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Development of imidazolinone herbicide tolerant borage (*Borago officinalis* L.)

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ABSTRACT

Borage (*Borago officinalis*) is an annual herb that produces a high level of gamma-linolenic acid (GLA) in its seed oil. Due to the recognized health benefits of GLA, borage is now commercially cultivated worldwide. However, an herbicide-tolerant variety for effective weed management has not yet been developed. Here we report the generation and characterization of ethyl methanesulfonate (EMS) induced borage mutant lines tolerant to the herbicide imidazolinone. An EMS-mutagenized borage population was generated by using a series of concentrations of EMS to treat mature borage seeds. Screening of the M2 and M3 borage plants using an herbicide treatment resulted in the identification of two imidazolinone-tolerant lines. Sequence analysis of two acetohydroxyacid synthase (AHAS) genes, AHAS1 and AHAS2, from the mutant (tolerant) and wild type (susceptible) borage plants showed that single nucleotide substitutions which resulted in amino acid changes occurred in AHAS1 and AHAS2, respectively in the two tolerant lines. A KASP marker was then developed to differentiate the homozygous susceptible, homozygous tolerant and heterozygous borage plants. An *in vitro* assay showed that homozygous tolerant borage carrying the AHAS1 mutation retained significantly higher AHAS activity than susceptible borage across different imazamox concentrations. A herbicide dose response test indicated that the line with the AHAS1 mutation could tolerate four times the normally used field concentration of “Solo” herbicide.

1. Introduction

Borage (*Borago officinalis* L.), an annual herb, originates from the Mediterranean region. Historically, borage has been used for culinary and medicinal purposes [1]. In recent decades, borage oil has attracted great interest for medical and nutritional uses due to its high content of gamma-linolenic acid (GLA) [2–5]. GLA oil has shown positive effects in treating a number of clinical conditions such as atopic eczema, psoriasis and rheumatoid arthritis [3,4,6,7–9,10]. In addition, studies also suggest that consumption of borage oil can improve fatty acid metabolism and skin function [11], reduce atherosclerotic symptom [12], decrease serum total cholesterol and lower blood pressure [13]. Because of these health benefits, many nutraceutical supplements, functional foods and body-care products have now been enriched with borage oil, resulting in a surge of borage farming.

Weed management is a critical component in borage cultivation. Due to the lack of an herbicide tolerant variety, farmers have only limited tools, including seeding timing, stale seedbed and mechanical weeding, to control annual weed species in borage crops. However, failure of these techniques greatly impacts borage seed yields [14]. Consequently, few farmers are willing to cultivate borage on a large scale, and borage producers eagerly await an effective approach for weed management.

Using herbicide-resistant varieties has been proven to be one of the most effective methods for weed control. As such, many herbicides with different inhibition mechanisms have been exploited in field crop production. Among these, imidazolinone herbicides, which have broad spectrums of weed control and relatively low chemical application rates, have received much attention. This group of herbicides inhibits an essential enzyme, acetohydroxyacid synthase (AHAS), which catalyzes the biosynthesis of branched chain amino acids in plants [15]. Many imidazolinone-tolerant crops have been developed through mutagenesis and selection techniques [15–19]. This report describes the generation, identification and characterization of ethyl

Abbreviations: AHAS, acetohydroxyacid synthase; ai/ha, active ingredient per hectare; ALS, acetolactate synthase; BLAST, basic local alignment search tool; EMS, ethyl methanesulfonate; FAM, fluorescein amidite; FRET, fluorescence resonance energy transfer; GLA, gamma-linolenic acid; HEX, 5-hexadecanoyl fluorescein; KASP, kompetitive allele specific PCR; PDC, pyruvate decarboxylase; PVP, polyvinylpyrrolidone; RACE-PCR, rapid amplification of cDNA ends PCR; SNP, single nucleotide polymorphism; ThDP, thiamine diphosphate

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methanesulfonate (EMS)-induced mutations in borage plants resulting in tolerance to imidazolinone herbicide.

