (Livak and Schmittgen, 2001), where delta cycle threshold of *Katahdin* (control) was defined as 100% transcript presence.

## Greenhouse Trials, Tuber Sample Preparation, and Chipping Analysis

Two replications of 70 independent RNAi lines along with controls were moved from tissue culture to greenhouses. The plants were grown in two separate greenhouses in the Biotron greenhouse facility at the University of Wisconsin-Madison. Growth conditions were 70% humidity, 16-h day/8-h night, with a 19°C/15°C day/night temperature differential, and light during the day of 500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  photosynthetically active radiation. Plants were fertilized with 5 g of Nutricote (13-13-13, Type 100; Sun Gro Horticulture) at 20 and 60 d after planting. Tubers were harvested 110 to 130 d after transplanting when leaves had senesced naturally. Fresh tuber weights of all tubers larger than 5 g in a single pot were measured at harvest. Tubers from the same line were combined into a single sample and stored in the dark at room temperature for 1 week, then three to six tubers from each line were randomly selected and stored in a 4°C chamber with 60% to 70% humidity for up to 180 d. The remaining tubers were stored in the dark at room temperature (22°C, 50%–70% humidity). Samples for potato chips were taken by cutting slices (from apical to basal end of the tuber, 1.5 mm thick) from tubers. The remaining tuber samples were frozen in liquid nitrogen and used for quantification of invertase enzyme activities, sugar profile, and transcript analysis. Two independent replications (three plants for each line in one replication) of *Solanum raphanifolium* (Rap) clones 2 and 6 (accession 310998), H28-5, H28-6, H28-7, and H28-10 clones, and Hap-chc clone were grown in greenhouses. All plants were grown for 130 to 150 d, and plants were fertilized with 5 g of Nutricot (13-13-13, Type 100) at 30 and 60 d after planting.

Tuber slices were fried for 2 min at 191°C or until the cessation of bubbles. Chip color was visually determined on a 10-chip sample from each line with the use of the Color Standards Reference Chart for Potato Chips (Snack Food Association). The color intensity of potato chips was also validated using a D25LT colorimeter (HunterLab). Each sample consisted of 30 to 40 crushed potato chips. A Hunter value of 50 or higher is a widely accepted chip color score in the potato processing industry.

## Invertase, Sugar, and Acrylamide Analyses

Frozen potato samples of 0.50 to 0.75 g were homogenized on ice with a chilled mortar and pestle in 2 volumes of buffer containing 50 mM HEPES-KOH, pH 7.5, 5 mM  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 1 mM EDTA, 1 mM EGTA, 0.1% (w/v) Triton X-100, 10% (w/v) glycerol, 5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. Crude extracts were centrifuged 5 min at 15,000g to pellet cell wall debris and precipitated proteins. Samples of 1 mL were desalted using PD MidiTrap G-25 columns following the manufacturer's directions for

gravity flow (GE Healthcare). Eighty microliters of desalted extract was placed in 0.2-mL tubes and shaken rapidly for 30 min to minimize the activity of invertase inhibitor proteins. Enzyme assays contained 20  $\mu\text{L}$  of protein extract and 60  $\mu\text{L}$  of reaction buffer containing 133 mM Suc and 26.7 mM Na-acetate, pH 4.7, and were incubated at 30°C. After 60 min, 8  $\mu\text{L}$  of Naphosphate (1 M, pH 7.4) was added to stop the reaction, and tubes were heated at 97°C for 3 min to inactivate the enzyme. Control samples contained all additions but were neutralized and heated immediately. The Glc formed was quantified using the assay described by Bethke and Busse (2008). Protein content of desalted extracts was determined using the Bio-Rad protein quantification reagent (Bio-Rad Laboratories) following the manufacturer's directions for the microassay. Invertase activity was calculated as nmol Glc formed  $\text{min}^{-1} \text{mg}^{-1}$  protein. HPLC analysis of potato tuber sugars was according to published protocols (Bethke et al., 2009) using a column temperature of 20°C.

