JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Improving Potato Storage and Processing Characteristics through All-Native DNA Transformation

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The dominant potato (*Solanum tuberosum*) variety for French fry production in the United States is the 131-year-old Russet Burbank. Market penetration of the higher yielding and more uniform Ranger Russet variety is limited to about one-fifth of that of the Russet Burbank because of two storage deficits: black spot bruise sensitivity and high levels of cold-induced sweetening. Here, these trait weaknesses are turned into strengths by simultaneously lowering the expression of Ranger Russet's tuber-expressed polyphenol oxidase (*Ppo*), starch-associated *R1*, and phosphorylase-L (*PhL*) genes. This genetic modification was accomplished without inserting any foreign DNA into the plant genome. French fries from the intragenic potatoes also contained reduced amounts of the antinutritional compound acrylamide while, unexpectedly, displaying enhanced sensory characteristics.

KEYWORDS: Genetic engineering; acrylamide; intragenic crops; potato

INTRODUCTION

Highly successful breeding programs have limited the average varietal age for many agronomically important crops to 4−6 years only (*1*). In contrast, the dominant potato variety Russet Burbank was developed 131 years ago. Although suffering from multiple deficits, this potato's excellent storage characteristics solidified its position as the preferred "white-fleshed" variety for French fry processing in the United States. Efforts to develop improved germplasm were hampered by complex tetrasomic genetics and inbreeding depression (*2*). One alternative variety that is considered to be the grower's favorite but has not been able to challenge Russet Burbank is Ranger Russet. Market penetration of this 15-year-old variety has been limited to only ∼20% of that of Russet Burbank. Although Ranger Russet combines superior yield with disease resistance, adaptability, tuber uniformity, and high levels of starch, it is particularly sensitive to tuber discolorations that are linked to impact-induced bruise. This phenomenon is caused by leakage of polyphenol oxidase (Ppo) from damaged plastids into the cytoplasm. The subsequent oxidation of polyphenols triggers a precipitation of black melanin that greatly affects tuber quality during prolonged storage (*3*). Furthermore, Ranger Russet accumulates high levels of glucose and fructose during cold storage. These reducing sugars react with free amino acids during high-temperature processing of the potato. Accumulation of the resulting Maillard reaction products lowers consumer appeal by darkening French fries. Therefore, the quality of Ranger Russet is generally compromised if tubers are stored for longer than about 8 weeks.

One Maillard reaction product that was recently identified in processed potato products is acrylamide. This compound reacts with the amino-terminal valine of hemoglobin to form adducts. The extent of adduct formation is a good marker for exposure levels and implies daily intakes approximating 100 μg, about 36% of which is derived from fried and baked starchy foods (*4*, *5*). These intake levels are much lower than those that are known to cause toxicological effects including neurological symptoms, decreased fertility, and cancer in rodents (>2 mg/kg of body weight) (*6*). Indeed, a recent population-based study in Sweden found that long-term dietary exposure to acrylamide in amounts typically ingested had no measurable impact on cancer (*7*, *8*). Nonetheless, public and scientific concerns may justify attempts to limit the content of acrylamide in processed food.

Both the storage and nutritional characteristics of potato can be improved by employing methods in genetic engineering. Silencing of the *Ppo* gene was shown to lower the extent of black spot bruise sensitivity (*9*, *10*), and the down-regulated expression of the starch-associated *R1* or phosphorylase-L (*PhL*) genes lowered the accumulation of reducing sugars in cold-stored potato tubers (*11*, *12*). Here, the simultaneous silencing of the *Ppo*, *R1*, and *PhL* genes is shown to provide black spot bruise tolerance and greatly reduced levels of cold-induced sweetening. Interestingly, French fries derived from the modified tubers displayed a strongly enhanced visual appearance and improved aroma while accumulating much lower levels of acrylamide.

