DOI:10.1021/jf1032262

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Tuber-Specific Silencing of the Acid Invertase Gene Substantially Lowers the Acrylamide-Forming Potential of Potato

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Some popular processed foods including French fries contain small amounts of toxic acrylamide. Efforts to lower the accumulation of this reactive compound by modifying the production process have a negative effect on sensory characteristics and are not broadly applicable. This study optimized a method developed more than a decade ago to lower the accumulation of the acrylamide precursors glucose and fructose in cold-stored tubers. In contrast to the original application, which lowered hexose content by one-third through constitutive expression of an antisense copy of the cold-inducible acid invertase (*Inv*) gene, the current approach was based on tuber-specific expression of an *Inv*-derived inverted repeat. Stored tubers of transgenic plants contained as little as 2% of the reducing sugars that accumulated in controls. This decline in glucose and fructose formation is counterbalanced by increased sucrose and starch levels. However, it did not trigger any phenotypic changes and also did not affect the formation of free asparagine, ascorbic acid, phenylalanine, and chlorogenic acid. Importantly, French fries from the low-invertase tubers contained up to 8-fold reduced amounts of acrylamide. Given the important role of processed potato products in the modern Western diet, a replacement of current varieties with the low-hexose potatoes would reduce the average daily intake of acrylamide by one-fourth.

KEYWORDS: Acrylamide; potato; genetic engineering; cold-induced sweetening

INTRODUCTION

Cold-induced sweetening (CIS), the process in which starch is gradually converted into reducing sugars, represents one of the most important issues for the French fry industry (*1*). Although freshly harvested potatoes contain hardly any glucose or fructose, the levels of these compounds rapidly increase upon cold storage. Despite efforts to limit CIS by optimizing storage conditions, tubers may eventually contain more than 40 mg of reducing sugars/g of dry weight (DW) (*2*, *3*). When heated at temperatures above 120 °C in low-moisture environments, the accumulated glucose and fructose react with amino acids to produce Maillard products that darken French fries. One of these products is acrylamide ($CH_2CHCONH_2$), CAS Registry No. 79-06-1), a human neurotoxin, potent rodent carcinogen, and possible human carcinogen (*4*, *5*). Dietary intake levels of acrylamide have been rising since the early 1900s and are currently estimated at 1 μg/kg of body weight/day (*6*).

The acrylamide content in French fries could be lowered by replacing currently available varieties with potatoes that are naturally poor in acrylamide precursors. Although there are no such varieties that also display all other traits demanded by the industry, it is possible to incorporate CIS resistance into existing varieties through genetic engineering (*7*). One strategy that accomplishes this goal was first conceived with the elucidation of the molecular basis of a wild tomato (*Lycopersicon chmielewskii*) mutant producing high-sucrose fruits. The mutant was different from domesticated tomato (*Solanum esculentum*) in that its fruits lacked vacuolar acid invertase (Inv), an enzyme catalyzing the hydrolysis of sucrose into glucose and fructose (*8*). A similar mutation in carrot (*Daucus carota*) lowered the formation of hexose in roots (*9*).

As soon as the *Inv* gene was cloned, it was fused to the constitutive 35S promoter of cauliflower mosaic virus in the antisense orientation and inserted back into tomato (*10*). In contrast to the original wild tomato mutant, the resulting transgenic lines displayed low Inv activity levels in all tissues. Fruits accumulated less than half the reducing sugars and up to 10-fold more sucrose than controls. Application of the same approach to potato produced 95 lines, one of which (designated I-43) was also low in Inv activity (*11*). Tubers of line I-43 were found to accumulate only two-thirds of the amount of reducing sugars that is present in untransformed plants. Because the reduction in glucose and fructose was balanced by an increase in sucrose levels, the hexose-to-sucrose ratio of line I-43 was less than half that of controls.

In an effort to reduce the acrylamide content of French fries, we optimized the *Inv* gene silencing-based CIS resistance trait in potato. We demonstrate the efficacy of a silencing cassette comprising a tuber-specific promoter driving expression of an *Inv*-derived inverted repeat in lowering the accumulation of glucose and fructose during cold storage. Incorporation of the

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 Published on Web 11/04/2010

trait into potato is not associated with apparent deleterious effects on plant vigor and yield. Importantly, French fries from the low-Inv potatoes maintain a lighter color than control fries and accumulate up to 8-fold less acrylamide.

