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Received: 8 November 2021 | Accepted: 13 April 2022

DOI: 10.1002/csc2.20760

Crop Science

ORIGINAL RESEARCH ARTICLE

Crop Breeding & Genetics

Induced mutations in *ASPARAGINE SYNTHETASE-A2* reduce free asparagine concentration in the wheat grain

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Assigned to Associate Editor Owen Hoekenga.

Abstract

Acrylamide is a neurotoxin and probable carcinogen formed as a processing contaminant during baking and production of different foodstuffs, including bread products. The amino acid asparagine is the limiting substrate in the Maillard reaction that produces acrylamide, so developing wheat (*Triticum aestivum* L.) cultivars with low free asparagine concentrations in the grain is a promising approach to reduce dietary acrylamide exposure. A candidate gene approach was used to identify chemically induced genetic variation in *ASPARAGINE SYNTHETASE 2* (*ASN2*) genes that exhibit a grain-specific expression profile. In field trials, durum and common wheat lines carrying *asn-a2* null alleles exhibited reductions in free asparagine concentration in their grains of between 9 and 34% compared with wildtype sister lines. These plants showed no significant differences in spikelet number, grain size and weight, germination or baking quality traits. These nontransgenic variants can be deployed without regulatory oversight in elite wheat germplasm to reduce acrylamide-forming potential with no negative effects on quality or agronomic performance.

1 | INTRODUCTION

Acrylamide is a potent neurotoxin and causes cancer in rodents (Beland et al., 2013). For humans, acrylamide is classified as a probable carcinogen by the International Agency for Research on Cancer (IARC, 1994) and mutational signatures attributed to acrylamide exposure were detected in approximately one-third of assayed tumor genomes, including 19 different tumor tissues (Zhivagui et al., 2019). There is a strong and growing incentive to reduce potential sources of acrylamide exposure in humans, and regulatory agencies are beginning to specify limits in recognition of this threat (reviewed in Raffan & Halford, 2019).

A major source of acrylamide exposure is the consumption of processed foods that are rich in carbohydrates (Tareke et al., 2002). Acrylamide levels are highest in potatoes (*Solanaceae tuberosum*) and coffee (*Coffea arabica* L.), but wheat (*Triticum aestivum* L.) is also a major source of dietary acrylamide due to the high volume of bread products consumed in the human diet (Raffan & Halford, 2019). Acrylamide is a processing contaminant that accumulates in the high temperature and low moisture conditions during baking as a product of the Maillard reaction (Mottram et al., 2002; Stadler et al., 2002). Modifying production conditions such as baking at lower temperatures or adding chemical amendments

Abbreviations: ASN, asparagine synthetase; CRISPR/Cas, clustered regularly interspaced short palindromic repeats/CRISPR-associated protein; CSU, Colorado State University; EMS, ethyl methanesulfonate; IS, internal standard; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction; QTL, quantitative trait locus; SRC, solvent retention capacity; TILLING, targeting induced local lesions in genomes.

can reduce the level of acrylamide formation, but these are often impractical to implement (Claus et al., 2008). Because asparagine provides the carbon skeleton on which acrylamide is formed, free asparagine concentration is the limiting substrate for acrylamide formation (Surdyk et al., 2004) and, in wheat, free asparagine concentrations in the grain are highly correlated with acrylamide levels in baked products (R^2 > .99; Curtis et al., 2014). Therefore, one promising approach to reduce dietary exposure to acrylamide is to develop wheat cultivars with low free asparagine concentration in grains.

Free asparagine concentration is strongly influenced by environmental factors. Providing nitrogen (N) is abundant, asparagine accumulates in the grain when protein synthesis is limited, such as in response to biotic and abiotic stresses, including sulfur (S) deficiency (Lea et al., 2007). Therefore, growers can minimize free asparagine concentrations by keeping crops free from pathogens and ensuring sufficient S supply in the soil (Claus et al., 2006; Curtis et al., 2019; Wilson et al., 2020). The estimated heritability of free asparagine concentration ranges between .13 to .65 in different field studies and exhibits approximately three- to five-fold variation within different panels of wheat germplasm (Corol et al., 2016; Emebiri, 2014; Rapp et al., 2018). Free asparagine concentration is a costly trait to phenotype, so characterizing the genetic basis of this variation would allow breeders to apply marker assisted or genomic selection to lower free asparagine concentration. However, the trait is highly quantitative with a complex genetic architecture influenced by multiple small-effect quantitative trait loci (QTL; Emebiri, 2014; Navrotskyi et al., 2018; Rapp et al., 2018). Few of these QTL overlap between studies, likely a factor of the strong influence of genotype × environment interactions.

A complementary strategy to reduce free asparagine concentrations is to leverage knowledge of asparagine biosynthesis to target candidate genes. In eukaryotes, asparagine synthetase enzymes catalyze the adenosine triphosphate (ATP)-dependent assimilation of inorganic N in the form of ammonium into asparagine (Gaufichon et al., 2010). The wheat genome contains three homeologous copies of five asparagine synthetase genes that exhibit distinct expression profiles during development (Curtis et al., 2019; Oddy et al., 2021; Xu et al., 2017). The *TaASN2* genes are notable for their grain-specific expression profile, and a natural deletion of *TaASN-B2* is associated with a mild reduction in free asparagine concentrations in the grain (Oddy et al., 2021). By contrast, the *TaASN-A2* and *TaASN-D2* homeologous genes show higher transcript levels than *TaASN-B2* but exhibit no natural genetic variation among a collection of 14 wheat cultivars (Oddy et al., 2021). In addition, no QTL for asparagine concentration has been mapped within the vicinity of an asparagine synthetase gene (Emebiri, 2014; Rapp et al., 2018). Plants carrying clustered regularly interspaced short palindromic repeats/CRISPR-associated protein (CRISPR/Cas)-induced knockouts of all three *TaASN2* homeologs exhibit reductions in free asparagine concentrations of up to 90% (Raffan et al., 2021). In greenhouse trials, these plants showed no obvious reduction in performance except a lower germination rate (Raffan et al., 2021). However, field trials are often necessary to fully characterize the effect of a genetic variant. For example, potato lines carrying RNA interference (RNAi) constructs to suppress *StASN1* and *StASN2* transcripts exhibited significant reductions in tuber acrylamide levels compared with wildtype control lines in the greenhouse (Rommens et al., 2008), but subsequent field trials revealed these tubers were smaller and had a cracked appearance (Chawla et al., 2012). A more targeted approach using an RNAi construct specific to *StASN1* controlled by a tuber-specific promoter had a similar effect in reducing tuber asparagine concentrations without affecting tuber yield in field-grown plants (Chawla et al., 2012).