EXPERIMENTAL PROCEDURES

Plasmid Construction. The DNA segment used to produce a multigene silencing construct contained the 154-base pair 5′-untranslated trailer of the POT32 *Ppo* gene (coordinates 1−154 of Genbank accession no. AY566556), the 179-bp 5′-untranslated leader of the *R1* gene (homologous to accession no. Y09533), and the 267-bp 5′-untranslated leader of the *PhL* gene (coordinates 1989−2256 of accession no. AF143202). Basic vectors are as described previously (*9*). The 573-bp spacer element used to separate the inverted repeats of the various silencing constructs represents the intron of the potato ubiquitin-7 gene (coordinates 1215−1788 of accession no. U26831).

10.1021/jf062477l CCC: $33.50
Published on Web 11/22/2006

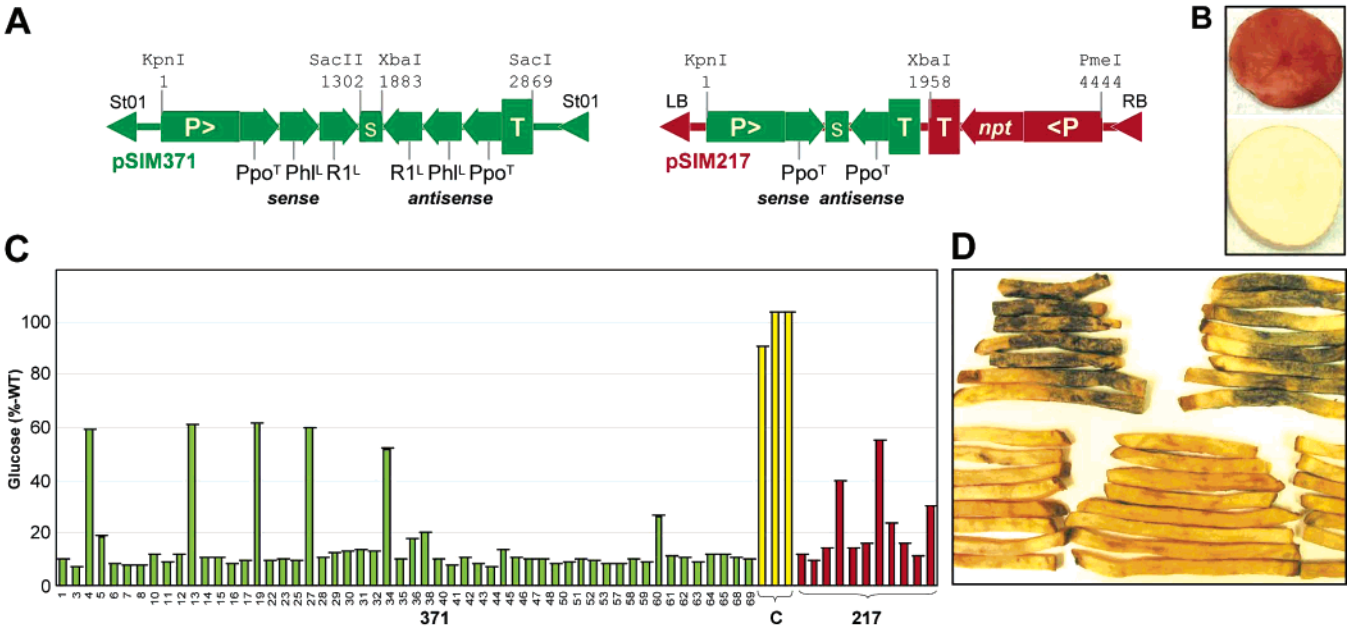

Figure 1. Mutigene silencing and bruise tolerance. (**A**) Diagram of constructs for multigene (pSIM371) and single-gene (pSIM217) silencing. P>, promoter of the Gbss gene; Ppo^T, trailer of the *Ppo* gene; PhL^L, leader of phosphorylase-L gene; $R1^L$, leader of *R1* gene; S, spacer from the ubiquitin-7 gene intron; T, terminator of the ubiquitin-3 gene. (**B**) Levels of Ppo tuber activity as assayed by pipetting a catechol solution onto cut tuber surfaces. Phenotypes are indicative for tubers of pSIM371 lines that displayed (top) and lacked (bottom) activity. (**C**) Ppo enzyme activities for 48 pSIM371 lines (green bars) and 11 of 50 pSIM217 benchmarks (red bars) that displayed reduced catechol-induced browning, as well as for untransformed Ranger Russet controls (yellow bars). Data are shown as percentage of average wild-type levels (ΔOD_{520} g^{-1} = 22.48 ± 0.98) and represent the mean ± SE of three independent experiments. (**D**) French fries from impacted untransformed (top) and pSIM217 (bottom) tubers. Note that the dark areas at the ends of intragenic fries represent the Russet skin and are not indicative of local discolorations.