MATERIALS AND METHODS

Plasmid Construction. Sense and antisense fragments of the cDNA of the *Inv* gene (Genbank Accession DQ478950) were amplified from a tuber poly(A)$^+$ mRNA-derived library of the potato variety Ranger Russet using the two primer pairs (1) 5′-CGG ATC CAC ACA TTC CTC CCG GAT CAA C and 5′-CGG GCC CCT CTA AAG TCC TTG ACA CCA ATG C and (2) 5′-CGG GCC CAG CGG ACC CAG TCC AGA CAC C and 5′-CAC TAG TCA TTA CAC ATT CCT CCC GGA TC. The amplified fragments corresponded to positions +61 to +727 (sense) and +560 to +57 (antisense), respectively, of the *Inv* gene. They were positioned as inverted repeat between regulatory elements from the potato variety Ranger Russet: the 2.2 kb tuber-specific promoter of the ADP glucose pyrophosphorylase (*Agp*) gene (Accession HM363752) and the 0.3 kb terminator of the ubiquitin-3 gene (Accession GP755544). Insertion of the resulting silencing cassette into a pSIM401-derived T-DNA region also carrying an expression cassette for the selectable marker neomycin phosphotransferase (*npt*) gene (*12*) yielded vector pSIM1632.

Transformation, Plant Growth, and Molecular Analysis. Stock plants of the variety Ranger Russet were maintained in magenta boxes containing 40 mLof half-strength M516 medium (PhytoTechnology, Shawnee Mission, KS) with 3% sucrose and 2 g/L Gelrite. Transformations were carried out as described previously (*13*). Kanamycin-resistant plants were confirmed to contain the T-DNA using a robust and reliable PCR method (*14*) with primers 5′-CAA GTG CAA TCA CAC TCT ACC ACA C and 5′-CTT GTA CAT AAT CAT CAG TGT CAC, which amplify a fragment comprising the junction between the Agp promoter and the *Inv*-derived inverted repeat. Transformants were grown for 3 months in Sunshine mix-1 (www.sungro.com) in 2 gal pots in a greenhouse that was controlled for temperature (18 °C minimum/27 °C maximum) and light (16 h photoperiod with an intensity of ~1500 μmol/m^2/s). Harvested tubers were stored for 2 months in a humidity-controlled chamber set at 5.5 °C. RNA was extracted with the Plant RNA Purification Reagent kit (Invitrogen, San Diego, CA), loaded onto a 1% agarose gel (20 μg/lane), and transferred to a Hybond N$^+$ nylon filter (GE Healthcare, Piscataway, NJ). The filter was hybridized with a 679 bp DNA fragment amplified from the *Inv* gene using the primer pair 5′-ACA CAT TCC TCC CGG ATC AAC and 5′-CTC TAA AGT CCT TGA CAC CAA TGC that had been labeled with digoxigenin according to the manufacturer's recommendations (Roche, Indianapolis, IN).

Processing and Sensory Evaluation. Tubers were washed, peeled, and cut into 7 × 7 mm strips. The potato strips were blanched for 9 min at 74 °C, dipped in 0.5% disodium acid pyrophosphate (SAPP) for 45 s at 77 °C, dried for 6 min at 77 °C, par-fried in soybean oil for 45 s at 191 °C, frozen overnight at −80 °C, and finish-fried for 3 min and 10 s at 168 °C. French fries (0.7 kg/sample) were spread on a tray and, after 3 min, rated for crispness according to an arbitrary scale of 1 (limp) to 9 (hard) after 3 min by a panel consisting of eight members (see ref *15* for details).

Sugar Determinations. Sugars were extracted by shaking approximately 150 mg of freeze-dried potato in 1 mL of 60% ethanol at 80 °C for 1 h. The supernatant was transferred to a fresh tube, and the pellet was re-extracted with 1 mL of ethanol for 30 min at 80 °C. The resulting solution was concentrated in a Speedvac to 60−70 μL, and 500 μg of ribose was added as internal standard. Sugar analyses were performed on an Agilent 1200 series HPLC system (Agilent, Santa Clara, CA), which consisted of an autosampler, a Zorbax carbohydrate column (0.46 × 15.0 cm), a solvent system of acetonitrile/water (75:25), and a refractive index detector, at a flow rate of 1 mL/min. Sugars were quantified using Agilent ChemStation software with external calibration.

Starch Assessments. Specific gravity, which is a reliable indicator for starch content, was determined by dividing the weight of tubers in air by their weight in water (*16*). Total starch levels were determined according to a method essentially developed by Jones and co-workers (*17*). Basically, 50 mg of freeze-dried potato powder was mixed in 1 mL of 60% ethanol by vortexing and then heating at 80 °C for 1 h. The supernatant was discarded to remove sugars, and pellet was re-extracted with 1 mL of 60% ethanol and heated for an additional 30 min. The supernatant was discarded, and the pellet was extracted for starch in 3 mL of 90% DMSO (Sigma, St. Louis, MO) by heating at 90 °C for 2 h in a constantly shaking water bath. The supernatant was transferred to a fresh tube. An aliquot was diluted in water and filtered through a 0.45 μm spin filter tube. The clear filtrate was analyzed on an Agilent 1200 series HPLC, which consisted of a vacuum degasser for efficient degassing of mobile phase, isocratic pump with autosampler, thermostated column compartment, and refractive index detector with automatic recycle valve. The HPLC separation was carried out on a 7.8 × 300 mm, Ultrahydrogel linear column (Waters Corp.) maintained at 60 °C with the mobile phase water at a flow of 1 mL/min. The refractive index detector (RID) temperature was at 40 °C. A standard curve was prepared using pure starch from potato (Sigma-Aldrich, St. Louis, MO). Total starch was quantified using Agilent ChemStation software with external standard calibration based on the exact amount of initial powder used.

