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Metabolic Profiling of Transgenic Potato Tubers Expressing *Arabidopsis* Dehydration Response Element-Binding Protein 1A (DREB1A)

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Supporting Information

ABSTRACT: Untargeted metabolome analyses play a critical role in understanding possible metabolic fluctuations of crops under varying environmental conditions. This study reports metabolic profiles of transgenic potato tubers expressing the *Arabidopsis DREB1A* transcription factor gene, which induces expression of genes involved in environmental stress tolerance. A combination of targeted and untargeted metabolomics demonstrated considerable metabolome differences between the transgenic lines and nontransgenic parent cultivars. In the transgenic lines, stimulation of stress responses was suggested by elevated levels of the glutathione metabolite, γ-aminobutyric acid (GABA), and by the accumulation of β-cyanoalanine, a byproduct of ethylene biosynthesis. These results suggest that the *Arabidopsis DREB1A* expression might directly or indirectly enhance endogenous potato stress tolerance systems. The results indicate that transgenesis events could alter the metabolic compositions in food crops, and therefore metabolomics analysis could be a most valuable tool to monitor such changes.

KEYWORDS: *transgenic crops, metabolomics, β-cyanoalanine, stress responses*

INTRODUCTION

Crop domestication involved the attempt to confer beneficial traits, such as yields, stress tolerance, shapes, and colors, to various plant species through selective breeding. During the past three decades, the development of genetic engineering techniques enabled breeders to transfer beneficial traits across species barriers directly into target crops. The first successes in plant genetic engineering were the introduction of herbicide tolerance and insect resistance into crops.[1,2] In response to global warming and climate change, massive changes in cultivation practices will be necessary to sustain crop productivity under environmental stresses such as recurrent drought and changes in the physical properties of soil, as well as biotic stresses from as yet unknown pests and pathogens.

It is expected that stress tolerance can be conferred on plants via the manipulation of endogenous defense systems involving the biosynthesis of secondary metabolites that function as antimicrobial compounds and insect deterrents and by the manipulation of structural elements that are involved in plant architecture.[3] One of the most promising means of engineering endogenous stress responses is the manipulation of transcription factor genes that epistatically regulate the metabolic activities that produce defense metabolites.[4] However, the production capacities of such defense metabolites in wild progenitors might become inactive during the domestication process.[5] It is therefore possible that the reinforcement of endogenous stress tolerance may result in the activation of unknown and/or latent stress responses, leading to unexpected metabolic changes and accumulation of undesirable metabolites. Thus, precautionary research is required to ensure the safety of genetically engineered crops.[6,7]

Implementation of metabolomics is a key to clarify biochemical constituents of crops. We performed our metabolite profiling study using a combination of untargeted metabolomics and quantitative analyses of targeted metabolites. Untargeted metabolomics is expected to provide global metabolome information together with specific metabolites for which levels fluctuate under certain conditions. The targeted analyses are designed to quantify selected metabolites of specific importance, including bioactive compounds and the marker metabolites identified through the untargeted metabolomics.

In this study, we characterized transgenic potato tubers[8,9] expressing the *Arabidopsis DREB1A* transcription factor gene under the control of either the CaMV35S promoter (transgenic potato genotype, 35S-3) or the *Arabidopsis rd29a* promoter (transgenic potato genotypes, D163 and D164). In *Arabidopsis*, DRE-binding protein/C-repeat binding factor (DREB/CBF) proteins bind the dehydration-responsive element/C-repeat

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(DRE/CRT) *cis*-acting elements and regulate the expression of both dehydration- and low-temperature-induced genes.[10] DREB proteins are classified into two subclasses: DREB1/CBF and DREB2. These genes act in response to low temperature and dehydration, respectively, and a number of transgenic studies have been directed toward engineering stress tolerance through the transgenic manipulation of *DREB* expression.[9,11−15] The *rd29a* gene is induced by desiccation, cold, high-salt conditions, and abscisic acid in *Arabidopsis*.[16] The transgenic approach using a combination of the *Arabidopsis rd29A* promoter and *DREB1A* successfully improved environmental stress tolerance in different plant species.[11,17]

The tuber metabolome profiles from transgenic and nontransgenic plants demonstrated considerable differences in their metabolic status, although no significant differences were found in the contents of the toxic compounds such as glycoalkaloids, α-solanine, and α-chaconine. The metabolite levels involved in the metabolism of glutathione and γ-aminobutyric acid (GABA) were clearly elevated in the transgenic lines, suggesting the possible stimulation of stress responses by the expression of *DREB1A*. In addition, β-cyanoalanine (β-CA) was detected in both transgenic and nontransgenic samples, and the accumulation levels were higher in the transgenic samples. β-CA is known to be a biosynthesis byproduct of ethylene (ET), which is a stress-related phytohormone.[18−20] It is therefore possible that *Arabidopsis DREB1A* expression might have directly or indirectly influenced endogenous stress responses, including ET biosynthesis, in the potato, resulting in the accumulation of β-CA. The current results indicate that variable environmental conditions could affect metabolic profiles of crops, irrespective of transgenic and nontransgenic backgrounds; the effect of transgenic manipulation on metabolic profiles should be closely monitored using holistic metabolic analyses.

