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Silencing of vacuolar invertase and asparagine synthetase genes and its impact on acrylamide formation of fried potato products

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Summary

Acrylamide is produced in a wide variety of carbohydrate-rich foods during high-temperature cooking. Dietary acrylamide is a suspected human carcinogen, and health concerns related to dietary acrylamide have been raised worldwide. French fries and potato chips contribute a significant proportion to the average daily intake of acrylamide, especially in developed countries. One way to mitigate health concerns related to acrylamide is to develop potato cultivars that have reduced contents of the acrylamide precursors asparagine, glucose and fructose in tubers. We generated a large number of silencing lines of potato cultivar Russet Burbank by targeting the vacuolar invertase gene *VInv* and the asparagine synthetase genes *StAS1* and *StAS2* with a single RNA interference construct. The transcription levels of these three genes were correlated with reducing sugar (glucose and fructose) and asparagine content in tubers. Fried potato products from the best *VInv*/*StAS1*/*StAS2*-triple silencing lines contained only one-fifteenth of the acrylamide content of the controls. Interestingly, the extent of acrylamide reduction of the best triple silencing lines was similar to that of the best *VInv*-single silencing lines developed previously from the same potato cultivar Russet Burbank. These results show that an acrylamide mitigation strategy focused on developing potato cultivars with low reducing sugars is likely to be an effective and sufficient approach for minimizing the acrylamide-forming potential of French fry processing potatoes.

Introduction

The presence of acrylamide in carbohydrate-rich foods cooked at high temperatures was first reported in 2002 (Rosen and Hellenas, 2002; Tareke *et al.*, 2002). Acrylamide is one of many compounds produced from reducing sugars and free amino acids during cooking as a product of the Maillard reaction (Mottram *et al.*, 2002; Stadler *et al.*, 2002), which gives many browned foods their desirable colour and flavour. Dietary acrylamide causes cancer and developmental defects in rodents and is a suspected human carcinogen (Hogervorst *et al.*, 2010; Lineback *et al.*, 2012; Vinci *et al.*, 2012). Potential food safety concerns related to dietary acrylamide were raised worldwide in 2002. Since then, government agencies, food processing companies and academic institutions have been engaged in multifaceted efforts to mitigate concerns related to dietary acrylamide (Bethke and Bussan, 2013; Gokmen and Palazoglu, 2008; Palazoglu *et al.*, 2010).

Raw potatoes, especially after cold storage, contain relatively high amounts of reducing sugars (glucose and fructose) and asparagine, the acrylamide precursors. As a result, processed potato products, including both French fries and potato chips, often have relatively high contents of acrylamide (Bethke and Bussan, 2013; Rosen and Hellenas, 2002; Tareke *et al.*, 2002). French fries and potato chips contribute a significant proportion of the average daily intake of acrylamide (Dybing *et al.*, 2005), especially in countries where potato-based fast foods and snack foods are common. Reducing sugars were thought to be the primary determinants of acrylamide content in fried potato products (Amrein *et al.*, 2003; Becalski *et al.*, 2004). Selection of potato cultivars with low concentrations of reducing sugars was recommended as a method to minimize acrylamide content in processed potato products (Amrein *et al.*, 2003). Genetic studies showed that accumulation of reducing sugars during cold storage, or cold-induced sweetening (CIS), is a complicated trait and is likely controlled by many genes (Menendez *et al.*, 2002). Nevertheless, the potato vacuolar invertase gene (*VInv*) was found to play the key role in reducing sugar accumulation during CIS (Bhaskar *et al.*, 2010). Silencing of the *VInv* can effectively suppress glucose and fructose accumulation in different potato cultivars and significantly reduce the acrylamide contents in both French fries and potato chips (Bhaskar *et al.*, 2010; Wu *et al.*, 2011; Ye *et al.*, 2010; Zhu *et al.*, 2014).

Asparagine is the most common free amino acid in potato tubers and typically accounts for about 30%–40% of total amino acids (Amrein *et al.*, 2003; Becalski *et al.*, 2004; Halford *et al.*, 2012; Muttucumaru *et al.*, 2014). The high concentration of free asparagine in potatoes relative to other starchy foods was considered to be one of the reasons that processed potato products are relatively high in acrylamide (Elmore *et al.*, 2005; FDA, 2006; Stadler and Scholz, 2004). However, potato cultivars have a relatively narrow range of asparagine content, ranging from 4 to 25 mg/g dry weight in tubers (Amrein *et al.*, 2003; De

Meulenaer *et al.*, 2008; Shepherd *et al.*, 2010; Whittaker *et al.*, 2010; Williams, 2005; Zhu *et al.*, 2010). By contrast, glucose and fructose contents in potato cultivars vary more widely, ranging from <0.04 to 4.8 mg/g dry weight in tubers grown and stored commercially for processing (Stark and Love, 2003). In addition, it has been largely unknown about the genetic factors that control the variation of free asparagine accumulation during potato growth and storage. Rommens *et al.* (2008) reported that asparagine synthetases (AS), which catalyse the ATP-dependent transfer of the amino group of glutamine to aspartate to generate glutamate and asparagine (Lea *et al.*, 2007), have a major impact on asparagine accumulation in potato tubers. Silencing of the potato asparagine synthetase genes *StAS1* and *StAS2* significantly reduced acrylamide formation in both French fries and potato chips (Chawla *et al.*, 2012; Rommens *et al.*, 2008).

Decreasing either *VInv* or *StAS1*/*StAS2* expression has been demonstrated to be a promising approach to suppress acrylamide formation in processed potato products. However, there is a complex relationship between asparagine and reducing sugar contents and acrylamide-forming potential (Muttucumaru *et al.*, 2008, 2014). Silencing of both *VInv* and *StAS1*/*StAS2* is one way to assess the impact of these genes on acrylamide-forming potential, as well as on plant growth. We produced a large number of transgenic lines of potato cultivar Russet Burbank in which all three genes (*VInv*, *StAS1*, *StAS2*) were silenced by a single RNA interference (RNAi) construct. Several lines with different levels of silencing of the three genes were evaluated for gene expression, acrylamide precursors and phenotypic changes in trials conducted in both greenhouse and field. The transcription levels of the three target genes were found to be correlated with the contents of reducing sugars and asparagine in tubers, and correlated with acrylamide formation in fries processed from the tubers. However, acrylamide contents of the fried products from the best triple silencing lines were not lower than those observed from the previously developed Russet Burbank RNAi lines in which only *VInv* was silenced (Zhu *et al.*, 2014). These results show that an acrylamide mitigation strategy focused on developing potato cultivars with low reducing sugars is likely to be an effective and sufficient approach for minimizing the acrylamide-forming potential of French fry processing potatoes.