Free Asparagine Evaluations. Free asparagine was extracted by homogenizing 250 mg of ground freeze-dried tubers with 5 μmol of sarcosine as an internal standard in 3.0 mL of 0.03 M triethylamine HCl buffer and adding (a) 150 μL 85 mM potassium hexacyanoferrate trihydrate ($K_4[Fe(CN)_6]\cdot 3H_2O$), (b) 150 μL of 100 mM zinc sulfate ($ZnSO_4\cdot 7H_2O$), and (c) 250 μL of 0.1 N NaOH with 3.0 mL of 0.03 M TEA buffer, pH 7.0, vortexing the mixture after each addition. The extract was centrifuged for 15 min at 4 °C at 40000 rpm, and the supernatant was transferred to a new tube. The pellet was resuspended in 5 mL of nanopure water and centrifuged. Supernatants were pooled with the first tube, and the final volume was adjusted to 12.5 mL with water. The extracted free amino acids were derivatized using the EZ:faast method according to the user's manual from the manufacturer (Phenomenex, Torrance, CA). Derivatized samples were analyzed by liquid chromatography−mass spectrometry (LC-MS) using an Agilent 1200 series HPLC system that was coupled to an Agilent 6300 series ion trap. Bruker's quantitative analysis software was used for quantification. For HPLC, we used a 25.0 × 0.3 cm EZ:faast AAA-MS column, and the mobile phase was 10 mM ammonium formate in water (A) and 10 mM ammonium formate in methanol 1:2, v/v (B), flow rate = 0.25 min/mL, with a gradient of 68−83% B in 13 min and 13−18 min 68% buffer B. MS was run in the positive mode with ESI and auto MS^n.

Quantifications of Ascorbic Acid. Ascorbic acid levels were quantified by HPLC, as described (*18*). Briefly, material was extracted from freeze-dried tuber powder with a 50% methanol buffer. LC-MS analysis of the extracts was performed with an Agilent 1200 series HPLC system that was equipped with a quaternary pump, a refrigerated autosampler, and a column heater, using DAD and MS ion trap detection. Typically, 5 μL of sample was injected into an Onyx column (Phenomenex, Torrance, CA) at 35 °C and a flow rate of 1 mL/min with a gradient elution of 0−1 min, 100% buffer A (10 mM formic acid, pH 3.5, with NH_4OH); 1−9 min, 0−30% buffer B (100% methanol with 5 mM ammonium formate); 9−10.5 min, isocratic 30% buffer B; 10.5−14 min, 35−65% buffer B; 14−16 min, 65−100% buffer B; and 16−15.5 min, isocratic 100% C. For MS analysis, ESI was used in the negative mode. The source was operated using 350 °C drying gas (N_2) at 12 L/min, 55 psi nebulizer gas, and a source voltage with a scan range of m/z 50−1000. Data analysis was performed using ChemStation software.

Acrylamide Determinations. Sample extraction and cleanup for acrylamide determinations were performed as described by the U.S. Food and Drug Administration (http://www.cfsan.fda.gov/dms/acrylamidi.html). Briefly, freeze-dried powder tissue was gently mixed with 1 mL of $^{13}C_3$-labeled acrylamide standard (200 ng/mL; Sigma-Aldrich) and 9 mL of water for 20 min and then centrifuged at 9000 rpm for 15 min in a Sorvall Legend XTR centrifuge (Thermo Scientific). A 5 mL aliquot of the clarified aqueous layer was transferred to a Maxi-Spin filter tube (0.45 μm, PVDF, Alltech Associates, IL, Deerfield, IL) and centrifuged at 9000 rpm for 2 min. One and a half milliliters of filtrate extract was loaded unto an OASIS HLB 6 cc solid phase extraction (SPE) cartridge, 200 mg packing (Water Corp., Milford, MA), that had been conditioned with 3.5 mL of methanol, followed by 3.5 mL of water. After the extract had passed the sorbent material, it was washed with 0.5 mL of water. Acrylamide was eluted by adding 1.5 mL of water to the sorbent bed. A second SPE purification involves the Bond Elut-Accucat cartridge (200 mg, 3 mL) from Varian (Chicago, IL), which was also conditioned with methanol