MATERIALS AND METHODS

Metabolic Profiling. Tubers were harvested from three lines of transgenic *Solanum tuberosum* cv. Desiree containing the *Arabidopsis DREB1A* gene under the control of either the *rd29A* promoter (*rd29A::DREB1A*) or the cauliflower mosaic virus 35S promoter (*35S::DREB1A*).[8,9] Plants were grown in a special netted house (semicontainment green house) that was operated without controlling the temperature and humidity. Cut tissues from a surface layer (2 mm thick) of tubers were immediately frozen in liquid N_2 and stored at −80 °C until use. A total of 12 samples (three replicates from each) were obtained from nontransgenic Desiree (NT), the *DREB1A* transgenic lines *rd29A::DREB1A* (D163 and D164), and a *35S::DREB1A* (35S-3) line. Metabolic profiling was carried out according to the method described previously[21] using the global unbiased metabolic profiling platform composed of three separate mass spectrometric platforms, as described below.[22] For sample extraction, 20 mg of each sample was thawed on ice and extracted using the automated MicroLab STAR system (Hamilton Co., Reno, NV, USA) in 400 μL of methanol containing recovery standards. The extracts were analyzed on an untargeted metabolomics platform that consisted of three independent instrumentation methods: ultrahigh-performance liquid chromatography−tandem mass spectrometry (UHLC-MS/MS^2) optimized for basic species, UHLC-MS/MS^2 optimized for acidic species, and gas chromatography−mass spectrometry (GC-MS). Ion peaks were matched to standard compounds stored in a reference library, and their relative levels were quantified.[22]

The UHPLC-MS analysis was performed using a Waters Acquity UHPLC (Waters Corp., Milford, MA, USA) coupled to an LTQ mass spectrometer (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA) with an electrospray ionization source. Two separate UHPLC-MS injections were performed on each sample: one optimized for positive ions and one for negative ions. Derivatized samples for GC-MS were analyzed using a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole MS unit operating at unit mass resolving power. Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular weight (m/z), preferred adducts, and in-source fragments, as well as their associated MS/MS^2 spectra. Comparison of experimental samples to process blanks (water only) and solvent blanks allowed the removal of artifactual peaks.[21,22]

Statistical analysis was performed using JMP (SAS, http://www.jmp.com) and R (http://cran.r-project.org/) to assess the differences in metabolite accumulation between the distinct lines. A log transformation was applied to the observed relative concentrations for each biochemical metabolite because the variance generally increases as a function of each biochemical metabolite's average response. A summary of the metabolites that achieved statistical significance ($p < 0.05$) is shown in Supplementary Table 1 (Supporting Information). A Welch two-sample t test was used to identify metabolites that differed between the nontransgenic (NT) samples and those from the three promoter constructs (35S-3, D163, and D164), according to the principle described by Oliver et al.[21] The false-positive rate associated with multiple comparisons was calculated using the false-discovery rate (FDR) method of Storey and Tibshirani;[23] q values for all tests are included in Supplementary Table 1 (Supporting Information). The q values for the vast majority of significant tests ($P < 0.05$) fell below the 10% FDR ($q < 0.10$). In the biochemical pathway analysis, all tests with significance of $P < 0.05$ were considered without restriction by q value.[21]

Determination of Glycoalkaloids and β-CA. Potato tuber tissues were extracted using hot methanol/water (70:30, v/v), and glycoalkaloids were analyzed using a liquid chromatograph (LC)−linear ion trap/time-of-flight mass spectrometer (TOF-MS; Nano Frontier LD, Hitachi High-Technologies Corp., Tokyo, Japan). Metabolites were separated using a Cadenza CD-C18 column (column temperature of 40 °C, 2 × 150 mm; Imtakt Corp., Kyoto, Japan) in a linear gradient elution using solvent A [H_2O containing 0.1% (v/v) formic acid] and solvent B [acetonitrile containing 0.1% (v/v) formic acid]. The elution was kept at an initial A:B ratio of 95:5 from 0 to 5 min and then linearly shifted to A:B = 5:95 from 5 to 50 min. Two major solanidine glycosides, α-solanine $[M + H]^+$ (m/z 868.6) and α-chaconine $[M + H]^+$ (m/z 852.6) with different sugar modifications, were distinguished by their different elution times of 27.9 and 28.3 min, respectively, in the ion selective mode of mass chromatograms (the lower detection limit was 4 ng for α-solanine). For the determination of glycoalkaloids, a calibration curve ($R^2 = 0.988$) was obtained using α-solanine (Sigma-Aldrich, St. Louis, MO, USA) as the reference standard (between 4 ng and 40 μg). The fragment ion (m/z 398.32) ascribed to solanidine (the alkaloid aglycone) in our LC-MS analysis was detected only at the elution times corresponding to α-solanine and α-chanonine (Supplementary Figure 1, Supporting Information), indicating the absence of differentially modified glycoalkaloids and hydrolysis of the glycoside moieties of the glycoalkaloids.

For β-CA determination, tuber tissues were frozen in liquid N_2 and homogenized with 80% (v/v) methanol, and ribitol (Sigma-Aldrich) was added to the homogenized samples. After centrifugation, the supernatant was filtered through a DISMIC-13P filter (Advantec, Tokyo, Japan) and subjected to methyloximation derivatization (at 30 °C for 90 min in anhydrous pyridine) and derivatization with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (Sigma-Aldrich). The derivatized samples were analyzed by GC-MS using a Saturn 2200 GC-MS system (Varian, Palo Alto, CA, USA) with a CP-SIL5 CB low bleed/MS column (15 m × 0.25 mm; Varian). The port temperature for split injections (a split ratio of 50%) was 270 °C, and helium was used as the carrier gas at a flow rate of 1.5 mL/min. The temperature program was started at 80 °C with a linear rise from 80 to 330 °C (8 °C/min) and maintained for the final 6 min at 330 °C. The peak areas of metabolites were automatically calculated using the 2200 Work-

station (Varian), and β-CA was determined in comparison to standard β-CA (Sigma-Aldrich).