For wheat, it will be important to evaluate the association between asparagine concentration and quality traits (Claus et al., 2006). Asparagine concentration showed a mild negative correlation with sedimentation volume (Z-sodium dodecyl sulfate; Corol et al., 2016; Rapp et al., 2018), a measure of the gluten quantity and strength in a flour sample. In a panel of European wheats, free asparagine concentration was also negatively correlated with 1,000-grain weight and hectoliter volume, and positively correlated with protein content (Rapp et al., 2018). A range of quality tests have now been optimized to more efficiently assay quality traits from small volumes of wheat grain. The Single Kernel Characterization System (SKCS) measures grain hardness, moisture, diameter, and weight (Gaines et al., 1996), the solvent retention capacity (SRC) assay is an indicator of gluten strength, gliadins, pentosans, and starch damage (Bettge et al., 2002), whereas mixograph analyses provide an indication of gluten strength and dough rheological properties (Bloksma & Bushuk, 1988).

In the current study, three backcross populations segregating for ethyl-methanesulfonate (EMS)-induced null alleles of *ASN-A2* were developed in durum and common wheat. In field trials, mutant lines exhibited significant reductions in free asparagine concentrations in their grain compared with

Core Ideas

- Three wheat *ASPARAGINE SYNTHETASE 2* knockout alleles were characterized in field experiments.
- Mutant alleles conferred significant reductions in grain free asparagine concentration.
- The alleles did not affect quality or agronomic traits.

wildtype sister lines but showed no significant changes in agronomic or quality traits. These nontransgenic *asn-a2* null alleles can be utilized without regulatory oversight in breeding programs to help develop wheat cultivars with reduced acrylamide-forming potential.

2 | MATERIALS AND METHODS

2.1 | Plant materials

Ethyl-methanesulfonate-mutagenized M_4 lines carrying null alleles in *ASN-A2* from the common wheat cultivar 'Cadenza' (T6-1048) and the durum wheat [*Triticum turgidum* L. subsp. *durum* (Desf.) Husn.] cultivar 'Kronos' (T4-2032 and T4-1388) were identified from an in silico targeting induced local lesions in genomes (TILLING) database (Krasileva et al., 2017) and provided by Dr. Jorge Dubcovsky, University of California-Davis. Each line was backcrossed twice to nonmutagenized wildtype plants of the corresponding cultivar (Kronos for lines T4-2032 and T4-1388; Cadenza for line T6-1048) to reduce the effect of background mutations. In 2019, selections were made of BC_1F_2 homozygous wildtype and *asn-a2* sister lines to grow in field experiments. In 2020, similar selections were made of homozygous wildtype and *asn-a2* sister lines using BC_2F_2 materials. These sister lines were grown in greenhouse conditions for quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) experiments to quantify *ASN2* transcript levels.

Seeds of the winter wheat cultivars 'Antero' PI 667743 (Haley et al., 2014), 'Hatcher' PI 638512 (Haley et al., 2005), 'Snowmass' PI 658597 (Haley et al., 2011), 'Snowmass 2.0' PVP 201900411, 'Brawl CL Plus' PI 664255 (Haley, Johnson, Westra et al., 2012), 'Byrd' PI 664257 (Haley, Johnson, Peairs, Stromberger, Hudson, Seifert, Kottke, Valdez, Rudolf, Bai et al., 2012), 'Denali' PI 664256 (Haley, Johnson, Peairs, Stromberger, Hudson, Seifert, Kottke, Valdez, Rudolf, Martin et al., 2012), 'SY Wolf', 'Winterhawk' PI 652927, and 'Canvas' PVP 201900408 were sown in field experiments in 2017 and 2018 and were sourced from the Colorado State University (CSU) wheat breeding program from Dr. Scott Haley.

2.2 | Field experiments

In the 2016–2017 field season, free asparagine concentration was measured in the grains of eight elite winter wheat cultivars grown in the CSU wheat breeding program's elite cultivar trial (Antero, Hatcher, Snowmass, Brawl, Byrd, Denali, SY Wolf, and Winterhawk). Grains were harvested as single replicates from 3.7- by 1.5-m) field plots in five trial locations (Fort Collins 40.653086, −104.999863; Akron 40.14858333, −103.1395472; Burlington 39.2999083, −102.2983472; Julesburg 40.8996, −102.2293278; Yuma 40.18595, −102.66099). In the 2017–2018 field season, asparagine concentration was measured from the grains of single replicates of four elite winter wheat cultivars (Snowmass, Snowmass 2.0, Byrd, and Canvas) from seven locations (Fort Collins; Akron; Julesburg; Orchard 40.479692, −104.071244; New Raymer 40.56793056, −103.8940111; Yuma; and Roggen 40.088177, −104.261670).

In the 2019 field season, $BC_1F_{2:3}$ sister lines derived from a single BC_1F_2 plant and homozygous for wildtype and mutant alleles of T4-2032, T4-1388, and T6-1048 were sown on 22 Mar. 2019 in Fort Collins as two-row plots 0.92-m long and with 23-cm spacing between rows using a randomized complete block design. Six genotypes were placed randomly within each block with five replications. Due to the loss of some samples some genotypes had fewer replicates. Free asparagine concentration was measured from the grains of these lines. This experiment was repeated in the 2020 field season in the same location using a randomized complete block design with six genotypes and six replications using $BC_2F_{2:3}$ sister lines derived from a single BC_2F_2 plant. Plants were sown on 25 Mar. 2020 and were phenotyped for a broader range of traits, including free asparagine concentration in grain; mean kernel weight (mg); spikelets per spike; germination rate 5, 7, and 14 days after sowing; seed diameter (mm); hardness index; protein concentration in grain; grain moisture (%); and solvent retention capacity of the whole grain meal.

2.3 | Phenotyping

Spikelet number was measured as the average number from 10 randomly selected spikes from each headrow. Germination tests were carried out using 50 seeds from each of the 36 headrows from the field experiment from field season 2019–2020. Seeds from each headrow were placed in two pots filled with compost from 24 cell seed inserts (25 seeds each) organized in five lines of five seeds each. Pots were placed in standard 1,020-seeds trays in the greenhouse with these conditions: 19.4 ˚C average day temperature, 18.3 ˚C average night temperature, 40% relative humidity, and 16-h daylength. Emerged seedlings were counted in each pot 5, 7, and 14 days after sowing.