2. Materials and Methods

2.1. EMS mutagenesis and screening of the mutant population for herbicide-tolerance

Approximately 3 kg of borage cv Biostar seeds were divided into 6 groups for mutagenesis treatments. The seeds (M1) were soaked in 0.5%, 1.0% or 1.5% (v/v) of EMS (Sigma-Aldrich, Milwaukee, Wisconsin, USA) solution for 8 or 16 h. After rinsing and drying, the mutagenized seeds were sown at the AgQuest research farm, Saskatoon, SK, in June, 2012. The M1 borage plants were grown to maturity and M2 seeds were harvested in groups according to the EMS treatments.

Herbicide tolerance screening was carried out in a growth chamber. M2 seeds were planted at 1–2 cm in 25 × 50 cm flats containing commercial potting mix (Sunshine Mix 3; Sun Gro.) under a 16 h light (22 °C) and 8 h dark (16 °C) cycle. Each flat contained 72 seeds. The group 2 herbicide, "Solo" (BASF Corp.), was applied over foliage when most plants were at the two-leaf stage. The spray solution included 84 g ai/ha (active ingredient per hectare) imazamox with the adjuvant Merge (BASF Corp.) at 0.5% (v/v). A moving nozzle cabinet sprayer with a flat-fan nozzle tip was calibrated to deliver 102 L/ha spray solution in a single pass. M2 plants were visually evaluated 21 days after spraying by comparing herbicide treated and untreated wild-type borage controls. Putative tolerant M2 plants were transplanted, self-pollinated and grown to maturity.

2.2. Cloning of borage AHAS genes and identification of point mutations responsible for imidazolinone tolerance

Total RNA was extracted from borage leaves using Trizol® according to the manufacturer's protocol (Invitrogen Corp, Carlsbad, California, USA). RACE-Ready cDNAs were synthesized by SMARTer RACE cDNA amplification (Clontech Laboratories, Mountain View, California, USA). The sunflower AHAS gene was used as a query in a blast search against a database of borage partial genomic sequences using CLC workbench software (CLC Bio, Aarhus, Denmark). Primers (Table S1) were designed based upon the borage DNA fragments having the highest homology with the query sequence. RACE-PCR reactions were carried out to amplify the missing ends of borage *AHAS1* and *AHAS2*.

After assembly of the missing ends of *AHAS1* and *AHAS2*, full-length AHAS genes were amplified by PCR using the proof-reading polymerase. The same, *AHAS1* and *AHAS2* genes from imidazolinone tolerant borage were also amplified using the same primer sets. By comparing AHAS genes from wild-type and tolerant borage plants using Vector NTI software (Invitrogen, Carlsbad, California, USA), the point mutations responsible for imidazolinone tolerance were identified in both AHAS genes.

2.3. Development of a KASP SNP marker linked to imidazolinone tolerance gene (AHAS1 mutation) in borage

Primers for KASP genotyping (Table S2) were developed following the manual from LGC genomics (LGC genomics, Teddington, Middlesex, UK), and 40 M3 borage plants from the *AHAS1* mutant tolerant line were randomly selected for KASP genotyping and imidazolinone screening. Leaf tissue was collected at the 2–4 leaf stage, then snap-frozen in liquid nitrogen and stored at −80 °C for genomic DNA extraction using the CTAB method [20]. The KASP genotyping assay was performed on a 96-well plate; each well contained about 10 μL of reaction mixture, including 5 μL of genomic DNA, 5 μL of 2X master mix (LGC Genomics, Teddington, Middlesex, UK), and 0.14 μL of primer mix. Four homozygous susceptible controls and two controls lacking DNA were also included. The KASP assay thermal cycling program was as follows: at 25 °C for 30 s; hold at 95 °C for 15 s; 10 cycles X (95 °C for 20 s, 61 °C for 60 s); 30 cycles X (95 °C for 20 s, 55 °C for 60 s); and a final hold at 25 °C for 30 s.