Results

Development of RNAi lines for triple silencing of *VInv*, *StAS1* and *StAS2* in Russet Burbank potato

Two potato asparagine synthetase genes, *StAS1* and *StAS2*, were identified on chromosomes 6 and 4, respectively. *StAS1* and *StAS2* contain 1770- and 1677-bp coding sequences, respectively, including a common 184 amino acid glutamine amidotransferase type 2 (GTTASE_TYPE_2) domain (Figure 1). *StAS1* and *StAS2* share approximately 74% DNA sequence similarity, and the encoded proteins share 81% amino acid similarity.

We designed a single RNAi construct to silence *StAS1*, *StAS2* and *VInv*. This construct contains a 311-bp cDNA fragment from the *StAS1* gene and a 311-bp cDNA fragment from the *VInv* gene (Bhaskar *et al.*, 2010). The *StAS1* fragment was selected from a region that shares 79% sequence similarity with the corresponding region of *StAS2* (Figure 1), which represents the region with the highest sequence similarity between the two genes. This construct was driven by the CaMV 35S promoter.

We developed 127 RNAi lines of potato cultivar Russet Burbank (triRBK lines). The expression of *StAS1, StAS2* and *VInv* in each line was assayed by quantitative real-time PCR (qRT-PCR) using leaf tissues as an initial test for effectiveness of the silencing construct. Twenty lines that represented a range of expression for the three genes in leaf tissue were selected for tuber-based studies. Finally, seven RNAi lines (triRBK4, 21, 28, 29, 39, 53, 69) that showed a representative range of expression for the three genes in cold-stored tubers (14 day at 4 °C) were selected for field evaluation and in-depth analyses of tubers.

Expression of *VInv*, *StAS1* and *StAS2* in RNAi lines

The seven selected RNAi lines were grown in the field in 2013. We monitored the expression levels of *VInv*, *StAS1* and *StAS2* in field-grown tubers at harvest and 1, 3 and 6 months after harvest. qRT-PCR was performed using tissues collected from both apical and basal ends (bud end and stem end) of the tubers. The *VInv* expression relative to untransformed Russet Burbank controls was generally lower in stem-end compared to bud-end samples (Table S1). The highest level of *VInv* silencing was observed in triRBK28, which at harvest showed 2% and 14% of the expression level in untransformed controls at the stem and bud end, respectively (Table S1). *VInv* expression relative to the reference gene *Actin97* reached the lowest level in all lines at 1 month of storage and then gradually increased (Figure 2a,b).

Expression levels of *StAS1* and *StAS2* in RNAi-silencing lines relative to Russet Burbank controls were also generally lower in the stem end compared to the bud end, although there were some exceptions (Table S1). Line triRBK28 had the highest levels of *StAS1*/*StAS2* silencing, and expression of these two genes was only 7% and 35%, respectively, of that in the Russet Burbank control (Table S1). Expression of *StAS1* and *StAS2* relative to expression of *Actin97* decreased with increasing time during storage and reached the lowest level at 3–6 months after harvest (Figure 2c–f). Silencing of *StAS1* was always more effective than silencing of *StAS2* in both ends of tubers of the same RNAi line. Four lines, triRBK53, triRBK4, triRBK21 and triRBK28, had *StAS1* expression that was significantly less in tuber stem and bud-end tissues than the expression in Russet Burbank controls ($P < 0.05$), and two of these lines, triRBK28 and triRBK21, had reduced expression of *StAS2* as well ($P < 0.05$).

The levels of silencing of the three genes were moderately correlated among different RNAi lines. Expression of *StAS1* was better correlated with expression of *VInv* ($R = 0.41$ using leaf-based data of all 127 lines and $R = 0.67$ using tuber-based data from 20 selected lines, $P < 0.001$) than with *StAS2* ($R = 0.35$ using leaf data and $R = 0.58$ using tuber data, $P < 0.01$).

Phenotype of the selected RNAi lines

We evaluated the phenotype of the seven selected RNAi lines in replicated field trials at the Hancock Agricultural Research Station, Wisconsin, in 2013 and 2014. Transplants were used in the 2013 field trial. All seven RNAi lines exhibited normal growth and tuber phenotypes when grown in the greenhouse (data not shown) and in the field from transplants (Figure S1). Tuber yields of all seven RNAi lines were not significantly different from the yield of Russet Burbank control in 2013. Tubers from the RNAi lines showed similar sizes, shapes and numbers as compared to the control (Figure S1).

Seed tubers, which were harvested from greenhouse-grown plants, were used in the 2014 field trial. Surprisingly, triRBK21 and triRBK28, which showed the highest level of silencing, showed a stunted and chlorotic plant phenotype shortly after

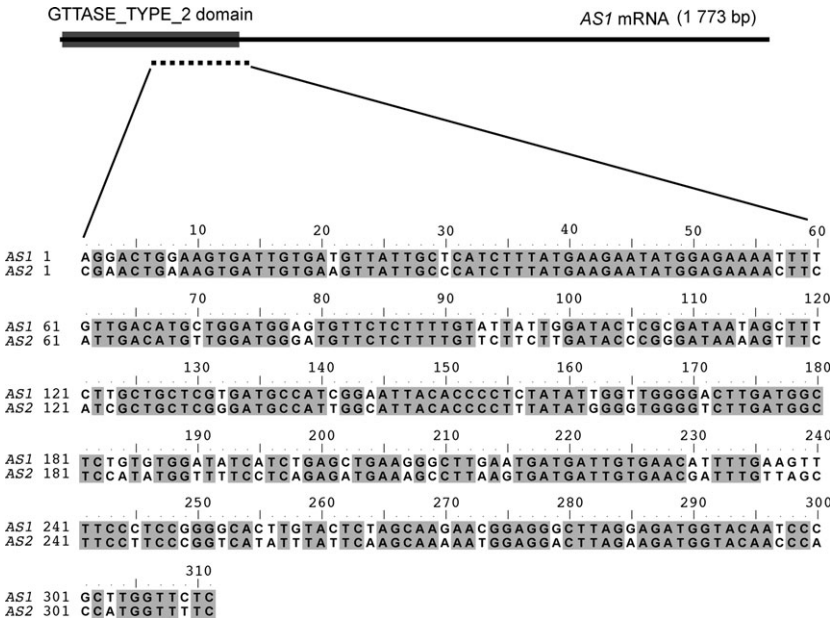

Figure 1 The structure of the potato gene *StAS1*. The GTTASE_TYPE_2 domain is located at 5′ of the gene. The majority of the 311-bp fragment targeted for RNAi (dotted line) overlaps the GTTASE_TYPE_2 domain. The 311-bp sequence of *StAS2* is shown in comparison with the *StAS1* target sequence.