γ-Glutamyl-β-CA was prepared as described by Watanabe et al.[24] Briefly, a reaction mixture (100 μL) containing 1 unit of γ-glutamyl transpeptidase (Sigma-Aldrich), 10 mM Tris-HCl (pH 8.5), 10 mM β-CA, and 10 mM glutamic acid was incubated for 1 h at 37 °C. The reaction was stopped by adding 100 μL of ethanol, and the formation of the reaction product, γ-glutamyl-β-CA $[M - H]^-$ (m/z 242.2), was confirmed using the LC-ESI-TOF-MS (Nano Frontier LD, Hitachi High-Technologies Corp.) under the same conditions as used for the glycoalkaloid determination.

Semiquantitative RT-PCR Analysis. A detailed comparison of gene expression levels was carried out using semiquantitative RT-PCR for genes involved in ET biosynthesis and β-CA metabolism. These genes include β-CA synthase, Cys synthase, 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS), ACC oxidase (ACO), γ-glutamyl transpeptidase (GGT), and nitrilase (NIT).

Gene-specific primers for γ-glutamyl transpeptidase (*St-GGT1*) and nitrilase (*St-NIT4*) were designed by referring to genomic sequences deposited in The Solanaceae Genomics Resource (http://solanaceae.plantbiology.msu.edu/). For *GGT1* transcript amplification, we identified a putative *GGT1* sequence (PGSC0003DMT400077372) in the potato, using the *Arabidopsis* GGT1 amino acid sequence at the Potato Genome Sequencing Consortium Data Release. This gene is named *St-GGT1*. The PCR amplification was designed to amplify the fragment encompassing the region from 693 to 1322 of the predicted open reading frame, which corresponds to exon2 thru exon4 of the predicted *St-GGT1* coding sequence. Another putative *GGT* sequence (*St-GGT2*), PGSC0003DMT400077372, was identified in the potato. These two *GGT* sequences are 65% identical. A potato nitrilase (*St-NIT4*) candidate (PSGC0003DMT400067695) was identified at the Potato Genome Sequencing Consortium Data Release, referring to the amino acid sequence of *Arabidopsis NIT4B* (At5g22300) involved in β-CA metabolism in *Arabidopsis*.[25]

In the potato, at least three *ACS* genes are expressed: *St-ACS1A*, *St-ACS1B*, and *St-ACS2*.[26] In this study, we used a primer set to simultaneously analyze both *St-ACS1A* and *St-ACS1B* and a separate set for *St-ACS2*.[26] Potato *ACO* gene (*St-ACO1*, *St-ACO2*, and *St-ACO3*) transcript levels were analyzed according to the sequences reported by Zanetti et al.,[27] whereas only the *St-ACO3* transcript accumulation was detectable in the samples used in this study. β-CAS (*StCAS1*, Genbank ID AB027000) and CS (*StCS1*, Genbank ID AB029511; *StCS2*, Genbank ID AB029512) gene transcripts were analyzed using the primers designed from the deposited sequences (Supplementary Table 2, Supporting Information). The primer sets for the analyses of *actin* (Genbank ID U60488) and *DREB1A* (Genbank ID AB007787) genes are listed in Supplementary Table 2 (Supporting Information).

Total RNA was isolated using Plant RNA Isolation Reagent (Life Technologies, Tokyo, Japan), and genomic DNA contamination was eliminated using an RNase-free DNaseI Set (Takara Bio, Kyoto, Japan). First-strand cDNA was synthesized in a 10 μL reaction mixture containing 250 ng of total RNA using an oligo$(dT)_{16}$ as the reverse primer. The reverse transcription reactions using AMV Reverse Transcriptase XL (Takara Bio) were carried out at 45 °C for 10 min and 90 °C for 5 min and then chilled to 5 °C for 5 min. PCR was carried out using 0.2 μL of the reverse transcription products as the template in a 10 μL of reaction mixture containing 1 mM $MgCl_2$, 0.2 mM dNTP mixture, 0.025 unit/μL PrimeSTAR HS DNA polymerase (Takara Bio), and 0.2 μM concentrations of primers. The PCR programs were prepared for each primer set (Supplementary Table 2, Supporting Information) with a PCR cycle at 94 °C for 10 s, a specifically determined annealing temperature for 30 s (Supplementary Table 2, Supporting Information), and a temperature of 72 °C for 30 min, followed by an extension period of 30 s at 72 °C. Aliquots of RT-PCR reactions were collected at five-cycle intervals to monitor the linear amplification phase. The potato actin gene was amplified using a specific primer set (Supplementary Table 2, Supporting Information) as the internal reference under the same PCR conditions for the genes of interest. RT-PCR products were resolved by 2% (w/v) agarose gel electrophoresis and visualized by staining with SYBR Green I (Lonza, Rockland, ME, USA).

RESULTS

Metabolome Comparison. Untargeted metabolomics approaches offer advantages to characterize unintended metabolic differences among transgenic and nontransgenic crops.[28,29] We detected a total of 165 metabolite candidates from the potato samples (Supplementary Table 1, Supporting Information). Among them, 113 metabolites match named structures of authentic standard compounds, and 52 represent currently unknown structural identities. The metabolites were mapped onto general biochemical pathways, as illustrated in the Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/) and the Plant Metabolic Network (http://www.plantcyc.org/). Many of the significant changes and trends observed are common to the three transgenic lines (35S-3, D163, and D164), although there may have been some differences in the magnitude of the responses (Supplementary Table 1, Supporting Information). There were some compounds that seem to be specifically different in the D163 or D164 lines (which are very similar in genotypic sense) or in 35S-3.