Plant Transformation and Genotyping. Production of transgenic and intragenic plants was carried out as described (*9*). Transformed plants were genotyped for the presence or absence of P-DNA, T-DNA, and backbone DNA by employing Polymerase Chain Reactions (PCR) with appropriate primer sets (*9*).

Tuber Assays. Phosphate levels in potato starch were determined by using AOAC method 995.11, Phosphorus (Total) in Foods (45.1.33 *Official Methods of Analysis of AOAC International*, 17th ed.). Samples were prepared by dry ashing in a muffle furnace followed with an acid digestion. The dissolved samples were then neutralized and treated with a molybdate−ascorbic acid solution and compared to a series of phosphorus standards (treated similarly). A dual-beam spectrophotometer was used for the colorimetric analysis at 823 nm. A glucose oxidase/peroxidase reagent (Megazyme, Dublin, Ireland) was used to determine the glucose levels of cold-stored tubers. Tubers were screened for Ppo activity by pipetting 0.5 mL of 50 mM catechol in 50 mM MOPS (pH 6.5) onto freshly cut tuber surfaces and assaying for color development. The levels of Ppo enzyme activity were compared with wild-type levels by mixing pulverized tubers (1 g) for 1 h in 20 mM catechol and 50 mM 3-(*N*-morpholino)propanesulfonic acid buffer at pH 6.5 (5 mL). After precipitation of the solid fraction, the change of OD_{520} was determined over time.

RNA Analysis. Gene expression levels were assessed by performing quantitative real-time RT-PCR as described (*11*).

Processing. Field-grown tubers were either processed immediately or first stored in humidity-controlled chambers set at 4 °C. Tubers were washed, blanched for 8 min at 74 °C, cut into shoestring strips, dipped in a 1% sodium acid pyrophosphate solution at 71 °C, dried at the same temperature until 14 ± 2% weight loss was achieved, fried at 200 °C for 40 s to attain 64 ± 2% first-fry moisture, and frozen for 20 min at −26 °C, shaking the tray two to three times in the first 6 min. Processed fries were then finish-fried at 168 °C for 3 min and 15 s.

French Fry Assays. Color was assessed by using an E30-FP Agtron Process Analyzer (Agtron, Reno, NE) whereby a lighter color is reflected by a higher number and values above 40 are generally considered to be acceptable. Samples were biochemically analyzed for acrylamide levels by employing liquid chromatography−mass spectrometry/mass spectrometry (LC-MS/MS). Total polyphenol levels were determined as described (*13*). Sensory evaluations of French fries were performed by a panel of eight professionally trained experts at the optimum time of 3 min out of the fryer.

RESULTS AND DISCUSSION

In an attempt to turn the storage deficits of Ranger Russet into strengths, we employed a multigene silencing approach. For this purpose, a DNA segment was produced that contains fragments of three "undesirable" genes. One of the fragments was derived from the 5′-untranslated trailer of the *Ppo* allele POT32 that is predominantly expressed in mature tubers (*3*). The other two fragments represent the 3′-untranslated leaders of the *R1* and *PhL* genes, the down-regulated expression of which is known to slightly lower the extent of cold-induced sweetening (*11*, *12*).