Quality evaluations were run at CSU's wheat quality laboratory using approved methods from the American Association of Cereal Chemists (AACC, 2000). The Single Kernel Characterization System (SKCS 4100; Perten Instruments) was used to measure mean kernel weight (mg), diameter (mm), and hardness index.

Protein concentration was measured by near-infrared spectroscopy (NIRS) on whole-grain and wheat-meal samples using a Foss NIRSystems model DA1650 (Foss North

America). A validation set of 25 samples were analyzed using the combustion method and used to adjust NIR calibrations if necessary (Leco Model FP-428; Approved Method 46-30). Samples of grain were ground on a UDY mill (UDY Corporation, Fort Collins, CO) equipped with a 0.5-mm sieve. Grain protein concentrations are presented as 12% moisture basis, and whole grain meal protein concentrations on a 14% moisture basis. Dough-mixing properties were assayed using a 10-g mixograph according to AACC Method 54-40A, which was optimized for water absorption based on whole-grain meal protein concentration obtained from the NIRS according to the equation:$\text{Predictedabsorption} = 4.27 + (1.69\text{protein})$, where protein represents the whole grain meal protein concentration (obtained with NIRS) at 140 g kg^{-1} moisture basis. The prediction equation was determined from experiments with the hard red winter wheat cultivar 'Cheyenne' (PI 553248; B. Seabourn, personal communication, 2000).

Dough-mixing properties were determined using the computerized mixograph and MIXSMART software (v. 1.0.484 for Windows, National Manufacturing). The following computerized mixograph parameters were used in the analyses: midline peak time, midline peak height, midline peak width, midline right slope, and midline right width (the midline curve width 2 min after peak).

Solvent retention capacity analysis was performed using whole wheat grain meal for water, sucrose, lactic acid, and carbonate solvents, using a method described previously (Bettge et al., 2002).

2.4 | Asparagine quantification

2.4.1 | Sample preparation

Following harvest, 2–3 g of seeds from each plot were milled using a hand mill and 95–105 mg of whole grain meal were placed in 2 ml Eppendorf tubes for asparagine quantification. Homogenized whole grain meal samples (~100 mg) were accurately weighed and mixed with 1 ml of cold 80% methanol in water. Fifty micrograms of isotopically labeled 13C4 L-asparagine (99%, Cambridge Isotope Laboratories) as internal standard (IS) were added from 1 mg ml^{-1} stock solution to each sample. Samples were vigorously vortexed at 4 ˚C for 1 h, and then sonicated for 30 min before an additional 1-h vortexing at 4 ˚C followed by centrifugation at 3,000 rpm for 15 min at 4 ˚C. Seven-hundred fifty microliters of supernatant were recovered, and solvent was removed under N. To the dried extract, 600 µl of methyl-tert-butyl ether (MTBE), 300 µl of methanol, and 100 µl of water were added. Samples were vigorously vortexed for 1 min, and then 500 µl of water were added to induce phase separation. The upper organic layer containing unwanted lipids was removed and discarded. To ensure all lipids were removed, another 1 ml of MTBE was added to the samples, vortexed for 1 min, centrifuged again at 3,000 rpm for 10 min, and the upper organic layer was again discarded. The solvent in lower layer was removed under N. The dried samples were resuspended in 300 µl of water before the addition of 700 µl of acetonitrile and vortexed for 1 min and then incubated at 4 ˚C for 1 h before centrifugation at 17,000 g and 4 ˚C. Nine hundred microliters of the upper supernatant were recovered, dried under N gas, resuspended in 50 µl of 50% (v/v) methanol in water and stored at −20 ˚C until analysis. Samples for a calibration curve for quantification were prepared in 50% (v/v) methanol in water at concentrations ranging from 0 to 200 µg ml^{-1} with IS and were analyzed in triplicates.

TABLE 1 Ions used for quantification

	Quantification $[M+H]^+$	Confirmation	Confirmation
	m/z		
Asparagine	133.02	87.05	74.02
Internal standard	137.03	90.06	76.03

2.4.2 | Liquid Chromatography-Mass Spectrometry Analysis

Each sample (2 µl) was injected onto a Waters Acquity Ultra-performance liquid chromatography (UPLC) system in a randomized order with a pooled quality control (QC) injection after every six sample injections and separated using a Waters Acquity UPLC ethylene bridged hybrid Amide column (1.7 µM, 2.1 by 30 mm) at a constant flow rate of 0.4 µl min^{-1}. The elution solvents were A, acetonitrile with 0.1% (v/v) formic acid; B, water with 10 mM ammonium formate and 0.1% formic acid. The solvent gradient started with 0.1% B at 0 min, increased to 80% B at 1.5 min, held at 80% B until 2.0 min, reequilibrated to 0.1% B at 2.25 min, held at 0.1% B for 2.5 min. The total run time was 4.75 min. The column and samples were held at 45 and 4 ˚C, respectively. The column eluent was infused into a Waters Xevo G2 TOF-MS with an electrospray source in positive mass spectrometry (MS) fullscan mode with target enhancement at 133 m/z. The dwell time was 0.2 s. The collision energy was set at 0. Calibration was performed using sodium iodide with 0.001 mg ml^{-1} mass accuracy. The capillary voltage was held at 2,200 V, source temperature at 150 ˚C, and N desolvation temperature at 350 ˚C with a flow rate of 800 L h^{-1}. The ions used for quantification are listed in Table 1.

2.4.3 | Quality Control

Quality control (QC) samples were pooled from final extracts of all the actual samples and were injected after every six

actual samples. Mean values and standard deviations for QC can be found in the result sheet. The coefficient of variation of eight QCs was 8.4%.

2.5 | Statistical analysis

A two-way analysis of variance (ANOVA) for free asparagine concentration in elite Colorado wheat germplasm was performed using R Studio (2022.02.0, Build 443) using genotype and environment as independent variables. Tukey's Honestly Significant Difference post-hoc test was used to perform pairwise comparisons between environments and between genotypes. Environment and genotype group means were calculated and plotted using the R package 'ggplot2'. A separate one-way ANOVA contrast was performed to test the association between the *TaASN-B2* deletion and free asparagine concentration in these cultivars. Regression analysis was performed using data from elite Colorado germplasm grown in 2017–2018 to determine the correlation between free asparagine concentration and three factors: grain protein concentration, flour protein concentration, and plot yield. One-way ANOVA analyses were performed to quantify the effect of *ASN-A2* mutations on variation in agronomic and quality traits within families segregating for *asn-a2* null alleles, and two-way ANOVAs using Type III Sum of Squares were performed using the computer software JASP (JASP, 2019) to determine the effect of genotypic variation in *ASN-A2* across all families. Raw data and statistical tests from these analyses are provided in Supplemental Tables S1 and S2.