Several measurements indicated the presence of stress responses in the transgenic samples. The accumulation levels of some metabolites, such as those involved in the GABA shunt or glutathione pathways, seem to point to the activation of oxidative stress responses in the transgenic samples.[21] Thus, the increases in GABA and 2-pyrrolidone levels, as well as Gln levels, suggested that the GABA shunt in the transgenic lines (Figure 1) was stimulated. In addition, marked accumulation of γ-glutamyl amino acids (Figure 2) suggests the activation of the

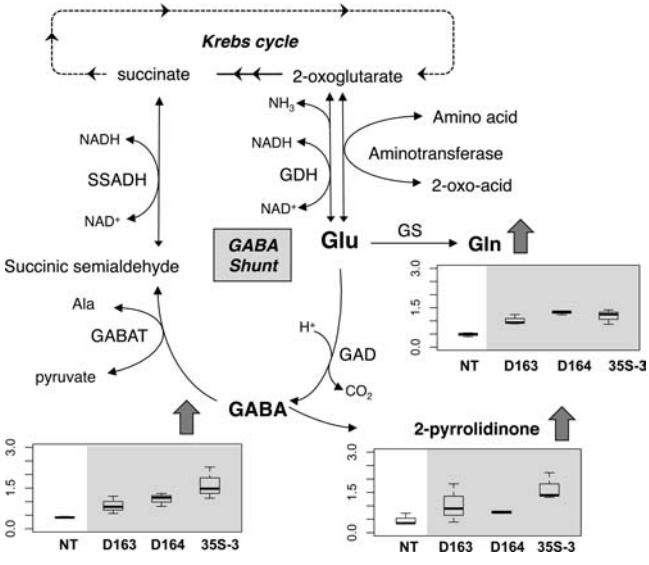

Figure 1. Relative comparison of metabolite levels in the glutamate/glutamine pathway. Metabolite levels in the transgenic (D163, D164, and 35S-3) and nontransgenic (NT) potato tubers are shown by the box plots. The box shows first quartile, median, and third quartile values. The whiskers represent upper and lower data. The *y*-axis is the median scaled value. Results from the transgenic lines are shown in the shaded area of the panels. GS, glutamine synthetase; GABAT, γ-aminobutyrate aminotransferase; GAD, glutamate decarboxylase; GDH, glutamate dehydrogenase; SSAD, succinic semialdehyde dehydrogenase.

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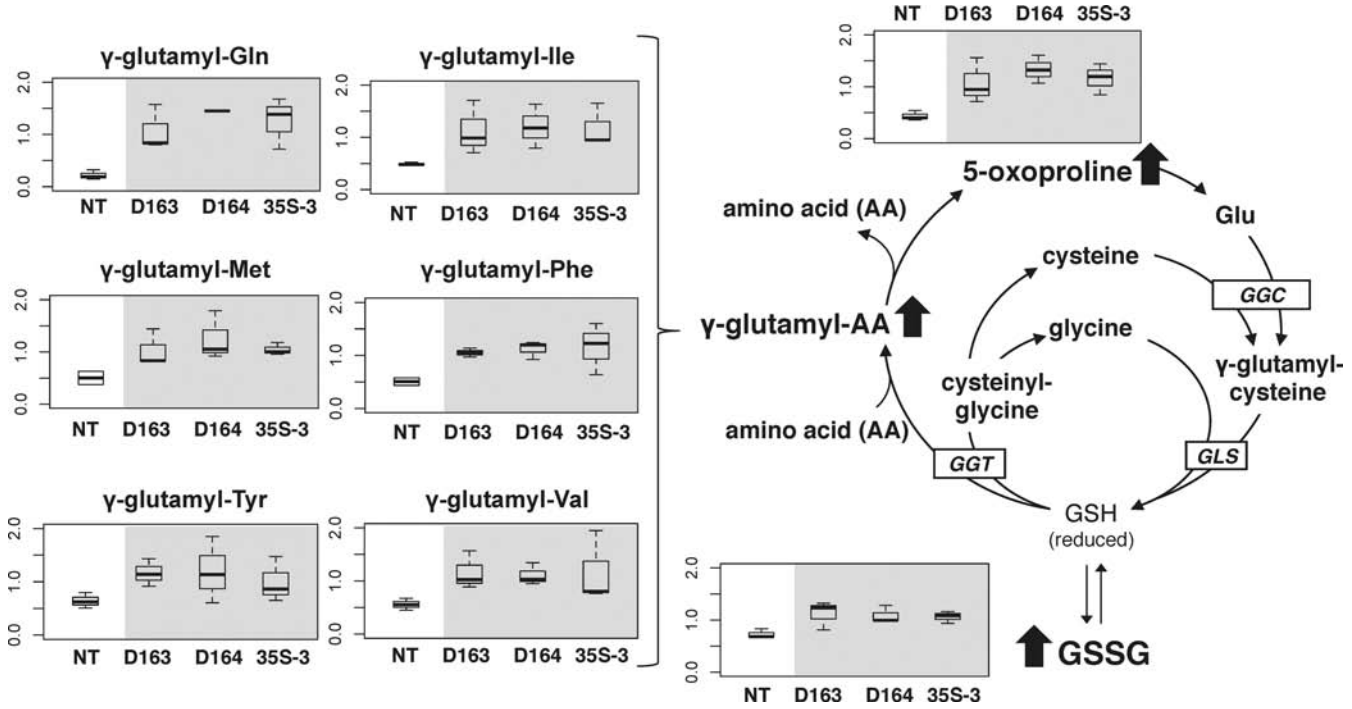