Two copies of the "triple-gene fragment" DNA segment were inserted as inverted repeat between the tuber-specific promoter of the granule-bound starch synthase (*Gbss*) gene (*14*) and the terminator of the potato ubiquitin-3 (*Ubi3*) gene (**Figure 1A**). Positioning of the resulting silencing construct between two tandemly repeated potato St01 elements that function as T-DNA border alternatives created an all-native potato transfer (P-) DNA (*15*). A plasmid carrying this P-DNA, designated pSIM371, was introduced into an *Agrobacterium* LBA4404 strain (*16*) also harboring a conventional binary vector. This second "LifeSupport" vector, pSIM368, carried both the bacterial neomycin phosphotransferase (*nptII*) positive selectable marker gene and the cytosine deaminase (*codA*) gene for negative selection inserted between conventional T-DNA borders (*3*).

The resulting double-vector strain was used to infect 21900 Ranger Russet stem explants. Upon cotransfer of the P-DNA and T-DNA, the explants were subjected to kanamycin for 5

days to select for transient *nptII* gene expression. Proliferation of cells containing stably integrated *nptII* T-DNAs was subsequently prevented by transferring explants to media containing 5-fluorocytosine (5FC), a chemical that is converted into toxic 5-fluorouracil (5FU) by the *codA* gene product (*17*). A total of 3822 shoots that survived the double selection were genotyped by PCR for the presence of the desired P-DNA and the absence of foreign DNA. This analysis found ~85% of plants to contain plasmid backbone DNA. After elimination of both this large group of backbone-DNA-containing plants and an additional group that still carried the T-DNA, 256 marker-free and all-native DNA (intragenic) plants were selected for propagation and planting in the greenhouse.

Tubers from 1-month-old intragenic plants were prescreened for Ppo activity by pipetting a catechol "indicator" solution onto cut surfaces (**Figure 1B**). Ppo activity levels were then biochemically quantified in tubers that displayed reduced catechol-induced browning. These analyses demonstrated that 48 intragenic lines (19%) were effectively silenced for Ppo, often at levels below 15% of untransformed controls (**Figure 1C**). Both the frequency and extent of silencing were comparable to those of plants that had been transformed with pSIM217, a construct designed to only down-regulate the expression of the POT32 *Ppo* gene (**Figure 1A**,**C** and data not shown). To confirm that reduced Ppo activity levels would provide black spot bruise tolerance, tubers were physically impacted and, after 2 weeks, used for processing. French fries obtained from both pSIM371 and pSIM217 tubers were found to be free of discolorations, including visible white spot impact damage. In contrast, control fries developed extensive black spot bruise symptoms that covered about half of the fry surface (**Figure 1D** and data not shown).

Phenotypes of the 48 intragenic and bruise-tolerant lines were further analyzed by growing them in the field. Untransformed controls and three groups of transgenic "benchmark" lines were included in this trial. The first group of benchmarks consisted of five of the above-described *Ppo*-silenced pSIM217 lines. A second group of five lines was successfully silenced for the *PhL* gene through expression of the silencing construct of pSIM216 (*11*), whereas the *R1*-targeted silencing construct of pSIM332 had been used to produce a third group of gene-suppressed lines (**Figure 2A**).

The extent of cold-induced sweetening and its impact on tuber quality was assessed by storing field-grown tubers for 3 months at 4 °C. Tuber analyses demonstrated that 43 of the bruise-tolerant pSIM371 lines accumulated 30−60% of wild-type glucose levels. These levels were generally lower than those obtained with the benchmark lines that had been silenced for only one of the starch-associated genes (60−90%) (**Figure 2B**). Thus, our results confirm that a single multigene silencing construct can be used effectively to down-regulate the expression of multiple genes. Furthermore, they indicate an either additive or synergistic effect of the combined *R1*/*PhL* gene-silencing approaches.

Reduced glucose levels are known to lower processing-induced fry darkening (*18*). However, it was not clear to what extent the overall appearance of French fries from the 43 intragenic "bruise-tolerant and low-glucose" lines would be different from that of untransformed controls. Spectrophotometrical analysis demonstrated that the fries from Ranger Russet and Russet Burbank tubers were relatively dark, with Agtron levels between 42 and 46 (**Figure 2C** and data not shown). These levels barely met the minimum requirement for commercial applications, which is generally set at values >40. In contrast, the much lighter fries from five selected intragenic lines, 371-23, -28, -30, -38, and -68, surpassed the target that was set by us for the commercial application of a modified potato (>55), reaching levels between 58 and 60 (**Figure 2C** and data not shown). Intermediary Agtron values of about 50 were obtained with the pSIM216 and pSIM332 benchmark lines.