2.6 | Genotyping assays

Genomic DNA was extracted from seedling leaf tissue using the cetyl trimethylammonium bromide (CTAB) method (Murray & Thompson, 1980). Kompetitive allele-specific PCR (KASP) assays were developed to genotype the G468A mutation in line T4-1388 (Supplemental Figure S1) and the G446A mutation in line T4-2032 (Supplemental Figure S2). Primers for each assay are listed in Supplemental Table S3. Each reaction consisted of 0.14 µl of KASP primer mix, 5 µl of KASP Master mix (LGC Genomics) and 5 µl of template DNA (50 ng μl^{-1}). A 100-µL KASP primer mix stock was prepared using 46 µl of nuclease-free water, 30 µl of common primer (100 µM), 12-µl specific primer with FAM (100 µM) and 12-µl specific primer with VIC (100 µM). The KASP thermal cycling conditions were: 94 °C for 15 s; 10 cycles of: 94 °C for 15 s, 61 °C for 1 min; 26 cycles of: 94 °C for 20 s, 55 °C for 1 min; 30 °C for 1 min.

The G585A mutation in line T6-1048 was genotyped using a cleaved amplified polymorphic sequences (CAPS) marker (Supplemental Figure S3). Primers ASN-A2_1048_F3 and ASN-A2_1048_R3 (Supplemental Table S3) were used to amplify a 1,063-bp product using the conditions 95 °C for 30 s; 35 cycles of: 95 °C for 15 s, 53 °C for 30 s, 68 °C for 1 min; 68 °C for 5 min. Each reaction consisted of 2.5 µl of 10X Standard *Taq* Reaction Buffer (NEB), 0.5 µl 10-mM dNTPs (Invitrogen, Life Technologies), 0.5 µl of 10-µM Forward Primer, 0.5 µl of 10-µM Reverse Primer, 5 µl of Template DNA (50 ng μl^{-1}), 0.125 µl of *Taq* DNA Polymerase (NEB), and nuclease-free water to a total reaction volume of 25 µl.

Amplified DNA was digested at 37 °C for 2 h with the restriction enzyme *Sty*I-HF (NEB) and products were run on a 0.8% (w/v) agarose gel stained with Invitrogen SYBR Safe DNA Gel Stain (Life Technologies). Template DNA carrying the mutant allele (A at nucleotide 585) showed a 1,063-bp band, whereas the wildtype allele (G at nucleotide 585) showed two fragments of 946 and 117 bp (Supplemental Figure S3). Heterozygous individuals had a characteristic banding pattern of 1,063, 946, and 117 bp. The presence and absence of *ASN-B2* was determined using an assay described previously (Oddy et al., 2021).

2.7 | qRT-PCR

Developing grains from six biological replicates of each genotype were harvested at two time points (21- and 28-d post anthesis) and immediately frozen in liquid N. Grains were ground to a fine powder using a pestle and mortar and RNA was extracted using a standardized extraction protocol modified from the cetyl trimethylammonium bromide (CTAB) method (Chang et al., 1993). RNA was quantified using a NanoDropTM 1000 Spectrophotometer (Thermo Fisher Scientific). To remove genomic DNA contamination, 1 µg RNA was treated with SuperScriptTM IV VILO ezDNase Enzyme (Thermo Fisher Scientific) in a 10-µl solution and incubated for 4 min at 37 °C. The DNase-treated RNA samples were quantified using a NanoDropTM 1000 Spectrophotometer before cDNA synthesis. cDNA was synthesized by mixing the ezDNase enzyme treated RNA samples in nuclease-free water with SuperScriptTM IV VILO Master Mix (Invitrogen), according to the manufacturer's instructions, and placing in a thermocycler using the following program: 25 °C for 10 min; 50 °C for 30 min; 85 °C for 5 min. Samples were diluted to 10 ng μl^{-1} according to the RNA-equivalent of starting DNase-treated sample before synthesis.

Quantitative reverse transcriptase-polymerase chain reaction was performed using the QuantStudioTM 3 System (Applied Biosystems) set to standard curve mode in 96-well 0.1-ml block. Each reaction contained 10-µl SYBR Green Master Mix (Applied Biosystems), 1 µL of forward and reverse primer (10 mM) and 5 µl cDNA (10 ng μl^{-1}). The expression of each target gene was measured as $2\exp(\Delta CT)$

relative to *ACTIN*. Primers for *ASN-A2*, *ASN-B2*, and *ASN-D2* were described previously (Oddy et al., 2021). The qRT-PCR primers for *ASN-A2* amplify a region of the gene in exon 1, upstream of all the induced EMS mutations used in the materials of this study.

Graphs were plotted in R using ggplot2 and student t-tests were performed to calculate statistical significance of pairwise differences in expression between genotypes.

3 | RESULTS

3.1 | Characterization of *asn-a2* null alleles

Two EMS-mutagenized lines in tetraploid Kronos (T4-1388 and T4-2032) and one in hexaploid Cadenza genetic backgrounds (T6-1048) were identified that carry point mutations predicted to introduce premature stop codons in the *ASN-A2* coding region (Figure 1a). All three lines are predicted to encode C-terminally truncated ASN-A2 proteins lacking the entire ASN-synthetase domain (Figure 1b), so are highly likely to encode nonfunctional proteins. Both Kronos and Cadenza carry a complete *ASN-B2* gene (Supplemental Figure S4), so T4-1388 and T4-2032 mutant plants carry one functional *ASN2* gene (*TdASN-B2*) and T6-1048 mutant plants carry two functional *ASN2* genes (*TaASN-B2* and *TaASN-D2*). Segregating $BC_1F_{2:3}$ and $BC_2F_{2:3}$ populations for each of these null alleles were developed by backcrossing to the corresponding wildtype parental line, selecting mutant alleles using genotyping assays described in Supplemental Figures S1, S2, and S3.

In wildtype plants of all three families, *ASN-A2* was the most highly expressed homeolog in grain tissues, and transcript levels rose between 21 and 28 d after anthesis (DAA; Figure 1c). Plants carrying *asn-a2* mutations exhibited significantly lower *ASN-A2* transcript levels ($P < .05$) than wildtype plants at 28 DAA in all three families (Figure 1c). By contrast, *ASN-B2* and *ASN-D2* transcript levels were not significantly different ($P > .05$) between wildtype and *asn-a2* mutant genotypes at either timepoint (Figure 1c).