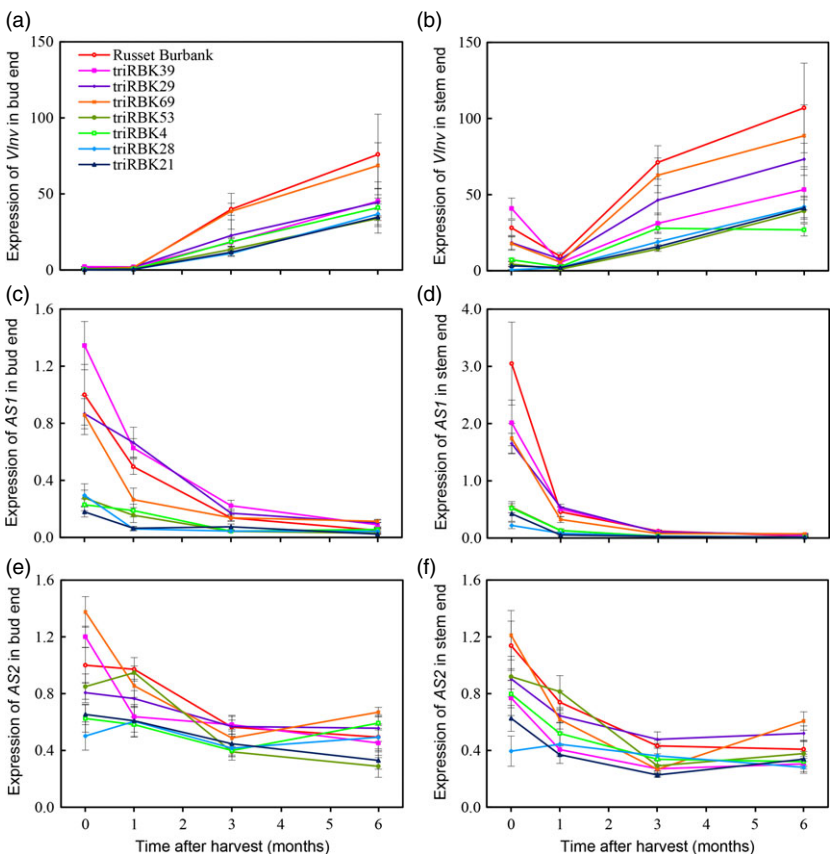

Figure 2 Expressions of *Vlnv*, *StAS1* and *StAS2* in bud end (a, c and e) and stem end (b, d and f) of tubers from seven RNAi lines and Russet Burbank control harvested from a replicated field trial in 2013. Gene expression is expressed relative to the expression of the reference gene *Actin97*. Data were collected from potato tubers at harvest and 1, 3 and 6 months after harvest. Harvested tubers were stored for 50 days at 13 °C and cooled over 24 days to a final storage temperature of 9 °C, which is a storage temperature profile typical of that used in commercial practice. Bars represent mean ± standard error of seven independent tuber samples.

emergence with significantly reduced plant vigour and tuber yields (Table 1 and Figure S2a). Lines triRBK4 and triRBK53 showed less severe reductions in yield than triRBK21 and triRBK28 (Table 1). Unambiguous abnormal phenotypes were not observed in the other three lines that had comparable yields as the control (Table 1). Interestingly, tubers from all RNAi lines, including triRBK21 and triRBK28, showed similar shape compared to those from the control (Figure S2b). Reductions in yield reflected reductions in tuber length and the total number of tubers from the RNAi lines (Table 1).

Table 1 Tuber phenotypes associated with seven RNAi lines and Russet Burbank control in a replicated field trial in 2014

Genotype	Yield (kg)/hill	Tuber number/hill	Tuber length (cm)
Russet Burbank	1.77 ± 0.23	9.7 ± 1.6	9.8 ± 0.6
triRBK69	1.55 ± 0.11	10.5 ± 1.5	9.3 ± 0.6
triRBK53	1.18 ± 0.06**	9.9 ± 1.4	8.6 ± 0.7*
triRBK39	1.51 ± 0.05	10.3 ± 3.0	8.8 ± 0.3
triRBK4	1.18 ± 0.17**	10.9 ± 1.4	8.2 ± 0.5**
triRBK29	1.59 ± 0.15	10.3 ± 1.4	9.6 ± 0.3
triRBK21	0.40 ± 0.23***	4.8 ± 1.8**	8.2 ± 1.4**
triRBK28	0.63 ± 0.19***	6.8 ± 0.9*	8.6 ± 0.2*

Values are means ± standard deviation from three replicate plots. Differences in means between the RNAi lines and the Russet Burbank control are indicated as different at $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***) level.

Acid invertase activity and reducing sugar accumulation in RNAi lines

We measured the acid invertase activity of tubers of the RNAi lines at harvest in 2013 and during storage in 2013 and 2014. Acid invertase activity was well correlated with *VInv* expression at stem end of tubers at harvest and 1, 3 and 6 months after harvest ($R = 0.83$–0.92). Lines triRBK21 and triRBK28, which had the lowest level of *VInv* expression, contained the lowest amounts of acid invertase at harvest and during storage (Figure 3). The reduction of acid invertase activity was especially distinct at the stem ends, with four lines showing significant reduction ($P < 0.001$) compared to the control. The acid invertase activity in these four lines remained low during long-term 9 °C storage with activity <0.55 units/mg protein (Figure 3b). The bud end of tubers showed much less acid invertase activity than the stem end (Figure 3a). The RNAi lines had similar levels of acid invertase activity at the bud end of the tubers compared to the control at harvest. However, tubers from triRBK28 and triRBK21 showed less acid invertase activity at the bud end during 9 °C storage ($P < 0.05$, Figure 3a).

