Research Article

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Expression and comparison of sweet corn CYP81A9s in relation to nicosulfuron sensitivity

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Abstract

BACKGROUND: Nicosulfuron, a sulfonylurea herbicide widely used for grass weed control in corn production, injures some sweet corn hybrids and inbreds. A specific cytochrome P450 (P450), CYP81A9, is suggested to be responsible for sensitivity to nicosulfuron and other P450-metabolized herbicides. Corn CYP81A9 enzymes were expressed in *E. coli* and investigated to find the factor(s) associated with their function and variation in metabolizing nicosulfuron.

RESULT: Recombinant expressed CYP81A9s from tolerant sweet corn inbreds produced an active form of P450, while CYP81A9 from a sensitive inbred produced an inactive form. Nicosulfuron bound to tolerant CYP81A9s, and produced reverse-type I ligand, while sensitive CYP81A9 showed no interaction with nicosulfuron. Investigation of 106 sweet corn inbreds showed variation in nicosulfuron injury. A survey of sweet corn CYP81A9 sequences showed mutations in codons for amino acids at 269, 284, 375, and 477 occurred in sweet corn inbreds with complete loss of P450 function (with mean injury >91%) and amino acid changes at 208 and 472 occurred in inbreds with moderate and complete loss of P450 function (with mean injury >14%).

CONCLUSION: Our results support that CYP81A9 enzyme is responsible for metabolizing nicosulfuron in sweet corn, and different types of amino acid changes in CYP81A9 sequence are associated with variation in nicosulfuron injury. Therefore, a careful selection of the tolerant allele will be critical for improving tolerance to nicosulfuron and several other P450-metabolized herbicides.

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

Keywords: *Zea mays*; *nsf1*; cytochrome P450; CYP81A9; nicosulfuron herbicide; P450-metabolized herbicide

1 INTRODUCTION

Cytochrome P450 metabolism of herbicides is an important mechanism of herbicide selectivity in plants.[1,2] Cytochrome P450s (or CYPs) are heme-dependent proteins capable of converting toxic herbicides into nontoxic compounds, typically called Phase I metabolism. The extent of detoxification in plant species varies widely depending on the levels and identities of P450 alleles expressed in species and types of herbicides.[1,3] To date, few herbicide-metabolizing P450s in plant species have been isolated and characterized. For example, Jerusalem artichoke CYP81B1 was expressed in yeast and characterized for hydroxylation of multiple fatty acids.[4] Soybean CYP71A10 was isolated and characterized in metabolizing four phenylurea herbicides including chlorotoluron.[5,6] Wheat CYP71C6 was expressed to show the function in chlorotoluron and triasulfuron metabolism.[7] Tobacco CYP71A11 and CYP81B2 were isolated to show their function in chlorotoluron metabolism.[8]

Nicosulfuron is a sulfonylurea postemergent herbicide used for control of specific broadleaf and grass weeds in corn (*Zea mays L.*).[9,10] Nicosulfuron inhibits acetolactate synthase (ALS) and, as a result, the synthesis of leucine, valine and isoleucine in plants.[11] Upon registration, use of nicosulfuron grew rapidly due to its low application rate (25 g ha^{-1}) and low presumed impact on the environment. While nicosulfuron is reported to be safe for most field corn hybrids, a number of studies have described large variation in the sensitivity of sweet corn lines to nicosulfuron.[12–15]

While herbicide-metabolizing P450s in corn have not yet been heterologously expressed and characterized, early genetic studies indicated that metabolism of nicosulfuron in corn is controlled by a single gene, *Nsf1* (nicosulfuron-1).[16] Plants homozygous for the dominant allele (*CYPCYP* or *Nsf1Nsf1*) were tolerant to nicosulfuron and other classes of herbicides.[12,14,17,18] Nicosulfuron showed the highest level of crop injury and yield loss in sweet corn in mutant homozygous *cypcyp* lines compared to tolerant heterozygous *CYPcyp* or homozygous *CYPCYP* lines.[12] Homozygous *cypcyp* lines also showed significant cross-sensitivity to various types of postemergent herbicides including four ALS-inhibitors (foramsulfuron, nicosulfuron, primisulfuron, and rimsulfuron), two hydroxyphenyl-pyruvate dioxygenase (HPPD)-inhibitors (mesotrione

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and tembortione), a growth regulator herbicide combination (dicamba+diflufenzopyr), a protoporphyrinogen oxidase (PPO)-inhibitor (carfentrazone) and a photosystem II (PSII)-inhibitor (bentazon).[14,17,18]