3.2 | Effect of *asn-a2* mutations on free asparagine concentration

To assay the effect of the *asn-a2* mutations on free asparagine concentrations, mature grain was harvested from field-grown headrows of $BC_1F_{2:3}$ sister lines from each population as five biological replicates. Free asparagine concentration was significantly lower in *asn-a2* mutants than in wildtype sister lines in the populations derived from line T6-1048 (29% reduction, $P < .001$) and line T4-1388 (33% reduction, $P < .05$; Figure 2a). In the population derived from line T4-2032, asparagine concentration was 9% lower in the *asn-a2* mutant plants, but the difference was not significant ($P = .198$; Figure 2a). These results were consistent with BC_2F_2 populations grown in 2020 at the same location as headrows in six biological replicates (Figure 2b). Free asparagine concentration was significantly lower in *asn-a2* mutants compared with wildtype sister lines in the populations derived from line T6-1048 (28% reduction, $P < .0001$) and line T4-1388 (24% reduction, $P < .05$). In line T4-2032, although the reduction in asparagine concentration in *asn-a2* mutant plants was proportionally greater than in the other populations (34% reduction) these differences were not significant due to higher variation ($P = .071$; Figure 2b). Across both years of the experiment, genotype was highly significantly associated with free asparagine concentration ($P < .0001$).

3.3 | Effect of *asn-a2* mutations on agronomic traits

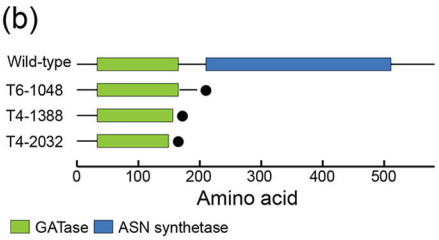
To determine the effect of the *asn-a2* mutations on plant growth and development, agronomic traits were analyzed in grains harvested from field-grown $BC_2F_{2:3}$ plants. Germination rates were above 84% in all samples and there were no significant differences between wildtype and *asn-a2* genotypes, measured 5, 7, and 14 d after sowing (Table 2). In line T6-1048, the germination rate was between 2.7 and 3% lower in *asn-a2* mutants than in wildtype sister lines, although the differences were not significant (Table 2). Conversely, germination rate was slightly higher in the *asn-a2* mutant than in wildtype sister lines in T4-1388 and T4-2032 populations (Table 2). Despite variation in spikelet number, grain weight, and diameter between Cadenza and Kronos genotypes, there were no significant differences in these traits between wildtype and *asn-a2* mutant sister plants in any of the three lines (Table 2).

3.4 | Effect of *asn-a2* mutations on quality and breadmaking traits

The effect of the *asn-a2* null alleles on quality traits were tested using the Single Kernel Characterization System (SKCS) and near-infrared protein analysis, which revealed there were no significant differences between wildtype and *asn-a2* sister lines for grain moisture, hardness, grain protein concentration, whole-grain meal protein concentration, or whole-grain meal ash concentration (Table 3). Solvent retention capacity analysis revealed no significant differences between wildtype and mutant genotypes in lactic acid, carbonate, water, or sucrose (Table 3), indicating these mutations have no effect on pentosan or gliadin content in the grains or on starch and gluten quality.

(a)

Line	Background	Mutation	Protein change
T6-1048	Cadenza	G585A	W195●
T4-1388	Kronos	G468A	W156●
T4-2032	Kronos	G446A	W149●



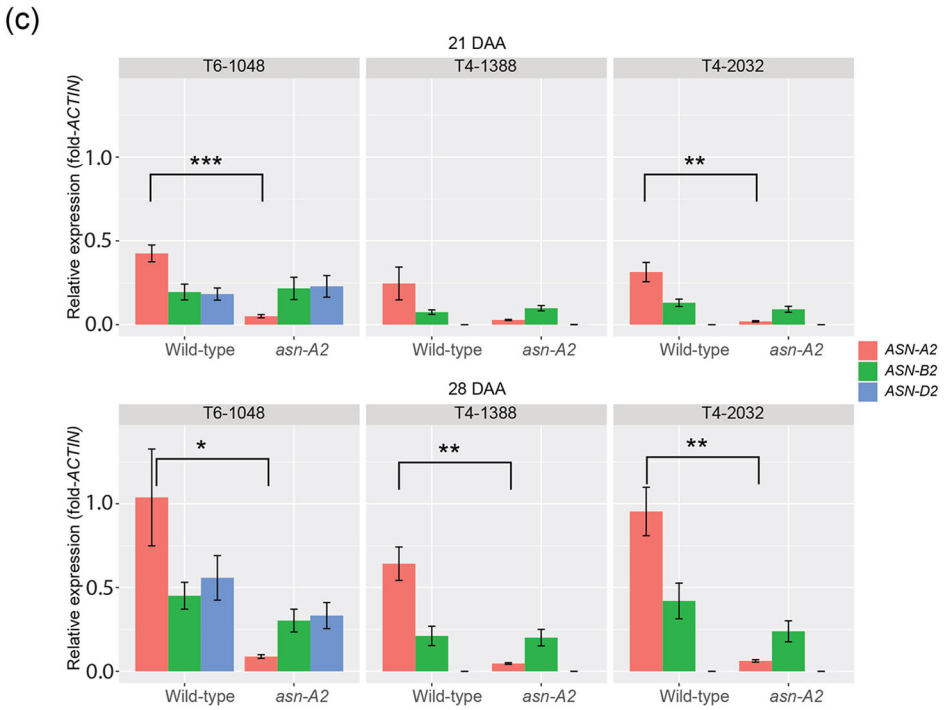


FIGURE 1 *ASN-A2* mutant characterization. (a) Details of the induced mutations in each line. Mutation position represents the mutated residue in the wildtype *ASN-A2* coding sequence. Protein mutations refer to the amino acid mutated where W = Tryptophan and ● = stop codon. (b) Schematic representation of the selected mutations in each line and their effects on protein translation. Glutamine amidotransferase (GATase) and ASN synthase conserved domains are highlighted in green and blue, respectively, and drawn to scale based on protein size. (c) Transcript levels of *ASN2* genes in grain tissues of wildtype and mutant lines at 21 and 28 d after anthesis (DAA). Error bars represent standard error of the mean ($n = 6$, except for T4-2032 wildtype samples, where $n = 5$). Student t-tests were performed for each gene between genotypes at each timepoint. *Significant at the .05 probability level. **Significant at the .01 probability level. ***Significant at the .001 probability level