We also quantified the glucose (Table 2), fructose (Table 3) and sucrose (Table 4) contents at both bud and stem ends of tubers from all RNAi lines harvested in 2013. The amounts of glucose and fructose were well correlated with the acid invertase activity ($R = 0.81$–0.95) and with the *VInv* expression level ($R = 0.78$–0.89) at stem end of tubers at harvest and 1, 3 and 6 months after storage. Accumulation of glucose and fructose at the tuber bud ends of RNAi lines was not significantly different from that in the control (Table 2 and Table 3, $P > 0.05$). However, significant reductions in reducing sugars were observed at the stem end of the tubers of all RNAi lines except triRBK29 ($P = 0.06$) 3 months after harvest (Figure 4a) and in all lines 6 months after harvest (Table 2 and Table 3, $P < 0.01$). By contrast, the RNAi lines contained similar amounts of sucrose at both ends of the tubers compared to the control ($P > 0.05$, Table 4).

Asparagine content of tubers from the RNAi lines

We measured the asparagine contents associated with the stem end of tubers of all RNAi lines harvested in 2013. Five tuber samples were collected from each of the seven RNAi lines and Russet Burbank control after 3 months of storage. Asparagine

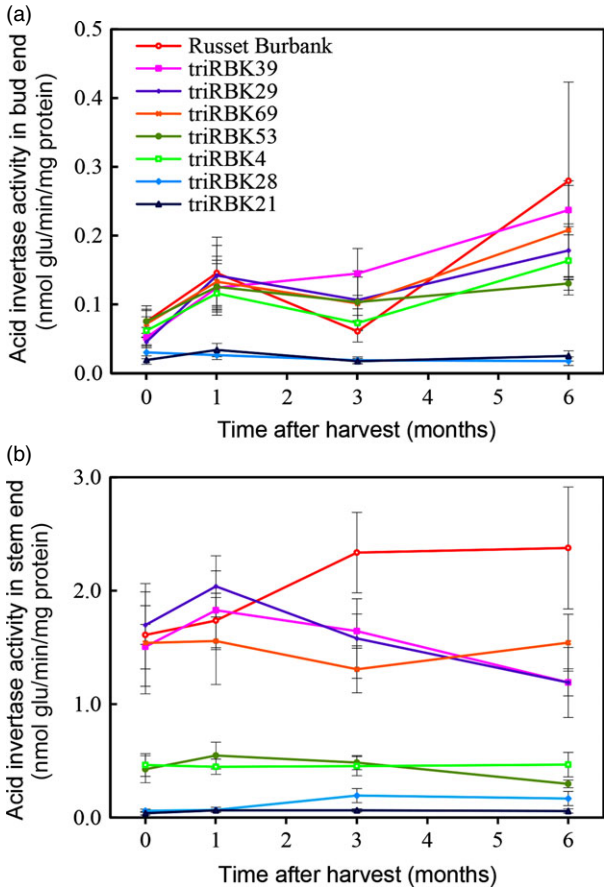

Figure 3 Acid invertase activity in bud end (a) and stem end (b) of tubers from seven RNAi lines and Russet Burbank control harvested from a replicated field trial in 2013. Data were collected from potato tubers at harvest and 1, 3 and 6 months after harvest. Harvested tubers were stored for 50 days at 13 °C and cooled over 24 days to a final storage temperature of 9 °C, which is a storage temperature profile typical of that used in commercial practice. Bars represent mean ± standard error of seven independent tuber samples.

contents in triRBK4, triRBK21, triRBK28 and triRBK53 were significantly lower than the control (Figure 4b, $P < 0.01$). The three remaining lines had a similar amount of asparagine as the control ($P > 0.05$). Differences in asparagine content mirrored the differences in *StAS1* expression at the tuber stem end. A strong correlation ($R = 0.91$) between asparagine content and the level of *StAS1* expression was found in stem end of the tubers 3 months after harvest, but a less strong correlation was found between asparagine content and the level of *StAS2* expression ($R = 0.45$).

Processing quality of tubers from the RNAi lines

We examined the colour of fried strips prepared from tubers of the seven RNAi lines and the control at harvest and after 1, 3 and 6 months of storage. Fried strips of tuber tissue were evaluated for the frequency of sugar-end defects. All RNAi lines, except for triRBK21 and triRBK28, had different degrees of defects at stem ends but were lighter than Russet Burbank control at each sampling period (Figure 5). Fried strips processed from triRBK21 and triRBK28, however, were free of sugar-end defects at all sampling periods.

Table 2 Glucose content in bud end and stem end of tubers from seven RNAi lines and Russet Burbank control harvested from a replicated field trial in 2013

			Postharvest storage		
Tuber end	Line	Harvest	1 Month	3 Months	6 Months
Bud end	Russet Burbank	0.13 ± 0.13	0.67 ± 0.79	0.14 ± 0.07	0.31 ± 0.44
	triRBK69	0.31 ± 0.34	0.21 ± 0.23	0.14 ± 0.04	0.25 ± 0.28
	triRBK53	0.31 ± 0.24	0.21 ± 0.08	0.21 ± 0.11	0.08 ± 0.03
	triRBK39	0.27 ± 0.23	0.37 ± 0.26	0.20 ± 0.06	0.22 ± 0.17
	triRBK4	0.20 ± 0.13	0.21 ± 0.13	0.24 ± 0.15	0.15 ± 0.09
	triRBK29	0.14 ± 0.12	0.26 ± 0.24	0.14 ± 0.08	0.27 ± 0.22
	triRBK21	0.19 ± 0.17	0.09 ± 0.04	0.16 ± 0.17	0.18 ± 0.17
	triRBK28	0.07 ± 0.02	0.05 ± 0.02	0.15 ± 0.13	0.07 ± 0.00
Stem end	Russet Burbank	3.23 ± 2.61	3.70 ± 1.77	2.85 ± 0.96	2.59 ± 1.45
	triRBK69	1.87 ± 1.87	1.70 ± 0.75	1.53 ± 0.65	1.88 ± 0.93
	triRBK53	1.82 ± 1.19	1.59 ± 0.71	1.51 ± 0.59	0.91 ± 0.44
	triRBK39	3.23 ± 1.76	1.78 ± 0.37	1.66 ± 0.45	1.16 ± 0.59
	triRBK4	1.52 ± 0.91	1.11 ± 0.29	1.82 ± 0.96	1.25 ± 0.42
	triRBK29	2.29 ± 1.13	2.48 ± 1.45	2.35 ± 1.14	1.40 ± 0.72
	triRBK21	0.45 ± 0.35	0.75 ± 0.51	0.56 ± 0.27	0.69 ± 0.27
	triRBK28	0.50 ± 0.36	0.43 ± 0.30	0.73 ± 0.49	0.63 ± 0.26

Data were collected from potato tubers at harvest and 1, 3 and 6 months after harvest. Values are means ± standard deviation of seven independent tuber samples. Glucose contents are shown as mg/g fresh tuber weight. Harvested tubers were stored for 50 days at 13 °C and cooled over 24 days to a final storage temperature of 9 °C, which is a storage temperature profile typical of that used in commercial practice.