One physiological disorder that was found in about 15% of untransformed Russet Burbank and Ranger Russet tubers, as well as in those from the benchmark lines, was sugar-end formation. Triggered by drought-stimulated sugar migration to the lower end of potato tubers, this stress response is manifested as darkly colored French fry "ends" (*18*). Unexpectedly, the intragenic French fries lacked such local discolorations (data not shown). Thus, this stress-induced defect, which can result in extensive product loss and reduced quality, is effectively addressed by simultaneously lowering *R1* and *PhL* gene expression.

The enhanced appeal of French fries from pSIM371 lines was confirmed by a panel of eight professionally trained individuals. By rating for color, color variation, and appearance defects, fries from tubers of five intragenic Ranger Russet lines were found to outperform those of both Ranger Russet and Russet Burbank (**Figure 2D**). In contrast, the visual appearance of the benchmark lines was not significantly different from that of untransformed controls (data not shown).

In addition to improved visual appearance and consistency, the intragenic fries from freshly harvested tubers also displayed a significantly enhanced overall aroma as sensed by the olfactory epithelium, which is located in the roof of the nasal cavity (**Figure 2E**). This observed improvement was even more dramatic in tubers that had been stored for 10 weeks at 4 °C. In fact, French fries from the cold-stored intragenic lines 371-28, -30, -38, and -68 still met or exceeded the sensory attributes of those from freshly harvested untransformed varieties. Although the biochemical basis of enhanced aroma was not studied as part of this work, it is possible that the combined silencing of the starch mobilization genes *R1* or *PhL* prevented the accumulation of certain Maillard reaction products that negatively contributed to flavor aroma. The aroma improvements were not detected in the single-gene-silenced pSIM216 and pSIM332 lines that lowered glucose accumulation only partially (data not shown). Apart from aroma, the fries from intragenic lines were not different from control and benchmark fries in terms of texture, toughness, or mealiness. Collectively, our data indicate that the simultaneous suppression of the starch mobilization gene *R1* or *PhL* enhances key quality and sensory attributes to deliver a more consistent and flavorful processed product.

Given that both *R1* and *PhL* genes impinge on the starch phosphorylation pathway, we performed a detailed chemical analysis of the tuber starch. From this study, we found that silencing of the *R1* and *PhL* genes triggered opposite effects on phosphate levels. Single-gene silencing of *R1* (pSIM332) resulted in lower levels of starch phosphate ($P = 0.0001$), whereas levels were increased in tubers silenced only for *PhL* (pSIM216; $P = 0.05$) (**Figure 2F**). Interestingly, tuber starch phosphate levels of three intragenic lines tested resembled those of the pSIM332 benchmarks, indicating that the action of *R1* appears, at least at this level, to be epistatic over *PhL*. The replacement of conventional potato tubers by tubers from the intragenic lines could potentially reduce phosphate levels in the wastewaters of French fry plants. Currently, these levels are at 25−40 mg of phosphate L^{-1}, about half of which is derived from potato starch. Prior to environmental release, wastewater