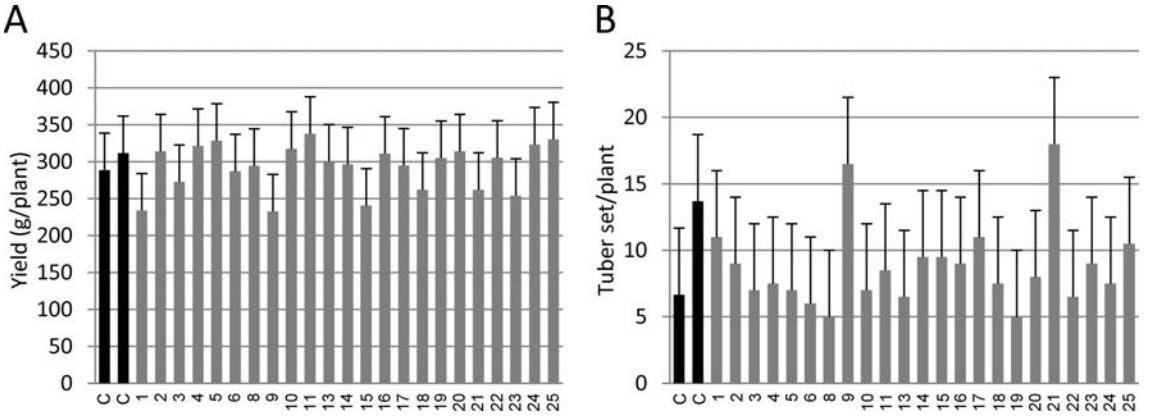

Figure 1. Yield, tuber set, and starch content. Tubers from two sets of pSIM401 control (C) lines (black bars) as well as plants representing 23 pSIM1632 lines (gray bars) were weighed (**A**) and counted (**B**). Results indicate the average for three plants per line ± SD.

(2.5 mL) and water (2.5 mL). Collected eluent from the OASIS SPE was loaded and eluted to the 1 mL mark before the remainder of the eluted portions was collected. The eluent was transferred to a 2 mL amber glass autosampler vial for LC-MS-MS.

Standards and sample portion extracts (20 μL) were analyzed on an Agilent 1200 HPLC equipped with a vacuum degasser, quaternary pump, autosampler, and temperature-controlled column oven coupled to a mass spectrometer. The Synergi 4 μ Hydro-RP 80A 250 × 2.0 mm LC column (Phenomenex) was maintained at 26 °C. The mobile phase was 0.5% methanol and 0.1% acetic acid in water at a flow rate of 0.2 mL/min. The MS determination of acrylamide and $^{13}C_3$-acrylamide was performed in electrospray ion source (ESI) positive mode. The source was operated using 350 °C drying gas (N_2) at 10 L/min, 35 psi nebulizer gas (N_2), isolation width 1.5, and source voltage with a scan range of m/z 30−80. For multiple reaction monitoring (MRM), m/z 72→55 for acrylamide and m/z 75→58 for $^{13}C_3$-acrylamide were monitored. Relative responses (peak areas) of acrylamide versus $^{13}C_3$-acrylamide (transitions m/z 72→55 and m/z 75→58, respectively) were used for calibration and quantification purposes. A standard curve was prepared from pure acrylamide in five different concentrations including $^{13}C_{13}$-acrylamide (Sigma-Aldrich) as internal standard. Data analysis was performed using Agilent ChemStation software and quantitative analysis was performed using Bucker's software.

Statistical Analyses. Data are presented as the means of the results of three experiments, and the error bars shown represent the standard deviation of the mean. Significance was determined using Student's two-tailed t test.

RESULTS AND DISCUSSION

To limit the CIS-associated accumulation of hexose, we designed a silencing cassette comprising the sense and antisense fragments of the *Inv* gene inserted between the tuber-specific promoters of the ADP glucose pyrophosphorylase (*Agp*) gene and the terminator of the ubiquitin-3 gene (see Materials and Methods). The cassette was positioned within the T-DNA of binary vector pSIM401, which already contained the *nptII* gene operably linked to the 35S promoter of cauliflower mosaic virus (*12*). An *Agrobacterium* LBA4404 strain carrying the resulting vector pSIM1632 was used to transform the French fry variety Ranger Russet, which is particularly prone to CIS. Twenty-three kanamycin-resistant plants, confirmed by the polymerase chain reaction (PCR) to contain at least one T-DNA integration event, were propagated in vitro to produce transgenic lines. Three plants of each of these lines were then grown in a temperature-controlled greenhouse together with transgenic pSIM401 controls. Most plants appeared phenotypically indistinguishable from each other during growth and senescence. However, plants of lines 7 and 12 displayed somaclonal variation and were not considered for further analyses.