Map-based approaches identified the *Nsf1* gene and sequenced it in field corn populations of W703A (sensitive inbred) and B73 (tolerant inbred).[19] Analyses of North American field corn lines sensitive to nicosulfuron showed that most contained a 392 bp insertion in their *Nsf1* coding sequences compared to lines tolerant to nicosulfuron that would result in a truncated nonfunctional P450 protein. Relatively few, including several sweet corn lines, lacked this 392 bp insertion and had other undefined molecular defects responsible for their nicosulfuron sensitivities.[19] In a sweet corn population created from a cross between Cr1 (a sensitive inbred) and Cr2 (a tolerant inbred) lines, the chromosomal location responsible for herbicide cross-sensitivity, including nicosulfuron, was closely linked to *Nsf1*.[17] An additional 45 sweet corn hybrids and 29 sweet corn inbreds had the same or closely linked gene to Cr1, conferring cross-sensitivity to multiple postemergence herbicides including nicosulfuron and mesotrione.[20] *Nsf1* appears to be a critical factor responsible for multiple herbicide tolerance in both field and sweet corn, yet a functional connection of *Nsf1* to herbicide tolerance has not been fully established.

The protein coding sequence of corn *Nsf1* has 521 amino acids that produces CYP81A9 (isoflavone 2′-hydroxylase-like precursor). While there are no functional characterizations of any herbicide-metabolizing P450s in corn, related studies in rice (*Oryza sativa*) lines with mutations in *bel* gene that its wildtype *Bel* encodes CYP81A6 (73% amino acid identity to corn CYP81A9) showed sensitivity to bentazon and sulfonylurea herbicides.[21] Other studies in rice barnyardgrass (*Echinochloa phyllopogon*) populations have shown that overexpression of the *CYP81A12* and *CYP81A21* genes confer tolerance to two other ALS-inhibiting herbicides, bensulfuron-methyl and penoxsulam.[22] Heterologous expression of these two grass P450s in yeast metabolized bensulfuron-methyl through O-demethylation.[22] In corn, transcriptome comparisons in nicosulfuron-treated and non-treated plants have indicated that *CYP81A9* (*Nsf1*), *CYP71C3*, and three other P450s are overexpressed in response to nicosulfuron.[23] Other members of the corn CYP81A subfamily include CYP81A16, which shows 97% amino acid identity to CYP81A9 and also annotated as CYP81A9, as well as CYP81A1, CYP81A2, CYP81A3 and CYP81A4, which are more distantly related (53%, 75%, 77%, and 70% identity to CYP81A9, respectively).

Even though single gene inheritance was confirmed in metabolism of multiple P450-metabolized herbicides in sweet corn, variations in their levels of herbicide sensitivity were observed depending on the genotypes of cultivars examined, the herbicide tested, the plant's environment, and the presence of other chemicals.[13,24–27] The number of *Nsf1* alleles is the predominant factor determining variation in herbicide sensitivity. Homozygous *Nsf1Nsf1* sweet corn showed the highest tolerance, and homozygous *nsf1nsf1* showed the highest sensitivity, while heterozygous *Nsf1nsf1* often showed intermediate response to several herbicides.[25,26] Yet, even in inbred lines with homozygous *nsf1* alleles, variation in sensitivity to nicosulfuron or mesotrione were observed, suggesting presence of another genetic factor.[20] Among the critical chemical parameters, increases in sensitivity were observed in the presence of insecticides such as terbufos followed by nicosulfuron application.[13] Addition of the chemical safener isoxadifen-ethyl to P450-metabolized herbicide tembotrione reduced plant injury in the heterozygous genotype.[28]

Given the potentially complicated mechanisms involved in detoxification of P450-metabolized herbicides in sweet corn, it has become increasingly evident that the functional basis of P450-mediated herbicide metabolism must be explained in corn. As a result, this study aims to (i) evaluate activities of CYP81A9 variants translated from different *Nsf1* alleles, (ii) investigate *Nsf1* in sweet corn inbred lines for the 392 bp insertion documented in field corn, and (iii) identify the extent to which changes in the enzyme associate with nicosulfuron phenotypic sensitivity.