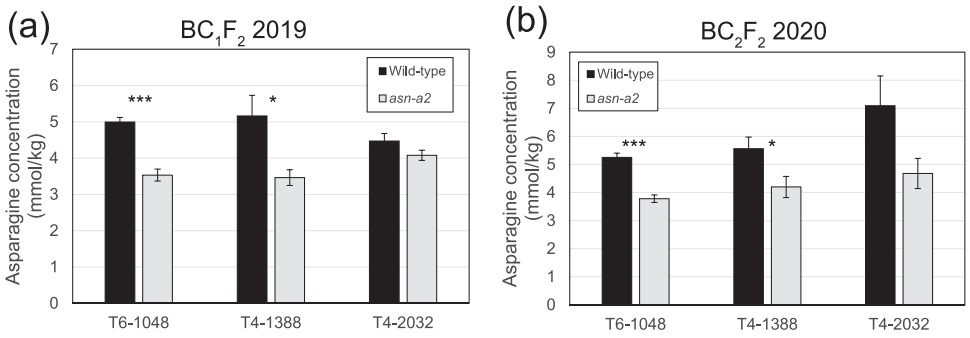


FIGURE 2 Free asparagine concentration in mature grain of wildtype and mutant *asn-a2* sister lines in (a) BC_1F_2 materials grown in 2019 and (b) BC_2F_2 materials grown in 2020. *Significant at the .05 probability level. ***Significant at the .001 probability level

TABLE 2 Effect of *asn-a2* mutations on agronomic traits in families derived from three mutant lines. Data represents the mean ± standard error for six biological replicates, except for kernel diameter in the family derived from mutant line T4-2032, where five biological replicates were used. Genotype effect represents significance of wildtype vs. *asn-a2* genotypes across all lines for each trait. Raw data and statistcal tests from these analyses are provided in Supplemental Tables S1 and S2

	T6-1048			T4-1388			T4-2032			Genotype effect
Trait	**Wildtype**	***asn-a2***	***P***	**Wildtype**	***asn-a2***	***P***	**Wildtype**	***asn-a2***	***P***	***P***
Germination 5d, %	89.0 ± 1.6	86.3 ± 1.2	.214	88.3 ± 1.7	89.3 ± 1.0	.629	84.7 ± 1.6	87.0 ± 1.6	.329	.862
Germination 7d, %	92.0 ± 1.8	89.0 ± 1.2	.198	91.0 ± 1.7	94.0 ± 1.3	.186	86.0 ± 1.8	91.0 ± 1.7	.070	.264
Germination 14d, %	92.7 ± 1.6	89.7 ± 1.1	.153	92.7 ± 1.6	94.7 ± 0.7	.277	86.3 ± 1.9	91.7 ± 1.5	.052	.315
Spikelet number	20.6 ± 0.1	20.2 ± 0.1	.131	15.1 ± 0.2	14.9 ± 0.2	.660	14.9 ± 0.3	14.9 ± 0.1	1	.301
Kernel weight, mg	24.9 ± 1.2	26.4 ± 0.7	.324	33.2 ± 0.8	32.8 ± 1.2	.772	33.8 ± 0.8	33.8 ± 0.9	.992	.667
Kernel diameter, mm	2.50 ± 0.05	2.5 ± 0.4	.439	2.8 ± 0.04	2.8 ± 0.06	.956	2.8 ± 0.04	2.83 ± 0.05	.870	.727

A mixograph was run on each sample to measure dough rheological properties and showed that *asn-a2* and wildtype sister lines exhibited no significant differences for peak mixing time, a measure of dough strength (Table 3). Peak value % was lower in *asn-a2* mutants than wildtype sister plants in all three lines, although this difference was significant only in line T4-2032 (Table 2). Right slope (% min^{-1}) values were inconsistent between lines and were slightly higher (less negative) in *asn-a2* mutants in lines T6-1048 and T4-1388, but significantly lower (more negative) in line T4-2032 (Table 3). Right-width % was not significantly different between genotypes in any line, indicating that the *asn-a2* mutations do not confer differences in mixing tolerance. Full mixographs for all lines are provided in Supplemental Figure S5.

3.5 | Free asparagine concentration in wheat cultivars adapted to the Great Plains

To better understand the opportunities to reduce acrylamide-forming potential in breeding programs, free asparagine concentration was measured in grain samples of elite winter wheat germplasm selected for their economic importance and acreage in Colorado and the Great Plains. Six winter wheat cultivars were assayed in five field locations in 2017 and four cultivars were assayed in seven locations in 2018. Free asparagine concentration was significantly positively correlated with grain protein concentration (R^2 = .39, P < .01; Supplemental Figure S6) and flour protein concentration (R^2 = .20, P < .05; Supplemental Figure S7), but was not correlated with plot yield (R^2 = .003, P = .81; Supplemental Figure S8; Supplemental Table S4).

Environment had a significant effect on free asparagine concentration (P < .001; Supplemental Tables S5 and S6), with high concentrations found in cultivars grown in Fort Collins 2016–2017 and Burlington 2016–2017, and low concentrations for cultivars grown in Julesburg in both 2016–2017 and 2017–2018 (Figure 3a). Genotype was also significantly associated with variation in free asparagine concentration (P < .01; Supplemental Table S6), with SY Wolf and Snowmass exhibiting the highest mean concentrations, and Canvas the lowest (Figure 3b). The cultivars Snowmass, Antero, and Hatcher carry the *TaASN-B2* deletion, whereas Brawl, Byrd, Denali, SY Wolf, and Winterhawk all carry a functional copy of this gene (Supplemental Figure S4). The deletion of *TaASN-B2* was not significantly associated with free asparagine concentration (P = .484; Supplemental Table S7) indicating that other genotypic differences are driving the observed variation in free asparagine concentration among these wheat cultivars.

