Appendix 7

Comparison of Inhibition of AtAHAS (S653N) and AtAHAS (S653N R272K) by Imidazolinones and Sulfonylurea

COMPARISON OF INHIBITION OF *At*AHAS (S653N) AND *At*AHAS (S653N R272K) BY IMIDAZOLINONES AND SULFONYLUREA

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SUMMARY

Recombinant wildtype *At*AHASL, *At*AHASL S653N, and *At*AHASL S653N R272K polypeptides were expressed in AHAS minus bacterial cells. The extracts were assayed for AHAS activity in the presence of various concentrations of imidazolinone (Pursuit – imazethapyr) and sulfonylurea (Glean – chlorsulfuron) herbicides to determine if the R272K mutation has an effect on AHAS activity and herbicide tolerance. No differences in enzyme activity or inhibition of AHAS by Pursuit or Glean were observed between the *At*AHASL S653N and the *At*AHASL S653N R272K polypeptides, indicating that the R272K mutation does not alter AHAS activity in the absence or presence of imidazolinones or sulfonylurea compounds.

INTRODUCTION

Soybean (*Glycine max* L.) plants have been developed that are tolerant to the imidazolinone class of agricultural herbicides. The herbicide-tolerant soybean plants derived from a single transformation event, referred to as Cultivance Soybean Event 127, were produced by introduction of an imidazolinone-tolerant acetohydroxyacid synthase (*ahasl S653N*) large subunit (*csr1-2*) gene from *Arabidopsis thaliana* into the soybean plant genome via biolistics using the PvuII fragment of transformation vector pAC321. As part of the molecular characterization of the DNA insert of Event 127, the open reading frame of the *AtAHASL* gene was sequenced. This resulted in the identification of a single base pair mutation from G to A that conferred an amino acid change of arginine to lysine at amino acid position 272 (R272K). The purpose of this study was to determine whether the R272K S653N double mutation in the *AtAHASL* polypeptide alters the level of herbicide tolerant activity to imidazolinones or sulfonylureas as compared to the *AtAHASL* S653N single mutation. Feedback inhibition by valine and leucine was irrelevant and not assayed in this study because the *At*AHASS small subunit, which confers end-product feedback regulation to the catalytic large subunit, *At*AHASL, was not co-expressed in this heterologous system. Likewise, the *At*AHASS small subunit was not co-expressed with *At*AHASL in Cultivance Soybean Event 127.

MATERIALS AND METHODS

Bacterial expression of recombinant *At*AHAS polypeptides

The open reading frame of wild type *AtAHASL*, *csr1-2* (*AtAHASL S653N*), and *AtAHASL 2653N R272K*, were cloned into the bacterial expression vector, *pTrcHis* (Invitrogen Life Technologies, Carlsbad, CA). After sequence confirmation, the plasmids were transformed into AHAS minus *JMC1 Escherichia coli* cells and stored at –80° C as glycerol stocks.

Bacterial glycerol stocks were streaked onto LB agar plates with appropriate antibiotic selection and grown overnight at 37°C. Two ml liquid LB media plus antibiotic was inoculated with a single colony and placed on a rotary shaker at 220 rpm overnight at 37°C. One half ml was used to inoculate 25 ml LB plus antibiotic in 125 ml flasks and shaken at 220 rpm at 37°C until the OD_{600} reached 0.5-0.6. For induction of protein expression, isopropyl thiogalactoside (IPTG) was added at a final concentration of 100 μM and the cells were incubated at 16°C overnight with shaking at 200 rpm. The 25 ml cultures were then divided into 3 6-ml aliquots and centrifuged at 6000 x g for 15 min. The pellets were immediately frozen at –80°C.

Preparation of bacterial extracts

The frozen bacterial pellets were resuspended in 6 ml phosphate buffered saline (PBS) supplemented with 1 mg/ml lysozyme, vortexed, and then incubated on ice for 60 min. The extract was centrifuged at 10,500 x g for 15 min at 4°C. Protein was precipitated with ammonium sulfate by incubating the supernatant with an equal volume of saturated ammonium sulfate, which was added slowly drop-wise (final concentration 50% saturation of ammonium sulfate). Samples were incubated on ice for 30 min with constant stirring. Samples were centrifuged at 17,500 x g for 15 min at 4°C and the supernatant was removed. The protein pellet was resuspended in 6 ml 2x AHAS assay buffer [100 mM Na-pyruvate, 200 mM KH_2PO_4 pH 7.3, 20 mM $MgCl_2$, 2 mM thiamine pyrophosphate (TPP), 20 μM flavin adenine dinucleotide (FAD)] and retained on ice.

AHAS assay

AHAS activity was measured as described by Singh *et al*. (1988). AHAS catalyzes the synthesis of the acetolactate (an acetohydroxy acid) by the condensation of two molecules of pyruvate. In this assay the acetolactate produced by AHAS is converted to acetoin in the presence of acid and acetoin is detected colorimetrically ($A^{530\ nm}$) after interaction with creatine and napthol.