Table 3 Fructose content in bud end and stem end of tubers from seven RNAi lines and Russet Burbank control harvested from a replicated field trial in 2013

			Postharvest storage		
Tuber end	Line	Harvest	1 Month	3 Months	6 Months
Bud end	Russet Burbank	0.04 ± 0.02	0.30 ± 0.35	0.08 ± 0.03	0.16 ± 0.15
	triRBK69	0.12 ± 0.17	0.13 ± 0.12	0.08 ± 0.01	0.15 ± 0.15
	triRBK53	0.14 ± 0.12	0.14 ± 0.04	0.14 ± 0.11	0.08 ± 0.02
	triRBK39	0.07 ± 0.05	0.20 ± 0.15	0.10 ± 0.03	0.19 ± 0.08
	triRBK4	0.08 ± 0.05	0.09 ± 0.04	0.12 ± 0.05	0.14 ± 0.08
	triRBK29	0.06 ± 0.03	0.10 ± 0.08	0.07 ± 0.04	0.16 ± 0.08
	triRBK21	0.03 ± 0.01	0.05 ± 0.02	0.08 ± 0.08	0.13 ± 0.13
	triRBK28	0.03 ± 0.02	0.04 ± 0.02	0.08 ± 0.08	0.05 ± 0.01
Stem end	Russet Burbank	2.77 ± 2.29	2.35 ± 1.24	1.90 ± 0.81	1.76 ± 1.19
	triRBK69	1.73 ± 1.74	1.05 ± 0.38	0.98 ± 0.56	0.92 ± 0.35
	triRBK53	1.77 ± 1.20	1.11 ± 0.54	0.74 ± 0.43	0.30 ± 0.11
	triRBK39	2.82 ± 1.80	1.25 ± 0.31	0.82 ± 0.38	0.82 ± 0.44
	triRBK4	1.32 ± 0.92	0.51 ± 0.08	1.18 ± 0.64	0.78 ± 0.32
	triRBK29	2.24 ± 1.12	1.71 ± 0.93	0.85 ± 0.43	0.77 ± 0.39
	triRBK21	0.32 ± 0.21	0.52 ± 0.31	0.28 ± 0.10	0.40 ± 0.19
	triRBK28	0.42 ± 0.38	0.36 ± 0.32	0.49 ± 0.38	0.48 ± 0.22

Data were collected from potato tubers at harvest and 1, 3 and 6 months after harvest. Values are means ± standard deviation of seven independent tuber samples. Fructose contents are shown as mg/g fresh tuber weight. Harvested tubers were stored for 50 days at 13 °C and cooled over 24 days to a final storage temperature of 9 °C, which is a storage temperature profile typical of that used in commercial practice.

We measured the acrylamide contents of the fried strips processed from tubers after 3 months of storage. Five samples from the stem ends of the fried strips, which corresponded to the samples used for asparagine analysis before frying, were used for acrylamide measurement. Samples from all seven RNAi lines contained significantly less amounts of acrylamide than the control (Figure 4c, $P < 0.001$). Remarkably, triRBK21 and triRBK28 contained only 84 and 89 μg/kg acrylamide, respectively, compared to 1233 μg/kg of the control (Figure 4c).

Table 4 Sucrose content in bud end and stem end of tubers from seven RNAi lines and Russet Burbank control harvested from a replicated field trial in 2013

			Postharvest storage		
Tuber end	Line	Harvest	1 Month	3 Months	6 Months
Bud end	Russet Burbank	2.03 ± 0.18	1.94 ± 0.24	1.57 ± 0.24	1.36 ± 0.24
	triRBK69	2.30 ± 0.32	1.98 ± 0.50	1.39 ± 0.24	1.19 ± 0.26
	triRBK53	2.60 ± 0.21	2.12 ± 0.20	1.43 ± 0.21	1.39 ± 0.16
	triRBK39	2.13 ± 0.22	1.65 ± 0.27	1.39 ± 0.11	1.31 ± 0.12
	triRBK4	2.06 ± 0.17	1.62 ± 0.16	1.37 ± 0.20	1.32 ± 0.14
	triRBK29	2.06 ± 0.15	1.86 ± 0.19	1.59 ± 0.20	1.39 ± 0.16
	triRBK21	1.99 ± 0.32	2.11 ± 0.24	1.45 ± 0.17	1.77 ± 0.52
	triRBK28	2.01 ± 0.28	1.45 ± 0.18	1.49 ± 0.25	1.74 ± 0.15
Stem end	Russet Burbank	1.46 ± 0.36	1.45 ± 0.49	1.19 ± 0.17	1.10 ± 0.28
	triRBK69	2.13 ± 0.34	1.86 ± 0.10	1.18 ± 0.35	1.23 ± 0.16
	triRBK53	1.12 ± 0.20	1.36 ± 0.21	1.54 ± 0.35	1.70 ± 0.14
	triRBK39	1.26 ± 0.07	1.08 ± 0.11	1.05 ± 0.18	1.17 ± 0.13
	triRBK4	1.14 ± 0.33	1.18 ± 0.18	1.13 ± 0.17	1.17 ± 0.07
	triRBK29	1.51 ± 0.31	1.23 ± 0.21	1.06 ± 0.36	1.17 ± 0.20
	triRBK21	1.70 ± 0.62	1.30 ± 0.13	1.23 ± 0.24	1.69 ± 0.41
	triRBK28	1.72 ± 0.47	1.29 ± 0.09	1.77 ± 0.35	1.44 ± 0.18

Data were collected from potato tubers at harvest and 1, 3 and 6 months after harvest. Values are means ± standard deviation of seven independent tuber samples. Sucrose contents are shown as mg/g fresh tuber weight. Harvested tubers were stored for 50 days at 13 °C and cooled over 24 days to a final storage temperature of 9 °C, which is a storage temperature profile typical of that used in commercial practice.