Table 1. Starch Content[a]

line	starch	line	starch
401-control	0.8425 ± 0.005	1632-11	0.8584 ± 0.005
1632-1	0.8906 ± 0.008*	1632-14	0.8481 ± 0.005
1632-2	0.8810 ± 0.005*	1632-15	0.8794 ± 0.006*
1632-3	0.8644 ± 0.005*	1632-16	0.8507 ± 0.006
1632-4	0.8812 ± 0.006*	1632-17	0.8720 ± 0.007*
1632-5	0.8696 ± 0.004*	1632-18	0.8632 ± 0.009*
1632-6	0.8459 ± 0.009	1632-19	0.8687 ± 0.009*
1632-7	0.8608 ± 0.004*	1632-20	0.8568 ± 0.005*
1632-8	0.8615 ± 0.006*	1632-21	0.8592 ± 0.005*
1632-9	0.8849 ± 0.006*	1632-22	0.8697 ± 0.007*
1632-10	0.8754 ± 0.007*	1632-23	0.8817 ± 0.007*

[a] Values show the average ± SD in mg/mg DW. Statistically significant differences from the control ($P < 0.05$) are indicated with an asterisk.

As shown in **Figure 1**, both the average number and total yield of greenhouse-grown tubers, harvested 3 months after planting, were similar among the various Inv lines and pSIM401 controls. Although this assessment still needs to be confirmed in the field, it indicates that tuber-specific silencing does not interfere with tuber set and tuber fill. The specific gravity, a reliable indicator for starch content, averaged 1.084 for pSIM1632 lines, which is higher than that for pSIM401 controls (1.060) (data not shown). Indeed, a biochemical assay demonstrated that introduction of the *Inv* gene silencing construct increased the accumulation of starch by 2.8%, from control levels of 0.843 mg of starch/mg of dry weight (DW) to, on average, 0.867 mg/mg of DW (**Table 1**). Line 1632-1 contained the greatest amount of starch, 0.891 mg/mg of DW, which implies a 6% increase in starch content.

Tubers were stored at 5.5 °C and, after 1 month, analyzed for fructose and glucose contents. As shown in **Figure 2**, tubers from almost all pSIM1632 lines (22 of 23) contained dramatically reduced amounts of fructose and glucose when compared to those of the pSIM401 controls. The greatest reductions were achieved in the high-starch lines 1632-1 and 1632-15, which contained only 2−3% of the reducing sugars that are formed in control tubers. Tubers of line 1632-13 were an exception in that they accumulated near-wild-type levels of glucose and fructose. Similar results were obtained after a 2 month cold-storage period, with controls accumulating 25−33-fold more glucose and fructose than the two best CIS-resistant lines (**Figure 2**). The high efficiency of gene silencing that can be deduced from this experiment is typical for inverted repeat-based approaches (*19*, *20*) and much higher than the ∼1% frequency associated with the previously employed antisense-based strategy (*11*). In that case, only 1 of 95 lines produced tubers with reduced hexose content. We also found our

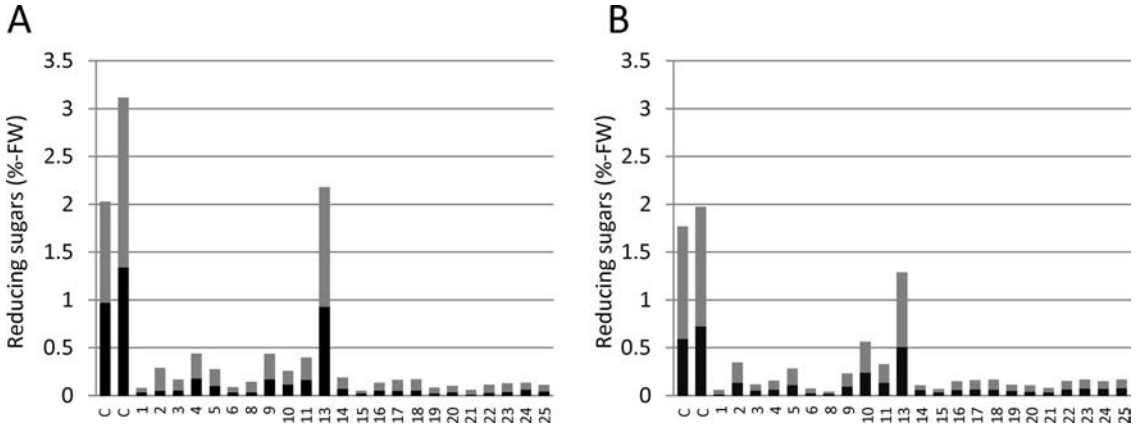

Figure 2. Reducing sugar content in tubers after storage at 5.5 °C. The total amount of glucose (black bar) and fructose (gray bar) was determined after 1 month (**A**) and 2 months (**B**) of cold storage and is shown as percentage of the fresh weight (FW) of tubers of two pSIM401 controls (C) plants as well as of 23 pSIM1632 lines.