2 EXPERIMENTAL METHODS

2.1 Experimental setup and plant materials

Phenotypic evaluation of nicosulfuron sensitivity was conducted at the University of Illinois Plant Care Facility. The seeds of 106 sweet corn inbred lines were obtained from three private companies (50 from company A, 43 from company B, and 13 from company C) for phenotypic evaluation of nicosulfuron sensitivity and sequencing of *Nsf1*. Sweet corn inbreds Cr1 and Cr2 and field corn inbred B73 also were included. Ten kernels of each corn inbred line were planted into one of three separate rows in a 30 by 30 by 7 cm flat. The planting was replicated four times with one non-treated control. At the three-leaf collar stage, nicosulfuron (Accent, DuPont) was sprayed at 70 g a.i. ha^{-1} using a spray chamber in which the nozzle traveled over the stationary flats with the spray solution pressure of 207 kPa. The herbicide rate was twice the recommended rate to simulate an overlap of herbicide application in the field. At 2 weeks after treatment (WAT), plant injury was assessed visually on plants of each replication compared to the non-treated control based on the percentage leaf area chlorosis or necrosis.

After plant injury assessment, leaf tissue was collected by bulking leaves of five random plants from the non-treated control. Tissue samples from sweet corn (Cr1 and Cr2) and the field corn inbred line (B73) were collected for protein expression and functional analyses of P450. Leaf tissues were frozen in liquid nitrogen immediately after removal from the plant and stored at −80 °C. Total RNA was extracted from Cr1, Cr2, and B73 using the RNeasy mini kit (Qiagen) with on-column DNase I digestion (RNase-Free DNase Set, Qiagen). Total RNA was reverse transcribed to cDNA using PrimeScript II First-Strand cDNA Synthesis Kit (Takara). Genomic DNA was extracted from all inbred lines using CTAB protocol.[29] Primers used for checking the 392 bp insertion in *Nsf1* (*Nsf1* insertion), sequencing of *Nsf1* (*Nsf1* isolation and *Nsf1* sequencing) and expressing protein (*Nsf1* expression) are as listed (Table 1).

2.2 Expression and characterization of P450

The coding region of *Nsf1* was isolated from cDNA of Cr1, Cr2, and B73 using PCR. The primer pair used for *Nsf1* expression was designed with His_4 tag at C-terminal, *Nde*I, and *Xba*I restriction enzyme recognition sites for ligation into the pCWori vector for protein expression (Table 1). The pCWori vector was provided by the lab of Dr. Mary Schuler, University of Illinois. After purification of PCR product, enzyme digestion with *Nde*I and *Xba*I, and ligation to pCWori vector, the final products were transformed to *E. coli* DH5α strain (Invitrogen), co-expressed with the pGro7 chaperone plasmid.

Separate protein expression of CYP81A9 from Cr1, Cr2, and B73 was carried out in three, 1000 mL TB culture in a 2.5 L Fernbach flask with 50 mg/L of ampicillin and 20 mg/L of chloramphenicol.

Table 1. Primer list used for molecular analyses of Nsf1

Primer name	Forward (5′-3′)	Reverse (5′-3′)
Nsf1 insertion	GTCCGAGCCAGAGGTCTACA	GTGGTGGACGTGGTCTCC
Nsf1 isolation	CCCTCTTCTTGCTCCACTACC	TTAAGAACACCACGCATAGCTG
Nsf1 sequencing		766 CGAGGATCTTGTTCCTCACG 775[a]
Nsf1.1		
Nsf1.2	653 AGGCCCACGAGTTCAAGCAG 672	
Nsf1.3	997 ATGTCACTGCTGCTGAACCA 1016	
Nsf1 expression	AAGGGAATTC**CATATG**GATAAGGCCTACATCGCCGC[b]	CTGC**TCTAGA**TTAATGGTCATGGTGGAGCCTCTTAAGAACACCAC

[a] Nucleic acid position of the primer in $Nsf1_{B73}$ coding sequence.
[b] Restriction enzyme recognition sites were indicated in bold.

When OD_{600} of cultures reached 0.8, expression was induced by adding 1 mM isopropyl-β-D-thiogalactiopyranoside (IPTG), 168 mg/L heme precursor δ-aminolevulinic (ALA), and 4 g/L l-arabinose. After a 36-h incubation at 27 °C with shaking at 180 rpm, cells were harvested, and protein was isolated using the method by Rupasinghe *et al.*[30] Further purification of P450 was conducted using Ni-NTA agarose affinity column (Qiagen) with slight modification from the method by Rupasinghe *et al.*[30] After purification, $CYP81A9_{Cr1}$, $CYP81A9_{Cr2}$, and $CYP81A9_{B73}$ were dialyzed to remove 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS) and imidazole for further functional analyses.