Discussion

The functional roles of *StAS1* and *StAS2* in potato

Asparagine is a nitrogen-rich amino acid and is thought to play an important role in the transport and storage of nitrogen (Lam *et al.*, 1994). Asparagine accumulation can be induced by abiotic and biotic stresses, such as mineral deficiencies, drought and salinity stress, toxic metals and pathogen attack (Lea *et al.*, 2007). The functions of asparagine synthetase genes have not been fully characterized; however, these genes have been implicated in responses to pathogen infections (Hwang *et al.*, 2011; Olea *et al.*, 2004). In addition, the asparagine synthetase genes are differentially regulated and may play different roles in different tissues or plant species (Chawla *et al.*, 2012; Herrera-Rodriguez *et al.*, 2002; Lam *et al.*, 1998; Tsai and Coruzzi, 1991).

Rommens *et al.* (2008) developed potato RNAi lines using a construct that contained a 0.4-kb DNA fragment from *StAS1* linked to a second 0.4-kb DNA fragment from *StAS2*. The construct had promoters for expression in the forward and reverse directions that were highly expressed in tubers relative to leaves. The 15 transgenic lines developed from this construct showed different levels of silencing of both genes (Rommens *et al.*, 2008). Interestingly, the transgenic plants were phenotypically normal when grown in a greenhouse but produced small, deformed and few tubers in the field (Chawla *et al.*, 2012). Separate silencing of *StAS1* and *StAS2* revealed that the development defects were caused by the down-regulation of *StAS2,* but not *StAS1* in tubers (Chawla *et al.*, 2012). We developed 127 RNAi lines using a construct containing a single 311-bp DNA fragment derived from *StAS1*. Seven RNAi lines with different levels of silencing of both *StAS1* and *StAS2* were evaluated in greenhouse and replicated field trials in 2013 and 2014. We observed no abnormal phenotypes with any of the RNAi lines when grown in a greenhouse, which agreed with the previous reports (Chawla *et al.*, 2012; Rommens *et al.*, 2008). Surprisingly, similar normal phenotypes were associated with all seven RNAi lines in the 2013 field trial when they were planted as transplants (Figure S1). By contrast, lines with the greatest extent of silencing, especially triRBK21 and triRBK28, were stunted and chlorotic in the 2014 field trial when they were planted as seed tubers (Figure S2a). Plants of these two lines produced shorter tubers and had a reduced tuber number compared to the control (Table 1), which resulted in the yield reduction (Figure S1).

These results suggest that *StAS1*/*StAS2* may play a role in response to abiotic stresses experienced during the early stages of growth that are absent or reduced when grown in a greenhouse. The function of *StAS1*/*StAS2* may be particularly important during early plant development, because transplants of the RNAi lines grew normally in the 2013 field trial (Figure S1). It has been well documented that the expression of asparagine synthetase genes is regulated by light (Herrera-Rodriguez *et al.*, 2002; Lam *et al.*, 1998; Wong *et al.*, 2004), and the light intensity is much greater and more variable under field conditions compared with greenhouse conditions. Our results do not support an essential role for wild-type expression of *StAS1* and *StAS2* in normal tuber development. Although field-grown triRBK21 and triRBK28 plants were stunted and chlorotic, tubers from these plants developed normally (Figure S1). In contrast, Chawla *et al.* (2012) showed that *StAS1*/*StAS2* double silencing lines produced small and cracked tubers with secondary growth. The different phenotypes observed from the *StAS1*/*StAS2* silencing lines by the two laboratories could be due to different potato cultivars used, different silencing constructs and/or different field growing conditions. Tuber asparagine contents of highly silenced lines described here were higher than the lowest amounts reported by

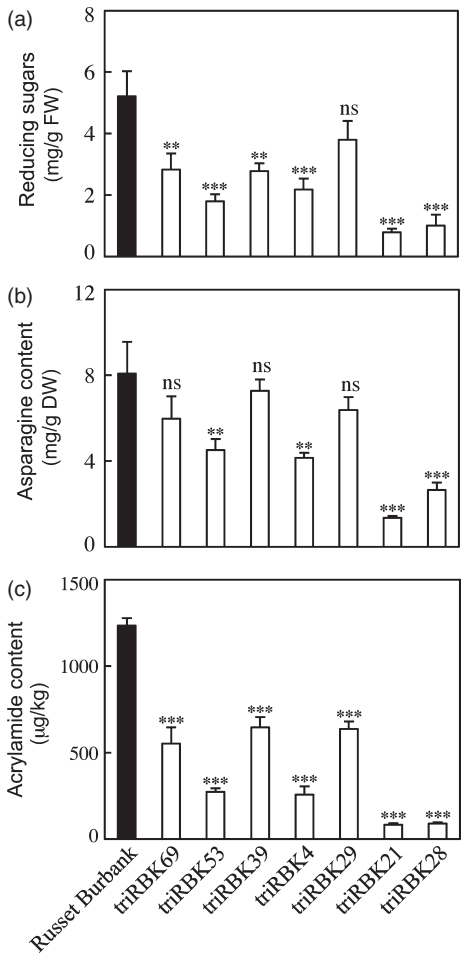

Figure 4 Tuber reducing sugar content (a), asparagine content (b) and acrylamide content of fried strips (c) of stem-end tissues of seven RNAi lines and Russet Burbank control harvested from a replicated field trial in 2013. Tissues were collected from tubers 3 months after harvest before (a, b) and after frying (c). Bars represent mean ± standard error of five independent tuber samples. Differences in means between the RNAi lines and the Russet Burbank control are indicated as not statistically different (ns) or different at $P < 0.01$ (**), or $P < 0.001$ (***) level.

Rommens *et al.* (2008), but comparable to those in *StAS1*-silenced lines reported by Chawla *et al.* (2012).