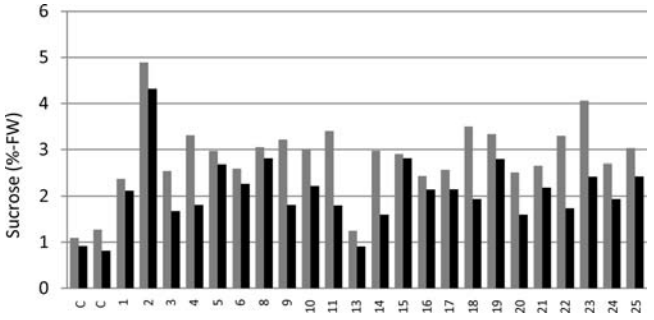

Figure 3. Sucrose content in tubers stored at 5.5 °C. Data were obtained after 1 month (gray bars) and 2 months (black bars) of cold storage.

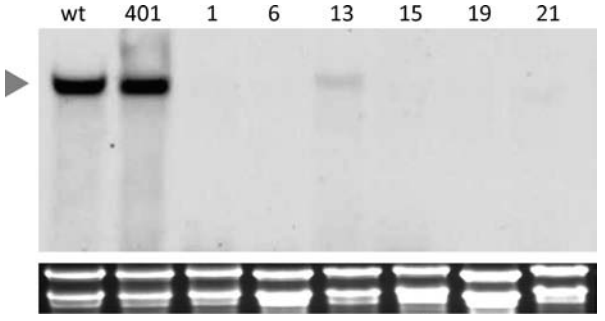

Figure 4. Transcript levels for the *Inv* gene in tubers stored at 5.5 °C for 2 months. wt, untransformed (wild type) potato; 401, transgenic control.

Table 2. Amounts of Phenylalanine and Chlorogenic Acid (Phenylpropanoids), Ascorbic Acid, and Asparagine in Potato Tubers[a]

line	phenylalanine	chlorogenic acid	ascorbic acid	free asparagine
401-control	0.48 ± 0.07	0.41 ± 0.07	0.63 ± 0.12	8.16 ± 1.16
1632-1	0.45 ± 0.06	0.45 ± 0.05	0.62 ± 0.09	6.65 ± 1.83
1632-2	0.65 ± 0.09	0.24 ± 0.04	0.61 ± 0.10	9.46 ± 1.07
1632-3	0.55 ± 0.08	0.44 ± 0.07	0.74 ± 0.16	9.64 ± 2.12
1632-4	0.50 ± 0.08	0.21 ± 0.14	0.66 ± 0.19	8.77 ± 1.94
1632-5	0.40 ± 0.07	0.38 ± 0.03	0.61 ± 0.09	6.73 ± 1.37
1632-6	0.38 ± 0.07	0.29 ± 0.10	0.61 ± 0.07	6.21 ± 1.81
1632-7	0.48 ± 0.06	0.22 ± 0.12	0.80 ± 0.13	9.03 ± 2.24
1632-8	0.51 ± 0.08	0.70 ± 0.23	0.64 ± 0.18	7.59 ± 1.08
1632-9	0.53 ± 0.05	0.30 ± 0.06	0.63 ± 0.12	9.28 ± 1.23
1632-10	0.47 ± 0.06	0.22 ± 0.11	0.65 ± 0.08	7.76 ± 0.96
1632-11	0.51 ± 0.07	0.23 ± 0.08	0.70 ± 0.20	8.22 ± 0.78
1632-14	0.41 ± 0.06	0.30 ± 0.04	0.73 ± 0.15	6.89 ± 1.35
1632-15	0.45 ± 0.05	0.29 ± 0.09	0.46 ± 0.11	7.33 ± 1.11
1632-16	0.41 ± 0.07	0.38 ± 0.04	0.69 ± 0.13	6.86 ± 1.36
1632-17	0.42 ± 0.06	0.23 ± 0.09	0.70 ± 0.09	7.75 ± 1.45
1632-18	0.45 ± 0.05	0.20 ± 0.14	0.77 ± 0.10	7.89 ± 1.67
1632-19	0.54 ± 0.07	0.29 ± 0.08	0.67 ± 0.11	8.85 ± 1.82
1632-20	0.50 ± 0.06	0.51 ± 0.19	0.67 ± 0.21	8.28 ± 0.98
1632-21	0.33 ± 0.08	0.29 ± 0.08	0.58 ± 0.11	6.29 ± 1.14
1632-22	0.50 ± 0.09	0.33 ± 0.06	0.71 ± 0.08	7.79 ± 1.23
1632-23	0.51 ± 0.07	0.45 ± 0.09	0.54 ± 0.08	8.87 ± 1.43
1632-24	0.52 ± 0.06	0.39 ± 0.06	0.67 ± 0.12	6.76 ± 0.93
1632-25	0.59 ± 0.08	0.33 ± 0.07	0.65 ± 0.14	7.16 ± 1.12