Figure 2. Relative comparison of the GSH pathway metabolite levels in the transgenic (D163, D164, and 35S-3) and nontransgenic (NT) potato tubers. The levels of oxidized glutathione (GSSG), 5-oxoproline, γ-glutamylphenylalanine, γ-glutamylisoleucine, γ-glutamyltyrosine, γ-glutamylmethioine, γ-glutamylglutamine, and γ-glutamylvaline are illustrated by the box plots. The box shows first quartile, median, and third quartile values. The whiskers represent upper and lower data. The *y*-axis is the median scaled value. Results from the transgenic lines are shown in the shaded area of the panels. GGC, GGT, and GLS represent γ-glutamyl cyclotransferase, γ-glutamyl transpeptidase, and glutathione synthase, respectively.

GSH recycling pathway in the transgenic lines. The increased accumulation of oxidized glutathione (GSSG) and γ-glutamyl amino acids was prominent in the transgenic potato tubers. 5-Oxoproline levels were also elevated in the transgenic lines (Supplementary Table 1, Supporting Information). Ascorbate, an important antioxidant, seemed to be dramatically elevated in the D163 and 35S-3 tubers (Supplementary Table 1, Supporting Information). However, it is not clear whether the *DREB1A* transgene expression led to oxidative damage or if these metabolic responses are part of a wider oxidative stress-protective mechanism in potato. A stress response was also suggested by the higher levels of β-CA accumulation in the transgenic plants, especially in the 35S-3 line [Supplementary Table 1 (Supporting Information) and Figure 3]. β-CA is produced as a byproduct of the ET biosynthesis.[30] ET is a phytohormone that plays crucial roles in a variety of physiological processes throughout plant development and in response to biotic and abiotic stresses.[31−36]

The sugar metabolism might also have been affected in the transgenic lines: the levels of sucrose, glucose-1-phosphate, glucose-6-phosphate, and fructose-6-phosphate were higher in the transgenic lines than in the controls. It is possible that the transport of carbon assimilation products might have been stimulated in the transgenic plants (Supplementary Table 1, Supporting Information). Glu biosynthesis, which is key to the acquisition of nitrogen (through the GS/GOGAT cycle), is influenced by the plants' carbon status. High levels of sucrose, relative to amino acid pools, are known to induce Glu synthase at the transcriptional level.[37]

Ethanolamine levels were enhanced in the transgenic lines (Supplementary Table 1, Supporting Information). It has been reported that ethanolamine is involved in the biosynthesis of

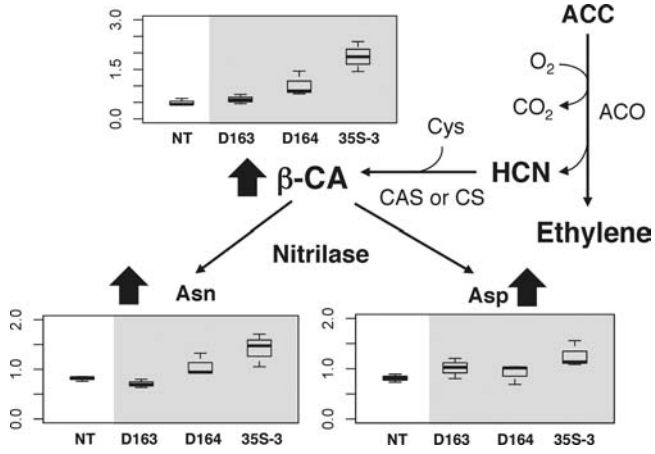

Figure 3. Relative comparison of the metabolite levels involved in the ET biosynthesis pathway. The transgenic (D163, D164, and 35S-3) and nontransgenic (NT) potato tubers were subjected to the metabolome profiling assay, and the results are shown by the box plots. Results from the transgenic lines are shown in the shaded area of the panels. 1-Aminocyclopropanecarboxylic acid (ACC) is the substrate of the ACC oxidase (ACO) reaction to produce ET, releasing HCN as a byproduct. Either β-cyanoalanine synthase (CAS) or cysteine synthase (CS) is responsible for the production of β-cyanoalanine (β-CA) from Cys and HCN, and nitrilases metabolize β-CA to Asp or Asn.

glycine betaine as an osmoprotectant in plants in response to high salinity.[38] However, our targeted analysis did not detect a significant increase in the glycine betaine levels in the transgenic lines (data not shown). The purine nucleosides, adenosine and guanosine, were elevated in all transgenic lines,

possibly reflecting an increase in the activity of purine salvage pathways. Also, some metabolite levels (e.g., methionine sulfoxide, quinate, tyramine, kynurenate, and dehydroascorbate) decreased in the transgenic lines, but the responses were not consistent among the three transgenic lines (Supplementary Table 1, Supporting Information).