TABLE 3 Effect of *asn-a2* mutations on quality traits in families derived from three mutant lines. Data represent the mean ± standard error for six biological replicates. Significant effects are highlighted in bold. Genotype effect represents significance of wildtype vs. *asn-a2* genotypes across all lines for each trait. Raw data and statistcal tests from these analyses are provided in Supplemental Tables S1 and S2. MB, moistuire basis; SRC, solvent retention capacity

	T6-1048			T4-1388			T4-2032			Genotype effect
Trait	**Wildtype**	***asn-a2***	***P***	**Wildtype**	***asn-a2***	***P***	**Wildtype**	***asn-a2***	***P***	***P***
Hardness index	74.4 ± 0.8	73.2 ± 1.0	.373	79.1 ± 1.0	77.2 ± 1.1	.239	79.4 ± 1.2	77.9 ± 1.7	.488	.110
Grain moisture, %	6.28 ± 0.06	6.17 ± 0.06	.231	6.16 ± 0.06	6.11 ± 0.06	.547	6.03 ± 0.04	6.08 ± 0.04	.472	.421
Whole grain meal ash (14% MB)	2.51 ± 0.08	2.43 ± 0.05	.410	2.14 ± 0.03	2.14 ± 0.03	.969	2.03 ± 0.02	2.09 ± 0.05	.286	.864
Whole grain meal protein (14% MB)	18.3 ± 0.2	17.8 ± 0.2	.163	18.0 ± 0.3	17.9 ± 0.3	.666	18.3 ± 0.2	18.6 ± 0.3	.457	.597
Grain Protein, % (12% MB)	18.7 ± 0.2	18.3 ± 0.2	.234	18.1 ± 0.3	18.1 ± 0.3	.946	18.9 ± 0.3	19.1 ± 0.3	.686	.778
Water SRC, %	77.8 ± 1.6	77.0 ± 1.4	.705	83.4 ± 0.8	84.5 ± 1.2	.488	85.0 ± 0.6	85.4 ± 0.8	.705	.818
Lactic acid SRC, %	101.3 ± 1.9	100.3 ± 2.1	.741	113.2 ± 1.8	112.2 ± 2.5	.752	116.6 ± 1.8	115.0 ± 2.2	.589	.486
Sucrose SRC, %	121.4 ± 1.5	118.7 ± 1.2	.195	130.9 ± 1.2	130.7 ± 1.4	.898	133.8 ± 1.8	134.1 ± 1.4	.880	.466
Carbonate SRC, %	105.8 ± 1.3	104.3 ± 0.9	.343	107.6 ± 2.0	107.8 ± 1.0	.908	110.8 ± 1.6	108.7 ± 1.3	.348	.338
Peak time, min	3.59 ± 0.12	3.34 ± 0.08	.099	3.64 ± 0.13	3.74 ± 0.16	.661	3.91 ± 0.16	3.56 ± 0.09	.088	.103
Peak value, %	47.3 ± 0.9	46.7 ± 0.4	.509	49.5 ± 0.5	48.7 ± 0.4	.280	48.4 ± 0.3	47.1 ± 0.3	**.010**	**.044**
Left slope, %/min	9.3 ± 0.6	9.5 ± 0.7	.759	10.0 ± 0.5	9.4 ± 0.6	.468	10.2 ± 0.7	10.8 ± 0.5	.510	.877
Right slope, %/min	−1.85 ± 0.23	−1.75 ± 0.21	.752	−3.79 ± 1.24	−2.68 ± 0.33	.408	−1.99 ± 0.22	−3.08 ± 0.13	**.003**	.931
Right width, %	11.9 ± 1.4	11.2 ± 0.7	.652	26.0 ± 2.6	24.2 ± 1.8	.587	25.6 ± 2.2	24.3 ± 2.4	.697	.427

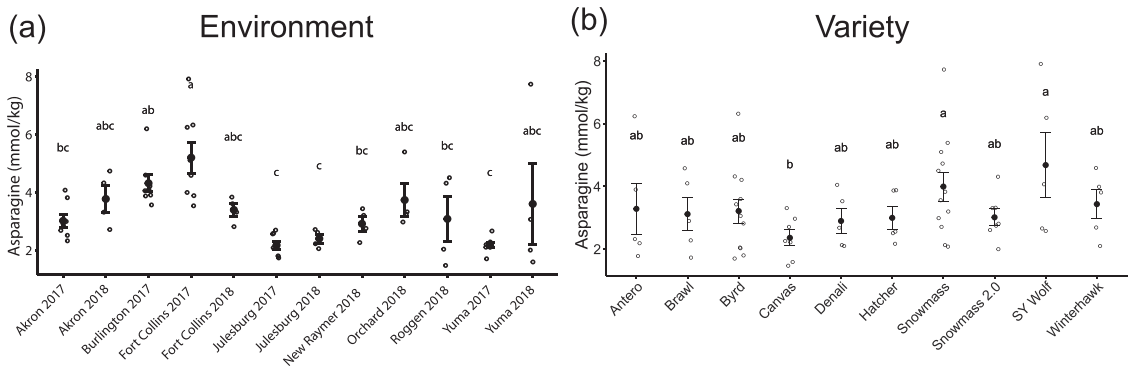


FIGURE 3 Mean free asparagine concentrations (± standard error) in elite winter wheat cultivars grown in field trials grouped by environment (a) and cultivar (b). Mean values not sharing a common letter indicate significant pairwise differences calculated by Tukey's post-hoc test ($P < .05$)

4 | DISCUSSION

4.1 | Induced *asn-a2* mutations confer reduced free asparagine concentration

Although natural genetic variation for free asparagine concentration exists in wheat germplasm collections, this is a highly quantitative trait determined by multiple small-effect QTL, complicating the identification, characterization, and deployment of natural variants (Corol et al., 2016; Emebiri, 2014; Rapp et al., 2018). As a complementary approach, in silico databases of chemically mutagenized wheat populations provide rapid access to novel genetic variation (Krasileva et al., 2017) that can be deployed in breeding programs without regulatory oversight, for example, to develop high-amylose wheat cultivars (Hazard et al., 2012). One drawback of mutagenized populations is the confounding effect of residual background mutations, since tetraploid and hexaploid M_4 plants carry, on average, 2,705 and 5,351 exonic point mutations, respectively (Krasileva et al., 2017). Even though two backcrosses were performed in the current study, the variable effect of these background mutations may account for phenotypic variation in line T4-2032 such that the differences in free asparagine concentration between wildtype and mutant sister lines were not significant (Figure 2). Despite this variation, consistent reductions in free asparagine concentrations in three independently derived mutant populations in both tetraploid and hexaploid wheat provide strong evidence that the observed phenotype is conferred by nonfunctional induced mutations in *ASN-A2*. Furthermore, transcript levels of *ASN-B2* and *ASN-D2* were not affected by *asn-a2* mutations in any line (Figure 1), suggesting the effects are due to reduced activity of *ASN-A2* itself, and not secondary effects on the activity of homeologous *ASN2* genes. However, these conclusions are based on results from just two environmental replications using sibling lines derived from individual mutant plants. It will be important to validate these findings using more extensive field trials replicated in both space and time and using independently derived populations segregating for variation in *TaASN2* genes. The introgression of the *asn-a2* null allele into Snowmass 2.0 has been initiated to characterize its effect in an elite winter wheat cultivar.