Targeted breeding to minimize potato acrylamide formation potential

Various strategies have been developed or proposed to reduce the acrylamide content in processed potato products (Bethke and Bussan, 2013; Gokmen and Palazoglu, 2008; Palazoglu *et al.*, 2010). Minimizing the amounts of reducing sugars and/or asparagine in raw potato tubers has significant advantages over changes in processing practices implemented for acrylamide reduction, because alterations in processing practices can be cost-prohibitive and may affect the flavour/quality of the final products. Silencing of either *Vlnv* or *StAS1* can significantly reduce the acrylamide content in French fries or potato chips (Bhaskar *et al.*, 2010; Chawla *et al.*, 2012; Rommens *et al.*, 2008; Wu *et al.*, 2011; Ye *et al.*, 2010; Zhu *et al.*, 2014). Except for that presented here, we are unaware of data that demonstrate the relative impact of these two precursors on acrylamide-forming potential in a single potato cultivar when both are strongly reduced. We have produced *Vlnv*-single silencing lines (Zhu *et al.*, 2014) and *Vlnv*/*StAS1*/*StAS2*-triple silencing lines from the same potato cultivar Russet Burbank. We applied the same procedure to evaluate all RNAi lines. The French fries processed from the best single silencing line (RBK1) contained 88 μg/kg of acrylamide at a position corresponding to the tuber stem end (Zhu *et al.*, 2014), which is comparable to 84 and 89 μg/kg from the two best triple silencing lines, triRBK21 and triRBK28. *Vlnv* expression was suppressed by a similar amount in the best single and triple silencing lines. Therefore, suppression of the *StAS1*/*StAS2* in Russet Burbank seems to have had little effect on acrylamide-forming potential in a *Vlnv*-suppressed genetic background. This result is consistent with the findings of Muttucumaru *et al.* (2014) that variation in fructose made the largest contribution to differences in acrylamide content, but variation in free asparagine contributed little (Muttucumaru *et al.*, 2014). Thus, an acrylamide mitigation strategy focused on developing potato cultivars with low reducing sugars will likely be the most effective approach for minimizing the acrylamide-forming potential of French fry processing potatoes. However, it is less clear whether this variety development strategy also applies to chipping potatoes as these already have very low tuber reducing sugar contents, and *Vlnv* expression during postharvest storage may differ substantially from that in Russet Burbank (Wiberley-Bradford *et al.*, 2014). Wild potato germplasm with extremely high level of CIS resistance is available (Hamernik *et al.*, 2009). Excitingly, transcription of *Vlnv* in germplasm derived from wild species *Solanum raphanifolium* was as low as the best *Vlnv*-silencing lines during cold storage at 4 °C (Bhaskar *et al.*, 2010). These germplasm stocks either possess allele(s) of *Vlnv* that are weakly expressed, or a strong suppressor(s) of *Vlnv*, which can potentially be introgressed into cultivated potato.

Experimental procedures

Development of RNAi construct for triple silencing of *Vlnv*, *StAS1* and *StAS2*

A 311-bp fragment of the *StAS1* (coordinates 328–638 of CK278037) was amplified using primers F: 5′-CACCAGGACTG GAAGTGATTGTGATG-3′ and R: 5′-ATTGGATCCGAGAACCAAG CGGGATTGTA-3′. Similarly, a 311-bp DNA fragment from the *Vlnv* (coordinates 1498–1808 of XM_006366103) was amplified using primers F: 5′-TAAGGATCCAGAGGCATTTTGGGACCATT-3′ and R: 5′-GCTCCATTCACTGCCTTTGT-3′. These two DNA fragments were amplified from a tuber mRNA-derived cDNA library of the potato variety Russet Burbank, were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, California) and linked with restriction enzyme *Bam*HI-HF (GGATCC, New England BioLabs, Ipswich, Massachusetts). The DNA segment was cloned into pENTR/D directional TOPO cloning vector (Invitrogen, Carlsbad, California) and then transferred into the pHellsGate8 vector with a constitutive CaMV 35S promoter. The DNA segment recombined in sense and antisense orientations was confirmed by restriction digestion (*Xho*I and *Xba*I) and sequencing of inserts.

Plant transformation and confirmation of silencing of *Vlnv*, *StAS1* and *StAS2*

Plant transformation was performed using methods described previously (Bhaskar *et al.*, 2008). The transgenic plants were first screened using the kanamycin resistance gene marker and confirmed by PCR using primers F: 5′-GGATGGAGTGTTCTC TTTTGTATTA-3′ and R: 5′-TTAGAAATGTAGAAGTAAACTGGCG