Targeted Analysis for Toxic Metabolites. Potato tissues are known to accumulate the toxic glycoalkaloids α-solanine and α-chanonine, which represent 95% of total glycoalkaloids in tubers.[39] The glycoalkaloid contents of potato tubers vary depending on environmental conditions during cultivation, the harvesting process, and the storage period. In most cases, the glycoalkaloid content remains within the range of 7−187 μg/100 g FW.[40] The levels of α-solanine and α-chanonine in NT were 14.6 ± 5.81 and 33.5 ± 9.78 μg/g FW, respectively. The levels of these glycoalkaloids in the D163 and D164 lines were comparable to those in NT, whereas in the 35S-3 line these levels were significantly lower than those in the NT. The fragment ion (m/z 398.32) ascribed to solanidine (the alkaloid aglycone) in our LC-MS analysis was detected only at the elution times corresponding to α-solanine and α-chanonine, indicating the absence of differentially modified glycoalkaloids (Supplementary Figure 1, Supporting Information). These results demonstrate that the expression of the *DREB1A* transcription factor in the potato did not affect the glycoalkaloid levels.

The neural toxicity of β-CA is well-known, particularly in the legume family plants.[41] In a study in which β-CA was intraperitoneal or orally administered to chicks and rats, the neurotoxic effects of β-CA were ascribed to its action toward the *N*-methyl-D-aspartate class of glutamate receptors.[42] Because the metabolome profiles are shown by relative comparison, β-CA contents were determined by a targeted analysis of the sampled tissues (Figure 4). The β-CA levels in the transgenic potato tubers were significantly higher than those in the NT tubers (1.80 ± 0.32 μg/g FW). In the transgenic lines, 35S-3, D163, and D164 had β-CA levels of 5.88 ± 0.86, 3.66 ± 0.50, and 3.38 ± 1.4 μg/g FW, respectively.

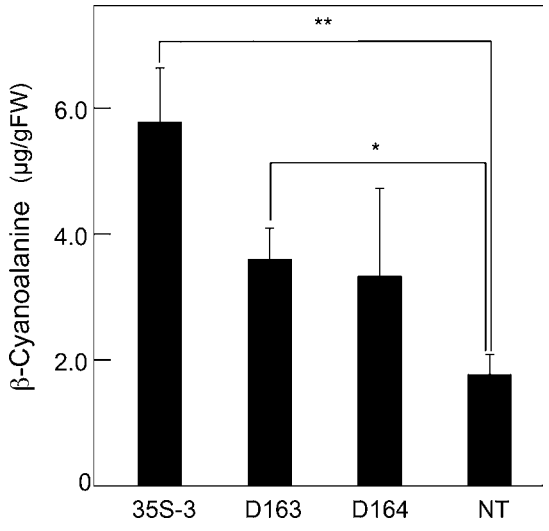

Figure 4. β-CA contents of transgenic (35S-3, D163, and D164) and nontransgenic (NT) potato tuber extracts. Error bars indicate the mean ± SD of three separate extracts. Asterisks indicate significant differences between NT and transgenic potatoes using a two-tailed Student's t test (*, $P < 0.05$; **, $P < 0.01$).

The toxic level of β-CA, given by the lethal dose at which 50% mortality (LD_{50}) is observed, is 134 mg/kg;[43] however, β-CA levels in potato tubers in our studies were far lower than toxic levels for human consumption.

Analysis of Gene Expression Involved in ET and β-CA Biosyntheses. It is possible that the elevated β-CA levels are caused by enhanced ET biosynthesis in the *DREB1A* transgenic potatoes. To test this hypothesis, we compared the transcript levels of the genes involved in ET biosynthesis in potato tubers (Figures 5 and 6). The initial step of ET biosynthesis is

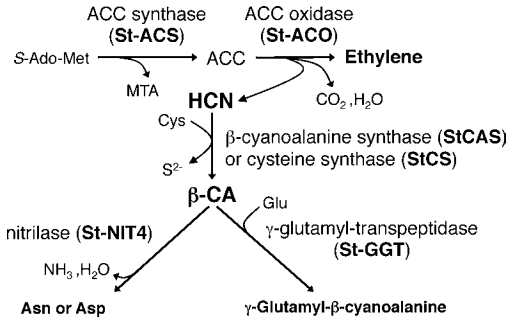

Figure 5. Schematic representation of the genes involved in β-CA production in potato. Transcript levels of ACC synthase gene (*St-ACS*) and ACC oxidase gene (*St-ACO*) were analyzed according to the sequence information reported by Destéfano-Beltrán et al.[26] and Zanetti et al.,[27] respectively. β-Cyanoalanine synthase (*StCAS*) and cysteine synthase (*StCS*) gene transcripts were analyzed according to the deposited sequences.[47] The genes responsible for the metabolic degradation of β-CA, γ-glutamyl transferase (*St-GGT1*, *St-GGT3*) and nitrilase (*St-NIT4*), were analyzed with reference to genomic sequences deposited in The Solanaceae Genomics Resource (http://solanaceae.plantbiology.msu.edu/).