[a] Values are shown in mg/g DW and represent the average of three measurements ± SD.

method to be more efficacious than the original approach because it triggered strong reductions in glucose and fructose contents, whereas the best line produced with the earlier antisense strategy still contained two-thirds of the reducing sugars that are formed in controls (*11*).

The reductions in hexose content were counterbalanced, in part, by increased amounts of sucrose. Tubers of lines 1632-1 and 15 contained 2−2.5-fold more sucrose than the pSIM401 control tubers (**Figure 3**). The correlation between total amounts of hexose and sucrose was −0.57, which is at least as strong as that associated with earlier studies in potato, tomato, and muskmelon (*10*, *11*, *21*). Subsequent RNA gel blot analyses of tubers of five pSIM1632 lines that accumulated the lowest levels of reducing sugars demonstrated that this new trait was associated with undetectable Inv transcript levels (**Figure 4**). In contrast, the only transgenic line that produced tubers with high levels of fructose and glucose, line 1632-13, was found to still weakly express the *Inv* gene (**Figure 4**).

Specific sugars are known to influence the biosynthesis of various compounds that inhibit or promote acrylamide formation (*22*−*24*). Whereas phenylpropanoids and ascorbic acid mitigate acrylamide formation during thermal processing (*25*), asparagine represents one of the main acrylamide precursors (*26*). To study whether CIS-resistant tubers contain altered amounts of these compounds, we biochemically analyzed both the pSIM1632 lines and their controls. This experiment demonstrated that there were no significant differences between low-hexose tubers and their pSIM401 controls (**Table 2**). The tubers contained, per gram of DW, 0.38−0.65 mg of phenylalanine 0.20−0.70 mg of chlorogenic acid, 0.54−0.80 mg of ascorbic acid, and 6.21−9.65 mg of free asparagine, indicating that sucrose, glucose, and fructose

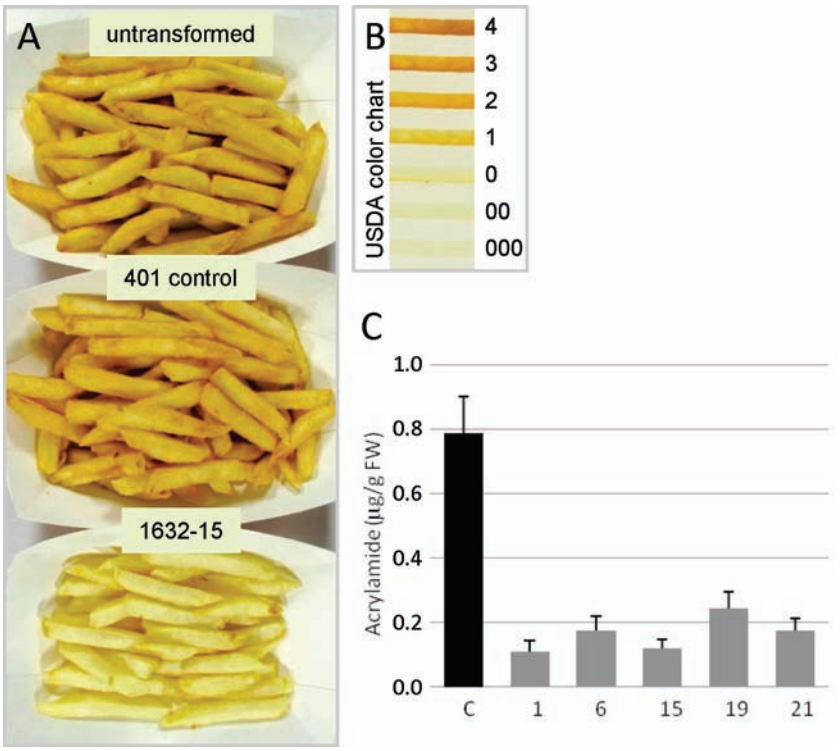

Figure 5. French fries. Typical fries from untransformed, transgenic control, and low-invertase tubers stored for 2 months at 5.5 °C are shown at the left (**A**); the USDA color chart used to assess fry darkness is shown at the right (**B**). Acrylamide levels in fries from 401 controls (black bars) and five 1632 lines (gray) represent the average of three analyzed samples ± SD (**C**).