Publication Date (Web): November 22, 2006 | doi: 10.1021/jf0624771

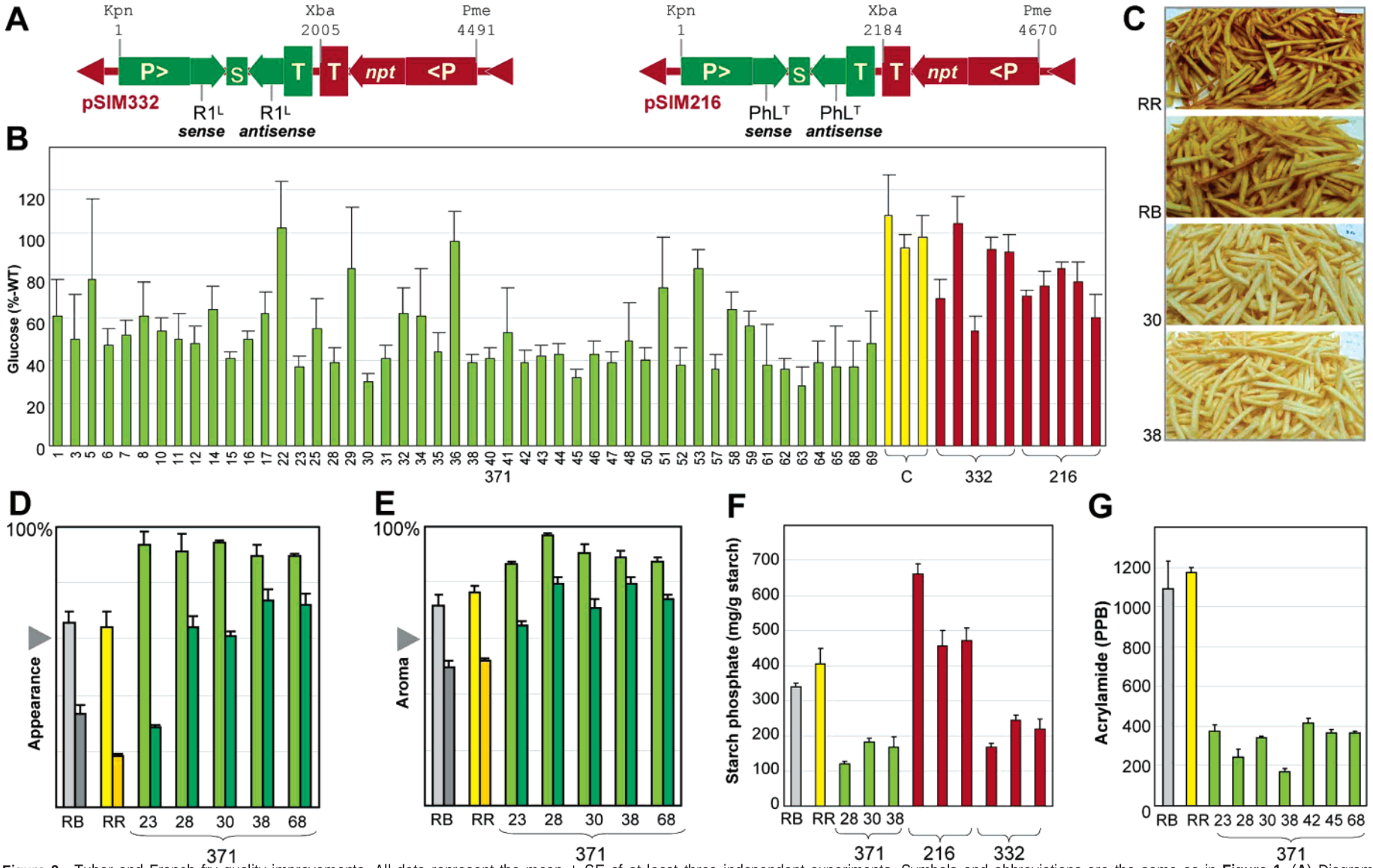

Figure 2. Tuber and French fry quality improvements. All data represent the mean ± SE of at least three independent experiments. Symbols and abbreviations are the same as in **Figure 1**. (**A**) Diagram of the single-gene silencing constructs pSIM216 and pSIM332. (**B**) Glucose levels in field-grown tubers after 3 months of cold storage for pSIM371 lines (green bars), untransformed Ranger Russet controls (yellow bars), and pSIM216 and pSIM332 benchmarks (red bars) as expressed as percentage of average wild-type levels (3.89 ± 0.15 mg of glucose/g of FW). (**C**) Color of French fries derived from cold-stored tubers of untransformed Russet Burbank (RB), Ranger Russet (RR), and intragenic lines 371-30 and -38. (**D**) Overall fry appearance of RB (gray bars), RR (yellow bars), and intragenic pSIM371 lines (green bars) based on visual assessments of color, color variation, and appearance defects by a professionally trained sensory panel. Rating was performed by using a scale from 0 to 100%. French fries were obtained from both freshly harvested tubers (left bars) and tubers that had been stored at 4 °C for 10 weeks (right bars). An arrow at 60% indicates the quality threshold for most French fry applications. (**E**) Overall aroma of fries from fresh (left bars) and cold-stored (right bars) tubers as assessed by a professional sensory panel. (**F**) Tuber starch phosphate levels. (**G**) Acrylamide levels in French fries of cold-stored tubers in parts per billion (PPB).