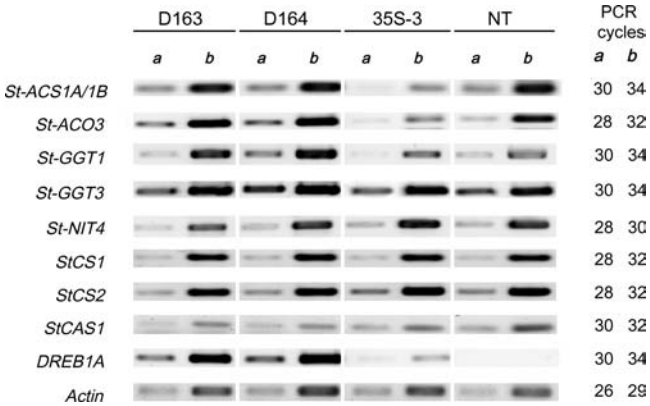

Figure 6. Transcript levels of genes related to ET and β-CA synthesis in the tubers from the *DREB1A* transgenic (35S-3, D163, and D164) and nontransgenic (NT) lines. Semiquantitative RT-PCR was performed using a gene-specific primer set for each gene listed in Supplemental Table 2 (Supporting Information) for *St-GGT1* (potato γ-glutamyl transferase-1), *St-GGT3* (potato γ-glutamyl transferase-3), *St-NIT4* (potato nitrilase-4), *StCS1* (potato cysteine synthase-1), *StCS2* (potato cysteine synthase-2), *StCAS1* (potato β-cyanoalanine synthase-1), *St-ACO3* (potato ACC oxidase-3), *St-ACS1A/1B* (potato ACC synthase-1A/1B), *DREB1A*, and *actin*. Two different PCR reaction cycles (shown by ***a*** and ***b***) were used for the analysis of each gene transcript level. The numbers shown on the right side correspond to those PCR cycles yielding the amplification products shown in lanes ***a*** and ***b***, respectively.

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catalyzed by ACS, yielding ACC and 5′-methylthioadenosine from *S*-adenosylmethionine (*S*-Ado-Met). ACC is then oxidized by ACO to give the end product, ET, releasing cyanoformic acid, which is spontaneously converted into HCN and CO_2.[18−20] The HCN is captured by β-CAS and CS to produce β-CA.

In potato plants, at least three *ACS* genes are expressed: *St-ACS1A* and *St-ACS1B*, which share 98% identity in their primary structures and are ubiquitously expressed in the potatoes, and the *St-ACS2* gene transcript, which has been detected in potato leaves, leaf petioles, roots, and tubers.[26] *St-ACS1A* and *St-ACS1B* are strongly induced in hypocotyls by indole-3-acetic acid and in leaves by wounding.[26] In our study, the transcript levels of *St-ACS1A/B* were comparable among the D163, D164, and NT lines, whereas those in the 35S-3 line were clearly decreased (Figure 6). The *St-ACS2* transcript was not detected in the tuber samples in this study.

ACO is known to be induced by various stresses in plants.[31−36] We analyzed the transcript accumulations of the potato *ACO* genes (*St-ACO1*, *St-ACO2*, and *St-ACO3*). It has been reported that the expression levels of *St-ACO1* and *St-ACO2* were higher in leaves and lower in roots and tubers.[27] We found that *St-ACO3* transcript levels were higher in the D163 and D164 lines than in the NT and 35S-3 lines (Figure 6), whereas *St-ACO1* and *St-ACO2* transcripts were not detectable in this study (data not shown).

In potato, β-CAS (*StCAS1*) and cysteine synthase (*StCS1* and *StCS2*) are involved in β-CA production.[44] There were no clear differences in *StCS1/2* and *StCAS1* transcript levels between the transgenic lines and the NT line (Figure 6). It has been reported that levels of β-CAS activity and the resulting protein accumulation were elevated by ET without corresponding transcript levels, suggesting that ET induces β-CAS protein accumulation at the posttranscriptional level.[44]

Increases in Asp and Asn levels in the transgenic lines (Figure 3) may be related to β-CA catabolism.[25,45] β-CA degradation occurs through GGT and NIT4 activity in *Arabidopsis*, producing γ-glutamyl-β-CA and Asp/Asn, respectively.[24] In the potato, we identified a putative *GGT* sequence (*St-GGT1*), using the *Arabidopsis* GGT1 amino acid sequence in the Potato Genome Sequencing Consortium Data Release (http://potatogenomics.plantbiology.msu.edu/). A potato nitrilase candidate (*St-NIT4*) was also identified in the same database, referred to the amino acid sequence of *Arabidopsis* NIT4B (At5g22300).[25] Figure 6 shows the higher levels of *St-GGT1* and *St-NIT4* transcripts in the D163 and D164 lines than in the 35S-3 line, suggesting possible stimulation of β-CA degradation in the D163 and D164 lines. However, γ-glutamyl-β-CA accumulation was not detectable in any of the potato lines in this study (data not shown). In contrast, the γ-glutamyl amino acid levels were consistently higher in the transgenic lines than in the NT line [Supplementary Table 1 (Supporting Information) and Figure 2]. It remains unclear whether the higher *St-GGT1* transcript levels were responsible for the accumulation of γ-glutamyl amino acids in the transgenic lines. In the metabolic profiling study, the levels of both Asp and Asn were likely to be higher in the transgenic potato tubers (Supplementary Table 1, Supporting Information), which may be ascribed to the stimulated metabolism of β-CA to Asp and Asn.