The reduction in free asparagine concentration in the *asn-a2* mutants ranged from 9 to 34% (Figure 2), comparable to the 16.2% reduction associated with a natural deletion of *TaASN-B2* in S-sufficient conditions (Oddy et al., 2021), but much lower than the 90% reduction observed in one CRISPR/Cas edited plant carrying mutations in all three *ASN2* homeologs (Raffan et al., 2021). The polyploid wheat genome provides functional redundancy, so it will be interesting to characterize isogenic materials with different combinations of *ASN2* alleles to reveal the relative effects of each homeolog and the extent to which they act additively to reduce free asparagine concentration. These alleles may also be combined with independent natural QTL elsewhere in the genome to further lower free asparagine concentration (Emebiri, 2014; Rapp et al., 2018). It will be essential that these materials are tested in multienvironment yield plots including variation in S supply, and in more diverse genetic backgrounds in order to fully characterize the effect of the environment and genotype × environment interactions for asparagine concentration (Emebiri, 2014; Rapp et al., 2018). Similar field trials using isogenic materials carrying higher-order combinations of *ASN2* alleles will reveal the extent to which it is possible to engineer reduced free asparagine concentration without introducing detrimental effects on agronomic or quality traits.

When integrating novel genetic variation, it is essential to determine possible pleiotropic effects on other traits. Most notably, CRISPR-edited plants with the greatest reductions in free asparagine concentration exhibited poor germination rates (Raffan et al., 2021), which may limit their utility for wheat breeders and growers. By contrast, the *asn-a2* mutant lines described in the current study exhibit no significant reduction in germination rate (Table 2), suggesting that the asparagine concentration in these seeds is sufficient for normal germination. A comprehensive quality analysis including

SRC, whole-grain meal, and mixograph analyses revealed that *asn-a2* mutant lines exhibit no detrimental effects for quality traits compared with wildtype plants (Table 3). These findings are consistent with previous analyses of the impacts of natural variation in free asparagine concentration on these traits, where only mild negative correlations between sedimentation volume (a proxy for the quality traits measured here) and free asparagine concentration were detected (Corol et al., 2016; Emebiri, 2014; Rapp et al., 2018). These results are encouraging and support the hypothesis that it should be possible to breed for mild reductions in asparagine concentration without compromising on baking quality or yield (Rapp et al., 2018).

4.2 | Reducing free asparagine concentration in other species

These findings show the potential to apply induced or natural variation in *ASN* genes to reduce asparagine concentrations in other species. Characterizing the expression profiles and natural variation of *ASN2* orthologs in barley (*Hordeum vulgare*; Avila-Ospina et al., 2015) and rye (*Secale cereale*; Raffan & Halford, 2021) could reveal opportunities to reduce free asparagine concentration in the grain of these species. However, because of their diploid genomes, the strategy to induce more subtle reductions in asparagine concentration by targeting different combinations of homeologous genes would not be possible, and instead, milder variants that reduce, but do not abolish, gene activity may be more valuable.

The duplication event that gave rise to *TaASN1* and *TaASN2* genes occurred only in the Triticeae lineage, meaning that the genomes of other monocots, including rice (*Oryza sativa*), maize (*Zea mays*) and sorghum (*Sorghum bicolor*), have no *ASN2* ortholog (Raffan & Halford, 2021). The rice genome contains two asparagine synthetase genes, so induced variation in either of these genes is likely to confer major changes in plant fitness not observed in wheat due to functional redundancy. However, in potato, while suppressing expression of both *StASN1* and *StASN2* had detrimental effects on growth and development (Chawla et al., 2012; Rommens et al., 2008), a more targeted approach focused only on *StASN1* reduced tuber asparagine content without other undesirable phenotypes, showing that it would be worthwhile exploring genetic diversity in the *ASN* gene family in other species.

4.3 | Characterizing the *ASN* gene family in wheat

Within wheat, reverse genetics approaches using either TILLING or CRISPR/Cas can also be applied to characterize other members of the *TaASN* gene family. In addition to their functional characterization, this approach may reveal novel combinations of alleles that could be beneficial for acrylamide reduction in wheat. Of note are *TaASN3.1* genes that are expressed during early embryo, ovule and grain development (Oddy et al., 2021; Xu et al., 2017). Searches of the in-silico TILLING database (Krasileva et al., 2017) revealed that there are multiple Kronos and Cadenza lines carrying point mutations encoding premature stop codons for all three homoeologous copies of *TaASN-3.1*. It would be interesting to develop these mutant materials to test the hypothesis that *ASN-3.1* contributes to free asparagine concentrations in the wheat grain. It is possible that combining these alleles with *ASN2* mutations could deliver further reductions in free asparagine concentrations in the grain. However, *TaASN3.1* genes are also expressed in leaf and stem tissues, so knockout alleles may have pleotropic effects on plant health and development.

5 | CONCLUSIONS

Nontransgenic, induced genetic variants in *ASN-A2* genes confer significant reductions in free asparagine concentration in wheat grains with no detrimental effects on agronomic or quality traits in field conditions. Although the use of these alleles in breeding programs will require commercial licenses from the intellectual property owners, they have the potential to reduce the acrylamide-forming potential of common wheat, either through recombination with the lines developed in this study, or by direct, targeted mutagenesis in elite wheat cultivars.

ACKNOWLEDGMENTS

This study was supported by funding from Colorado Wheat Research Foundation and the Colorado Wheat Administrative Committee. We are grateful to Forrest Wold-McGimsey for excellent technical support, to Dr. Jorge Dubcovsky for providing EMS-mutant lines for this study, and to Dr. Scott Haley for providing seeds from winter wheat elite lines grown in CSU winter wheat breeding plots. Asparagine quantification was performed at CSU, by Dr. Linxing Yao of the Analytical Resources Core: Bioanalysis and Omics Center (ARC-BIO).

AUTHOR CONTRIBUTIONS

Rocío Alarcón-Reverte: Investigation; formal analysis. Yucong Xie: Formal analysis; Investigation. John Stromberger: Investigation. Jennifer D. Cotter: Investigation. Richard Esten Mason: Funding acquisition; Supervision. Stephen Pearce: Conceptualization; Writing – original draft.

CONFLICT OF INTEREST

The authors state no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Alarcón-Reverte, R., Xie, Y., Stromberger, J., Cotter, J. D., Mason, R. E., & Pearce, S. (2022). Induced mutations in *ASPARAGINE SYNTHETASE-A2* reduce free asparagine concentration in the wheat grain. *Crop Science*, 1–13. https://doi.org/10.1002/csc2.20760