For acrylamide analysis, potato chips were processed from tubers stored at 4°C for 14 d without reconditioning. Potato tubers were cut axially to obtain slices and fried in vegetable oil for 2 min at 187°C or until the cessation of bubbles. Fried chips were allowed to cool at room temperature (22°C) for 5 to 8 min and were ground thoroughly with a mortar and pestle, and the powder was used for acrylamide analysis. Acrylamide analysis was performed in the Department of Bacteriology, University of Wisconsin-Madison, by a modified Environmental Protection Agency method described previously (Park et al., 2005). A *t* test (two-sample unequal variance, two-tailed distribution) was used to study whether the means of two groups were statistically different from each other.

## Field Trial of Selective RNAi Lines

A field trial of select RNAi lines, an empty vector control line, and *Katahdin* was conducted at the Hancock Agricultural Research Station at Hancock, Wisconsin, during the 2009 growing season. All the necessary regulatory approvals to perform the field evaluations of transgenic lines were obtained from the U.S. Department of Agriculture Biotechnology Regulatory Services prior to planting. Tissue culture plantlets that were 3 to 4 weeks old and approximately 14 to 18 cm in height were transplanted in the field in the first week of June. Rows were spaced 91 cm apart, and plants were spaced 61 cm apart within each row. A complete randomized design was used for three replicates of each line with nine plants per replicate. Standard cultivation and management practices were followed throughout the growing season. A 4.5- to 6.8-kg sample of tubers from each replicate was used for specific gravity measurements. Selected sample units were weighed in air and then reweighed while suspended in water. Specific gravity was calculated using the following formula: specific gravity = weight in air / (weight in air – weight in water). The total weight of tubers larger than 30 g from each replicate was used to determine yield.

## RNA-Seq and Data Analysis

Potato tuber samples of greenhouse-grown *Katahdin* (control) and RNAi line 1 were used for RNA-Seq analysis (Mortazavi et al., 2008). All tubers used in the experiment were stored at room temperature (22°C) for 15 d after harvest. Whole tubers were flash frozen in liquid nitrogen and ground to a fine powder without thawing. Total RNA was isolated using the Plant RNA Isolation Mini Kit (Agilent). Independent RNA extractions were performed for each of six different tubers from each line. The quality of RNA was verified by running the RNA extracts on 1.2% formaldehyde denaturing gels (formaldehyde loading dye; Ambion/Applied Biosystems). RNA quantity was determined using a Nanodrop (ThermoFisher Scientific). A final RNA sample of 300  $\mu\text{L}$  was obtained by pooling 50  $\mu\text{L}$  of RNA from each of six different tuber replicates. The RNA was converted to cDNA using the mRNA-Seq Kit from Illumina and sequenced on an Illumina GA2 sequencer, generating 41-bp fragments. Purity-filtered reads were aligned to the Potato Genome Sequencing Consortium version 3 draft assembly of *Solanum phureja* DM1-3 516R44 (<http://www.potatogenome.net/index.php/>; Visser et al., 2009) using the TopHat short-read alignment program (Trapnell et al., 2009). Expression levels were determined from 33,655 gene models constructed from the FGENESH (Salamov and Solovyev, 2000) ab initio gene finder (dicot matrix) using Cufflinks (0.8.2; <http://cufflinks.cbc.umd.edu/>), which generates expression levels in the form of reads/FPKM. The median FPKM values were 1.7 (vector control) and 1.6 (RNAi line 1). FGENESH models were assigned functional annotation by using the first informative best BLASTX hit of the



predicted model-translated nucleotide sequence against the UniRef100 database (release 15.14).

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Northern-blot hybridization of 29 independent RNAi lines using the *Vlnv* gene as a probe (C, nontransformed Katahdin).

**Supplemental Figure S2.** Specific gravity (A) and yield (B) of three field-grown RNAi lines, Katahdin, and a Katahdin line transformed with empty vector in a field trial with three replications of nine plants each.

**Supplemental Figure S3.** Illustration showing the color of potato chips made from Katahdin and RNAi lines that showed greater than 97% reduction of *Vlnv* transcript.

**Supplemental Figure S4.** Chip color score, measured as reflectance using a HunterLab D25LT instrument.

**Supplemental Figure S5.** Potato chip color derived from RNAi lines with different degrees of *Vlnv* gene silencing.

**Supplemental Figure S6.** Acrylamide content in potato chips derived from three field-grown RNAi lines and the Katahdin control.

**Supplemental Table S1.** Potato chipping scores and performance of tubers from greenhouse-grown RNAi lines.

**Supplemental Table S2.** Expression levels of genes involved in starch and sugar metabolism.

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