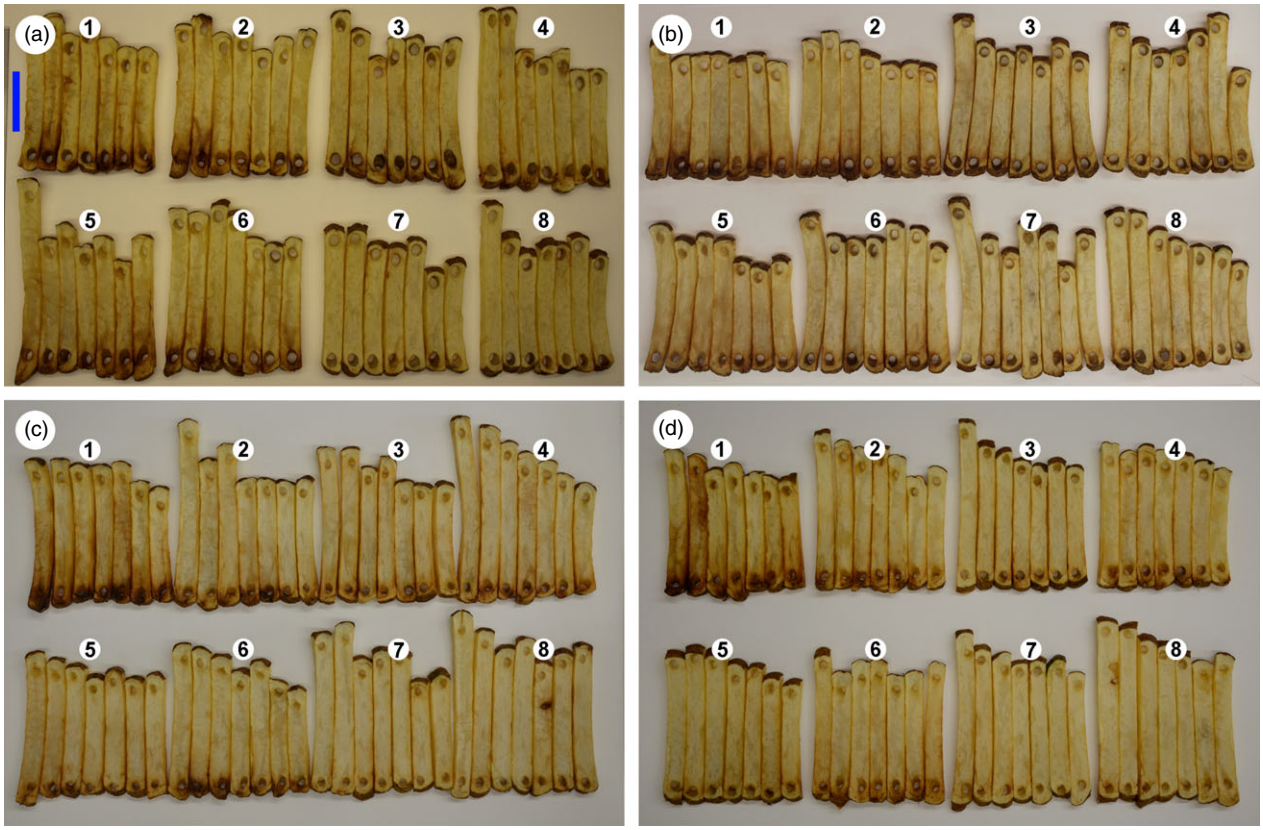

Figure 5 Fried potato strips processed from seven RNAi lines and Russet Burbank control harvested from a replicated field trial in 2013. Fried strips were prepared from tubers at harvest (a) and 1 (b), 3 (c) and 6 (d) months after harvest. Harvested tubers were stored for 50 days at 13 °C and cooled over 24 days to a final storage temperature of 9 °C, which is a storage temperature profile typical of that used in commercial practice. Numbers represent samples from Russet Burbank (1), triRBK69 (2), triRBK53 (3), triRBK39 (4), triRBK4 (5), triRBK29 (6), triRBK21 (7) and triRBK28 (8), respectively. Slices are positioned with the tuber stem-end portion nearest to the bottom of each photograph. Note: triRBK21 (7) and triRBK28 (8) are free of sugar-end defects associated with the stem ends after long-term storage. The vertical blue bar in (a) represents 5 cm.

-3′, which amplifies a 336-bp DNA fragment spanning the *StAS1* and *VInv* gene fragments within the construct.

Methods of quantitative real-time PCR (qRT-PCR) for gene expression analysis were according to published protocols (Zhu *et al.*, 2014). Expression of *VInv*, *StAS1* and *StAS2* relative to the reference gene *Actin97* was quantified by qRT-PCR using the SYBR Advantage qPCR Premix (Clontech, Mountain View, California) on MJ Research Opticon 2 (Bio-Rad Laboratories, Hercules, California). qRT-PCR primers for the *Actin97* and *VInv* were described previously (Zhu *et al.*, 2014). PCR primers for the *StAS1* were F: 5′-TCCTTCCACTCCTTATGACACTTTG-3′ and R: 5′-TGAAGTTGTGCT CCCCATTGC-3′ (amplicon size, 189 bp), and primers for the *StAS2* were F: 5′-CATTGGCATTACACCCCTTTAT-3′ and R: 5′-AATCCAG TCCGCCTGAGAGA-3′ (amplicon size, 296 bp).

Twenty independent RNAi lines were selected for further tuber-based studies based on the low expression levels of the three genes in leaf tissues of clones relative to that in leaf tissues of wild-type Russet Burbank. Tubers were harvested from plants that had senesced naturally and were stored at 4 °C for 14 days. The methods for collection of tuber tissues, RNA extraction, DNase treatment and reverse transcription of total tuber RNA were described previously (Zhu *et al.*, 2014). All data for qRT-PCR, acid invertase activity, sugar contents, asparagine and acrylamide contents were calculated using the Statistical Analysis System version 9.1 (SAS v9.1) (SAS Institute Inc, Cary, NC). Analyses of variance (ANOVA) were carried out using PROC GLM.

Field trial and postharvest storage

Based on relative expression of the *VInv*, *StAS1* and *StAS2* in leaves and in cold-stored tubers (14 day storage at 4 °C) from greenhouse-grown plants, seven RNAi lines covering a wide range of silencing of the three genes in tubers were selected for in-depth analyses, including triRBK4, triRBK21, triRBK28, triRBK29, triRBK39, triRBK53 and triRBK69. In the 2013 field trial, 3-week-old *in vitro* tissue culture plants of the RNAi lines and of Russet Burbank controls were grown in Metromix 360 (Sun Gro Horticulture, Vancouver, BC, Canada) in a greenhouse for 4 weeks and then transplanted to research plots at the Hancock Agricultural Research Station (HARS), Wisconsin, in three replicates for each line and eight plants for each replicate. After harvest, the 28 largest tubers from each line were divided into four groups that were assigned to each of four time points (at harvest and after 1, 3 and 6 months of storage). Harvested tubers were stored in the Hancock Storage Research Facility using a storage protocol similar to that used commercially. Tubers were stored initially at 13 °C for 50 days for wound healing and preconditioning. The storage temperature was decreased gradually from 13 to 9 °C at a rate of 0.1 °C per 8 h, which took a total of 24 days. Tubers were then held at

9 °C, a temperature typical for commercial storage of Russet Burbank. In the 2014 field trial, seed tubers harvested from greenhouse-grown plants were planted on 27 May 2014 at HARS, Wisconsin, in three replicates with 6–8 seed tubers in each plot. Research plots were managed using standard cultivation practices of potato.

Molecular, biochemical and fried product evaluation

At each of the four time points, at harvest and after 1, 3 and 6 months of storage (13–9 °C), tuber tissues were sampled from the bud and stem ends of seven tubers of the seven RNAi lines and Russet Burbank control as described previously (Zhu *et al.*, 2014). Gene expression was quantified by qRT-PCR as described above. Enzyme activity assay for acid invertase and high-performance liquid chromatography (HPLC) analysis for sugar content used the methods described previously (Bhaskar *et al.*, 2010; Zhu *et al.*, 2014). Fresh tuber tissues (~5 g from each tuber) were taken from stem end of five randomly selected tubers of the seven RNAi lines and Russet Burbank control for asparagine quantification using HPLC. The gas chromatography/mass spectrometry (GC/MS) method described previously (Bhaskar *et al.*, 2010; Zhu *et al.*, 2014) was used to quantify stem-end acrylamide in five fried strips, which corresponded one-to-one with those analysed for asparagine content.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Phenotypes of seven RNAi lines and Russet Burbank control harvested from a replicated field trial in 2013.

Figure S2 Leaf and tuber phenotypes of seven RNAi lines and Russet Burbank control harvested from a replicated field trial in 2014.

Table S1 Expressions of *VInv*, *StAS1* and *StAS2* in bud end and stem end of tubers from seven RNAi lines and Russet Burbank control harvested from a replicated field trial in 2013.