DISCUSSION

Our metabolic profiling study suggests that endogenous potato stress responses might be stimulated by the transgenic expression of *Arabidopsis DREB1A*, regardless of the promoters used. Kasuga et al.[17] have reported that *DREB1A* expression caused growth retardation in a study that employed the constitutive promoter CaMV35S. Thus, the constitutive expression of *DREB1A*, which is stress-inducible[10] and induces expression of its target genes, might not be favored under normal growth conditions. *DREB1A* expression elicited by the stress-inducible *rd29a* promoter successfully increased stress tolerance in the potato.[8,9] It should be noted that the *DREB1A* transcript was not detected in these transgenic potatoes without the presence of stress factors.[8,9] The high levels of *DREB1A* transcripts detected in D163 and D164 (Figure 6) suggest that these plants might have behaved as if they experienced certain environmental stresses, including the up-regulation of endogenous defense-response genes, because the potato plants used in this study were not grown in environmentally controlled conditions. The presence of a unique transcript fragment (PUT-157a-Solanum_tuberosum-42922 in The Solanaceae Genomics Resource), of which the known primary structure is 57% identical to *DREB1A*, suggests that a functional stress tolerance mechanism in potatoes is similar to the DREB1A-inducible stress response. Thus, it is possible that the metabolic responses we observed might have mirrored the outcome of interactive endogenous stress responses that were intensified by the transgenic expression of *Arabidopsis DREB1A*. However, regardless of the promoters used, *DREB1A* transcript levels may be expected to coincide with changes in the metabolic profiles. However, the *DREB1A* transcript levels (Figure 6) did not match the accumulated levels of some metabolites (e.g., the β-CA level was higher in 35S-3 line than in the rd29a line), indicating that gene transcript levels were not linearly related to the extent of metabolic fluctuations.

The prominent accumulation of γ-glutamyl amino acids (Figure 2) suggested the activation of the GSH recycling pathway in the transgenic potatoes (Figures 1 and 2). Oliver et al.[21] have reported that the levels of γ-glutamyl amino acids were dramatically increased in desiccation-tolerant *Sporobolus stapfianus* during dehydration and that two different desiccation-tolerant species, the bryophyte *Tortula rulalis* and the lycophyte *Selaginella lepidophylla*, also showed elevated levels of γ-glutamyl amino acids during dehydration. Oliver et al.[21] also reported that *S. stapfianus* leaves exhibited significant accumulation of *GGT* transcripts during dehydration. They hypothesized that increased levels of γ-glutamyl amino acids might be involved in the desiccation tolerance by providing protection from stresses caused by reactive oxygen species.

Involvement of γ-glutamyl amino acids in oxidative stress tolerance has not been extensively studied in plants. In the γ-glutamyl cycle in animals, GGT transfers the γ-glutamyl moiety from GSH to water or an amino acid, yielding either Glu or a corresponding γ-glutamyl amino acid, which is then transported back into the cytoplasm.[46] γ-Glutamyl amino acids also serve as the substrates of γ-glutamyl cyclotransferase (GGC), which converts γ-glutamyl amino acids into 5-oxoproline, releasing the corresponding amino acids.[46] 5-Oxoproline is hydrolyzed to Glu by an ATP-dependent enzyme, 5-oxoprolinase.[46] In *Arabidopsis*, it has been reported that the apoplastic GGT1 and GGT2 control the degradation of oxidized GSH in the extracellular space, and the vacuolar enzyme GGT4 is

responsible for the degradation of stored GSH conjugates to release Glu as well as Cys−Gly conjugates.[47]

It is not known whether plant *GGT* genes are involved in γ-glutamyl amino acid production. In this study, we observed a significant increase in γ-glutamyl amino acids and 5-oxoproline in the transgenic potatoes. It has been suggested that GGC and 5-oxoprolinase are primarily involved in GSH recycling.[48] However, the *GGC* gene remains unidentified and uncharacterized in plants.[49] The identification and characterization of *GGC* in plants is essential to understand the stimulated increase in γ-glutamyl amino acids and the physiological roles of γ-glutamyl amino acids and GSH metabolism, particularly in stress tolerance.

Because potatoes are grown for human consumption, a large concern is the presence of unfavorable metabolites. In our study, we found that there was no increase in potato glycoalkaloid levels in the transgenic tuber samples. However, our untargeted metabolomics revealed elevated levels of β-CA in the transgenic potatoes, which was confirmed by the targeted analysis. The natural occurrence of β-CA and γ-glutamyl-CA, of which the toxicological effects are identical, were first characterized in *Vicia angustifolia* and *Vicia sativa*.[41,50] The toxicity levels[43] and species-dependent toxicity[51] of β-CA are known, and the β-CA levels that accumulated in the current analytical samples (both transgenic and nontransgenic materials) should not be harmful to humans.

Although the number of analyzed plants was limited, the current results indicate that further research and analyses are required to confirm whether or not these differences are indeed significant and if they reflect a clear change in the metabolic profile from nontransgenic to transgenic potato tubers. It is possible that latent metabolic activities in both transgenic and nontransgenic crops may be reactivated, as suggested by the stimulated stress responses. The global metabolome profiles, particularly the profiles of unfavorable metabolites, could not be reasonably explained by the gene transcript levels alone. Untargeted metabolomics together with targeted analyses play a critical role in the clarification of metabolic fluctuations in crops. Further routine metabolome profiling is necessary to ensure food safety. In addition, metabolome profiling studies with transgenic crops conferred stress tolerance shed light on plant stress responses under environmental stresses, generating novel hypotheses that could contribute to further development of stress tolerance technologies.

■ ASSOCIATED CONTENT

Supporting Information

Additional figure and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

ACC, 1-aminocyclopropane-1-carboxylate; ACO, ACC oxidase; ACS, ACC synthase; CA, cyanoalanine; CAS, CA synthase; CN, cyanide; CS, cysteine synthase; ET, ethylene; GABA, γ-aminobutyric acid; GABAT, γ-aminobutyrate aminotransferase; GAD, glutamate decarboxylase; GDH, glutamate dehydrogenase; GGC, γ-glutamyl cyclotransferase; GGT, γ-glutamyl transpeptidase; GLS, glutathione synthase; GOGAT, glutamate synthase; GS, glutamine synthetase; HCN, hydrogen cyanide; NT, nontransgenic; SSAD, succinic semialdehyde dehydrogenase

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