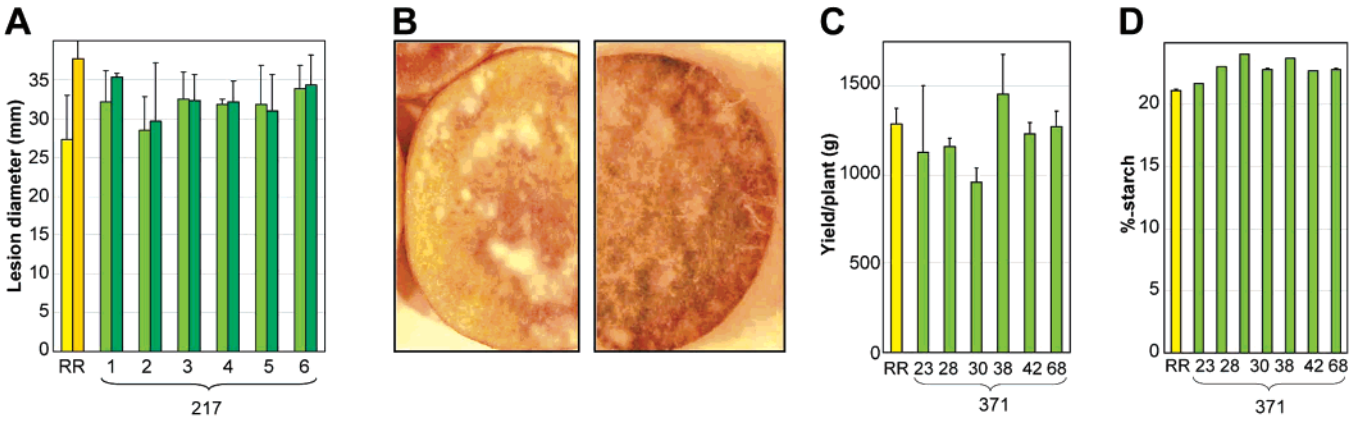

Figure 3. Agronomic characteristics. (**A**) Tuber response to infection with *Phytophthora infestans*. Tubers of both Ranger Russet (RR) control and PPO-silenced pSIM217 plants were subjected to 10^5 (left bars) and 10^6 (right bars) fungal spores and incubated at 18 °C. Disease progression was assessed by determining lesion size (millimeters) 5 days postinfection. All data represent the mean ± SE of at least three experiments. (**B**) Representative symptoms of infected pSIM217 (left) and control (right) tuber slices, 10 days after infection. (**C**) Yield of mid-sized to large (>100 g) tubers per plant. Data represent the mean ± SE of four replicated plots of 25 plants each. (**D**) Starch content. RR, Ranger Russet.

phosphates must be reduced, either by biological or physical postfactory treatment, at significant production expense. An alternative in planta method to reduce phosphate contamination would provide significant environmental and production advantages.

The low levels of reducing sugars in intragenic tubers prompted us to determine acrylamide levels in French fries obtained from these tubers. Acrylamide is a toxic Maillard compound that is largely derived from heat-induced reactions between the carbonyl group of reducing sugars and asparagine (*19*, *20*). Field-grown tubers from a second-year trial in Aberdeen, ID, were cold stored, processed, and biochemically analyzed. **Figure 2G** shows that French fries generated from intragenic tubers contained only about a third of the acrylamide that accumulates in control fries. Because consumption of French fries was recently estimated to contribute to ~16% of the total dietary intake of acrylamide (0.07 μg/kg of body weight/day) (http://www.cfsan.fda.gov), application of the "low acrylamide" fries would reduce the daily acrylamide intake by more than 10%. Further reductions may be obtainable by applying analogous acrylamide reduction strategies to oven-baked fries, potato chips, bread, and other starchy products, the consumption of which contributes to an estimated 59% of average intake levels (http://www.cfsan.fda.gov).