Pursuit (imazethapyr) and Glean (chlorsulfuron) stocks were diluted in water to 2x the desired final assay concentration. Fifty μl of the soluble protein extract was incubated with 50 μl of water or herbicide (0-1250 μM Pursuit and 0-1250 nM Glean) for 60 min at 37°C. The reaction was quenched by the addition of 20 μl 5% H_2SO_4 and incubated at 60°C for 15 min. Acetoin color was developed by incubating the quenched reactions with creatine (0.17%) and 1-napthol (1.7% in 4 N NaOH) for 15 min at 60° C. The absorbance was measured at 530 nm. Background samples were generated by pre-quenching with acid prior to assay incubation and the absorbances thus generated were subtracted from the test samples. All assays were conducted in triplicate and only mean values are reported here. The amount of activity obtained for each sample in the absence of inhibitor was assumed to be 100%. Samples assayed without inhibitor were used as control samples.

Sandwich ELISAs were performed in parallel to determine the relative quantities of expressed recombinant *At*AHAS protein. These assays indicated that expression and quantity of the recombinant *At*AHAS protein was comparable in each sample tested.

RESULTS

The AHAS activity of wildtype *At*AHASL was significantly inhibited by Pursuit and Glean (Figure 1). At the lowest concentration of Pursuit (9.75 μM), there was less than 20% of control activity remaining in the wildtype *At*AHASL extract. By comparison, *At*AHASL S653N and *At*AHASL S653N R272K were equally tolerant to Pursuit (Figure 1A). Likewise, the *At*AHASL S653N and *At*AHASL S653N R272K mutant polypeptides were equally tolerant to Glean, with an approximate 2.5 times higher IC_{50} than wildtype *At*AHASL (Figure 1B). These results indicate that there was no change in AHAS inhibition by imidazolinone or sulfonylurea that occurred by the addition of the R272K mutation to the S653N mutation of the *csr1-2* gene.

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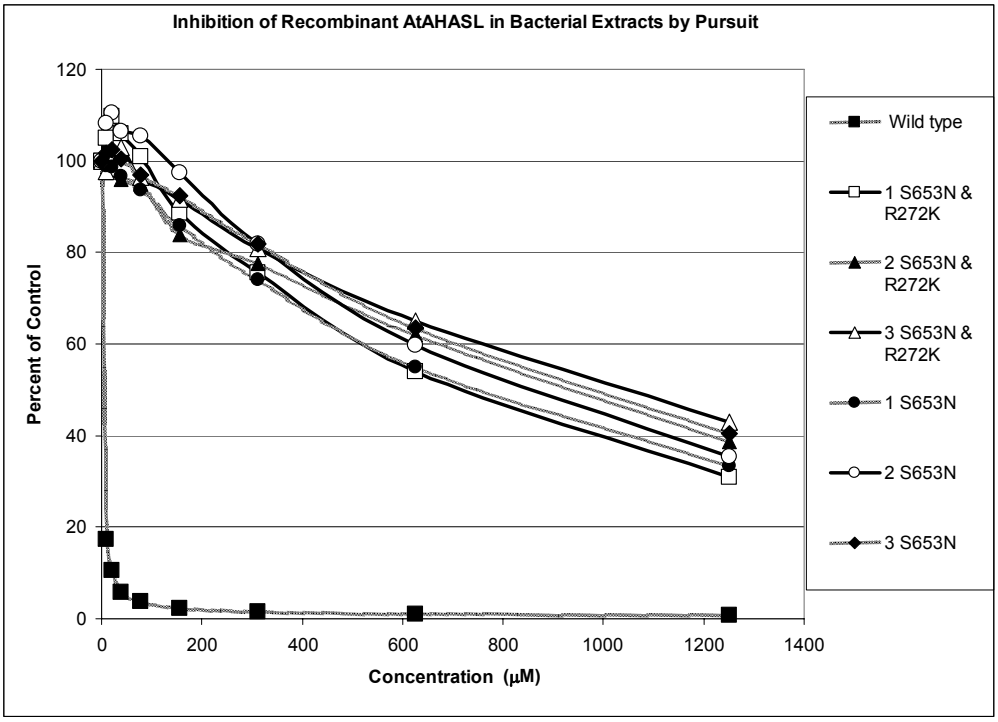
REFERENCES

Singh BK, Stidham MA, Shaner DL. (1988) Assay of acetyohydroxyacid synthase. *Anal. Biochem.* 171, 1730179.

Figure 1. AHAS assay of recombinant *At*AHAS expressed in *E. coli*. **A)** The assay was performed with and without (control) the indicated concentrations of Pursuit (imazethapyr). Independent cultures were assayed in triplicate and averaged. These data are representative of two independent experiments. **B)** The assay was performed with and without (control) the indicated concentrations of Glean (chlorsulfuron).

Legend: Wildtype- bacterial extract with expressed wildtype *At*AHAS; 1-3 S653N &R272K- 3 independent extracts with expressed *At*AHAS harboring the double mutation; 1-3 S653N- 3 independent extracts with expressed *At*AHAS harboring the single S653N mutation.

A



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