2.4. In vitro AHAS activity assays of tolerant and susceptible borage plants

2.4.1. Preparation of enzyme sources

At the 4–6 leaf stage, about 3–4 g of leaf material was harvested and snap-frozen in liquid nitrogen and stored at −80 °C. The *in vitro* assay was conducted according to the method of Yu et al. [21] with some modifications. About 1 g of the frozen material was ground to a fine powder in liquid nitrogen, then homogenized in 4 vol of cold extraction buffer containing 0.1 M K_2HPO_4 (pH 7.5), 10 mM sodium pyruvate, 0.5 mM $MgCl_2$, 0.5 mM thiamine pyrophosphate (TPP), 10 μM flavin adenine dinucleotide (FAD), 4 mM DTT, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10% v/v glycerol, and 4% w/v soluble PVP. The homogenate was filtered through two layers of miracloth and the filtrate was centrifuged at 30,000*g* for 20 min at 4 °C. The supernatant was brought to 30% saturation by drop-wise addition of solid $(NH_4)_2SO_4$ to allow gum-like substances to form and to be removed; the supernatant was then brought to 50% saturation with $(NH_4)_2SO_4$. The solution was allowed to stand on ice for 10 min with occasional stirring and any additional "gums" were removed. The sample solution was then divided into two technical replicates before centrifugation. After centrifugation at 100,000*g* for 20 min at 4 °C, the upper gummy protein layer was carefully collected as an enzyme source for the activity assay.

2.4.2. Enzyme incubation and colorimetric reaction

The gummy protein layer was re-dissolved in 1.4 mL incubation buffer containing 50 mM K_2HPO_4 buffer (pH 7.0), 100 mM sodium pyruvate, 10 mM $MgCl_2$, 1 mM TPP and 1 μM FAD. The amount of protein in each sample was determined by the Bio-Rad protein assay using a dye reagent (#500-0006, Bio-Rad, Mississauga, Ontario, Canada). The standards consisted of a series of concentrations of 0, 1, 5, 25, 125, 625 μM imazamox PESTANAL®, analytical standard (Sigma-Aldrich, Milwaukee, Wisconsin, USA) in 200 μL of the reaction mixture. The mixture was incubated at 37 °C for 1 h. To stop the reaction, 32 μL of 1 M H_2SO_4 was added, and decarboxylation was allowed to occur at 65 °C for 15 min. The samples were then incubated with 34 μL of creatine solution (1% w/v in 2N NaOH) and 68 μL of α-napthol solution (5% w/v in 2N NaOH) at 60 °C for 15 min. After cooling for 10 min at room temperature to maximize color development, the mixture was briefly centrifuged at 13,000*g*. The reaction solution of 200 μL was transferred to a 96 well microtiter plate and the absorbance at 520 nm was measured. The background level was determined by adding 32 μL of 5N NaOH to the reaction mixture after 1 h of incubation.

A unit of enzyme activity was defined as micromole of acetoin produced, and the specific activity of the AHAS enzyme in tolerant and susceptible borage was calculated on the basis of micromole acetoin produced per millgram protein per minute. Therefore, in order to quantify enzymatic levels, standard curves of acetoin were generated using a series of acetoin dilutions in the incubation buffer.

2.4.3. Herbicide type and dosage responses of AHAS1 mutant borage line

Homozygous *AHAS1* mutant plants were grown in a greenhouse under the same conditions described above. At the two to four leaf stage, "Solo" herbicide was applied over foliage at 2X (84 g ai/ha imazamox), 4X, 8X, 16X, 32X, 64X, 128X and 256X rates in an herbicide chamber. Sprayed M4 plants were visually evaluated at 21 days after herbicide application by comparing with untreated controls.

The homozygous *AHAS1* mutants were also tested with 8 types of group 2 herbicides including Solo (84 g ai/ha imazamox, BASF), Muster (45 g ai/ha ethametsulfuron-methyl, DuPont), Pursuit (102 g ai/ha imazethapyr, BASF), Everest 2.0 (116 g ai/ha flucarbazone, Arysta LifeScience), PrePass XC (20 g ai/ha florasulam, Dow AgroScience), Pinnacle SG (11 g ai/ha thifensulfuron, DuPont), Express SG (32 g ai/ha