As a cultivar, Ranger Russet has a strong disease resistance profile, which presumably would be maintained after the intragenic improvement. In contrast to Russet Burbank, it contains natural tolerance against a range of viral diseases, including potato viruses X and Y and potato leaf roll virus (*21*). Furthermore, it displays resistance against the fungal "early die" pathogen *Verticillium dahliae*, allowing growth of Ranger Russet without need for expensive methyl bromide-based fumigation and the inadvertent loss of nontarget soil microbes (*22*). Because a constitutive reduction in *Ppo* expression levels might negatively influence plant defense responses (*23*), we sought to silence the *Ppo* gene only in the cortex and pith of the tuber, retaining enzyme activity in the epidermis (*9*). Here, we further tested pSIM217 lines for their susceptibility to the fungal pathogen *Phytophthora infestans*, causal agent of potato late blight. Tubers from both the transgenic lines and untransformed controls were infected with 10^5 and 10^6 spores of the aggressive US-8 strain (*24*). This infection resulted in a similar progression of disease as observed in control tubers (**Figure 3A**), demonstrating that *Ppo* gene silencing did not enhance the susceptibility against this important potato pathogen. Interestingly, severe tuber blackening was observed in only control tubers (**Figure 3B**). This result indicates that some of the early *P. infestans* disease symptoms are associated with *Ppo*-mediated coloration. The possibility of less obvious infection suggests that broad utilization of intragenic Ranger Russet may require monitoring for late blight, perhaps by including border rows of untransformed potato as "sentinels".

Overall data from the two field trial seasons suggested that the incorporated traits had no negative effect on agronomic performance. Analyses of tubers from the second-year trial did not reveal any differences between untransformed and intragenic lines in terms of plant and tuber typeness (data not shown). Furthermore, yields of regularly sized (>100 g) tubers were compromised only in line 371-30 (**Figure 3C**). This line displayed some morphological abnormalities and is likely to represent a somaclonal variant. The only measurable agronomic distinction between all intragenic lines and untransformed Ranger Russet related to starch levels. Tubers of the pSIM371 lines contained increased levels of starch, from 217 mg/g wild-type levels to an average of 232 mg/g in the modified lines (**Figure 3D**). This 7% increase in starch concentration is generally considered to be beneficial for the production of French fries.

Our results demonstrate that a multigene silencing construct enhanced the performance of Ranger Russet in seven different ways: black spot bruise resistance, reduced cold-induced sweetening, reduced stress-induced sugar ends, enhanced fry aroma, reduced amounts of processing-induced acrylamide, reduced starch phosphate content, and increased starch. By replacing some of the acreage that is currently occupied by Russet Burbank, it will also be possible to increase yields and lower costs for disease control. Furthermore, Ranger Russet's greater environmental adaptability will allow an expansion of territory in regions not wholly suitable for Russet Burbank cultivation. We have shown that both the sensory and nutritional characteristics of potato can be improved by simultaneously silencing the tuber-expressed *Ppo*, *R1*, and *PhL* genes. By committing to all-native DNA transformation methods for the incorporation of output rather than input traits, we hope to address at least some consumer concerns about the genetic modification of food crops (*25*).

ACKNOWLEDGMENT

We thank Jaime Menendez-Humara, Hua Yan, Oleg Bougri, Joanna Lewis, Richard Pinegar, Cheryl Franklin-Miller, Fred Dalton, Janet Baker, and Jeff Henderson for excellent technical assistance. We are deeply appreciative for the support of the University of Idaho, especially Peggy Bain, Jeffrey Stark, Tom Salaiz, and Steve Love. We acknowledge Scott Simplot, Bill Whitacre, and Steve Vernon for continued support.

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Received for review August 29, 2006. Revised manuscript received October 31, 2006. Accepted November 2, 2006.

JF062477L