Table 3. French Fry Color[a]

	fry color (USDA chart)						
	000	00	0	1	2	3	4
untransformed	0	0	0	8 ± 2	87 ± 12	5 ± 1	0
pSIM401 control	0	0	0	12 ± 3	82 ± 9	6 ± 2	0
pSIM1632-1	2 ± 0	63 ± 19	35 ± 6	0	0	0	0
pSIM1632-6	6 ± 1	76 ± 14	18 ± 3	0	0	0	0
pSIM1632-12	0	0	0	19 ± 3	79 ± 10	2 ± 0	0
pSIM1632-13	0	0	0	14 ± 2	81 ± 11	5 ± 1	0
pSIM1632-15	6 ± 1	92 ± 9	2 ± 0	0	0	0	0
pSIM1632-19	0	34 ± 5	57 ± 12	9 ± 2	0	0	0
pSIM1632-21	0	23 ± 5	68 ± 13	9 ± 3	0	0	0

[a] Three samples of par-fried potato strips were finish-fried independently and rated for color using the USDA color chart. Data are the average ± SD.

were not rate-limiting in the biosynthesis of any of these compounds.

To determine the effect of CIS resistance on the acrylamide potential of stored tubers, we peeled three tubers of each line, cut the tubers into 7 × 7 mm strips, and processed the strips by blanching, par-frying, freezing, and finish-frying for 3 min. The resulting French fries of low-Inv lines were much lighter in color than pSIM401 fries but displayed a similar crispness as assessed by a sensory team (**Figure 5A**,**B**; **Table 3**; and data not shown). Acrylamide levels were around 0.8 μg/g of fresh weight (FW) for both controls and pSIM1632 tubers that were not reduced in Inv transcript levels. These levels plummeted to as low as 0.1 μg/g of FW in the light-colored fries from silenced tubers (**Figure 5C**). This up to 8-fold reduction in acrylamide content was due exclusively to impairment of sucrose hydrolysis because, as mentioned above, pSIM1632 tubers were not altered in the levels of typical phenylpropanoids, ascorbic acid, and free asparagine.

In conclusion, we present a method to efficiently lower the acrylamide potential of existing potato varieties. The method is based on transforming plants with an *Inv* gene-derived inverted repeat fused to a tuber-specific promoter. Tubers of 21 of 23 transgenic lines were greatly impaired in hydrolyzing sucrose into glucose and fructose during cold storage. Introduction of the CIS resistance trait may lower the average dietary intake levels of acrylamide by as much as one-fourth because (a) French fry consumption is associated with 28% of the acrylamide intake (http://www.usda.gov/oce/risk_assessment/risk_forums/DeNovi111907.pdf) and (b) the acrylamide-forming potential of modified lines was shown here to be up to 8-fold lower than that of controls. It should be mentioned that the light color of French fries from CIS-resistant potatoes may make it possible to transition from white-fleshed potatoes to yellow-fleshed potatoes. In that way, the low-acrylamide fries would display a desirable yellow-golden color while also containing large amounts of carotenoid antioxidants (*27*).

One advantage of silencing the *Inv* gene in a tuber-specific rather than constitutive manner is that it avoids pleiotropic effects on plant growth and development. Inv plays an important role in futile cycles involving the vacuolar hydrolysis and cytoplasmic synthesis of sucrose (*28*), and plants with constitutively low Inv activity levels are weak and stunted (*21*, *29*). This undesirable phenotype may explain, in part, the difficulties associated with recovering such plants from transformation. Our result also suggests that the Inv-dependent process of sucrose/hexose interchanges is not critical in dormant potato tubers. We are currently preparing to test various pSIM1632 lines in the field so that we can confirm that the genetic modification did not affect vigor, yield, or tuber quality.

Previous studies have shown that CIS resistance can be introduced into potatoes, to some extent, by silencing genes other

than the *Inv* gene. The down-regulated expression of the starch-associated water dikinase *R1* and phosphorylase-L (*PhL*) genes impaired starch degradation and limited the accumulation of soluble sugars by about 50% (*30*). French fries from these *R1*/*PhL* gene-silenced tubers formed, upon processing, one-third of the acrylamide that accumulated in untransformed control fries. An alternative approach achieved 2-fold reductions in the acrylamide-forming potential of potatoes by overexpressing an invertase inhibitor gene (*30*). It might be interesting to create potatoes that not only contain very low sugar levels but also lack the ability to produce asparagine (*31*). These potatoes contain hardly any precursors for acrylamide and would be expected to yield fries with negligible amounts of this neurotoxin.

ACKNOWLEDGMENT

We thank Kristi Fessenden, Artesia Stivison, Joshua Sutherland, and Heather Holloway for excellent technical support.

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Received for review August 19, 2010. Revised manuscript received October 20, 2010. Accepted October 23, 2010. This work was financially supported by J.R. Simplot Co.