Evaluation of P450 activity was performed using reduced carbon monoxide (CO) difference spectra and substrate binding assay. By adding approximately 1 mg of sodium dithionite (Sigma) and about 60 bubbles of CO at a rate of 1 bubble/s to the isolated $CYP81A9_{B73,}$ $CYP81A9_{Cr1}$, and $CYP81A9_{Cr2}$, absorbance was recorded between 400 nm and 500 nm after baseline correction using Cary 100 Bio spectrophotometer.[31] When cytochrome P450 is reduced by sodium dithionite and combined with CO, a maximum spectra absorbance at 450 nm is cytochrome P450's basic and distinctive characteristic. Its maximum absorbance can return to 420 nm when the enzyme is denatured and becomes biologically inactive.[32] The concentration of CYP81A9 was calculated with the peak size at 450 nm, based on the formula suggested by Guengerich *et al.*[33] For the substrate binding assay, different concentrations of nicosulfuron (Sigma) from 1.0 to 4.0 μM were added to 0.5 μM of $CYP81A9_{B73}$ and from 0.1 to 4.0 μM were added to 0.4 μM of $CYP81A9_{Cr2,}$ and absorbance was recorded between 350 nm and 500 nm. The reduced CO difference spectra and substrate binding assay were replicated three times.

2.3 Allelic evaluation of P450

The first investigation of genomic *Nsf1* on 106 sweet corn commercial inbred lines was to investigate the 392 bp insertion known to produce a truncated nonfunctional P450 protein. The investigation of 392 bp insertion also was done on 28 field corn inbred lines that showed over 50% injury to either mesotrione (another P450-metabolized herbicide) or nicosulfuron surveyed in the field and greenhouse, respectively. Using the *Nsf1* insertion primer (Table 1) and PCR Amplification Kit (Takara), size of PCR products was compared visually in 2% agarose gel.

After confirming absence of visible 392 bp insertion, isolation of genomic *Nsf1* from 106 sweet corn commercial inbred lines was conducted by PCR using PCR Amplification Kit (Takara) and *Nsf1* isolation primer pair covering 97% of full *Nsf1* coding sequence (Table 1). The PCR products were purified and sent to Roy J. Carver Biotechnology Center, University of Illinois for sequencing. Three *Nsf1* sequencing primers were used to cover most of the *Nsf1* sequence except the intron site (Table 1). The sequencing results were cleaned and aligned to compare for predicted amino acid changes among lines using Sequencer 5.4 (Gene Codes Corporation). Amino acid change information and nicosulfuron injury at 2 WAT were compared to identify association between the changes and injury levels. Statistical analyses involved analysis of variance (ANOVA) on phenotypic response to nicosulfuron, and calculations of means and standard errors were conducted using PROC GLM in SAS software 9.4 (SAS Institute Inc., Cary, NC, USA).

3 RESULTS

3.1 Biochemical characterization of P450

Isolated CYP81A9 from B73 (tolerant field corn inbred), Cr2 (tolerant sweet corn inbred), and Cr1 (sensitive sweet corn inbred) showed difference in their functional basis. When CO difference spectra results were compared, $CYP81A9_{B73}$ and $CYP81A9_{Cr2}$ showed absorbance peaks at 450 nm, whereas $CYP81A9_{Cr1}$ showed an absorbance peak at 420 nm (Fig. 1). Based on peak size, $CYP81A9_{B73}$, $CYP81A9_{Cr2}$, and $CYP81A9_{Cr1}$ separately expressed in 3 L culture of *E. coli* yielded up to 1.45 nmoL/mL, 0.44 nmoL/mL, and 0.34 nmoL/mL, respectively.

The substrate binding assay also showed differences among $CYP81A9_{B73}$, $CYP81A9_{Cr2}$, and $CYP81A9_{Cr1.}$ Gradual addition of pure nicosulfuron from 1.0 to 4.0 μM to 0.5 μM of $CYP81A9_{B73}$ and from 0.1 to 4.0 μM to 0.4 μM of $CYP81A9_{Cr2}$ increased absorbance at approximately 420 nm and decrease absorbance at approximately 390 nm (Fig. 2). The difference in absorbance between 420 nm and 390 nm increased up to 0.0042 and 0.0014 by addition of nicosulfuron to $CYP81A9_{B73}$ and $CYP81A9_{Cr2,}$ respectively. Absorbance did not change with addition of nicosulfuron to $CYP81A9_{Cr1}$ (data not shown).

3.2 Allelic evaluation of P450

Phenotypic evaluation of nicosulfuron sensitivity on 106 sweet corn inbred lines showed variation in injury response. Forty-nine lines showed injury less than 20%, hereafter called nicosulfuron tolerant. Five, 24, and 18 lines showed intermediate injury response to nicosulfuron from 20 to <40%, 40 to <60%, and 60 to <80% injury, respectively. Ten lines showed 80% or more

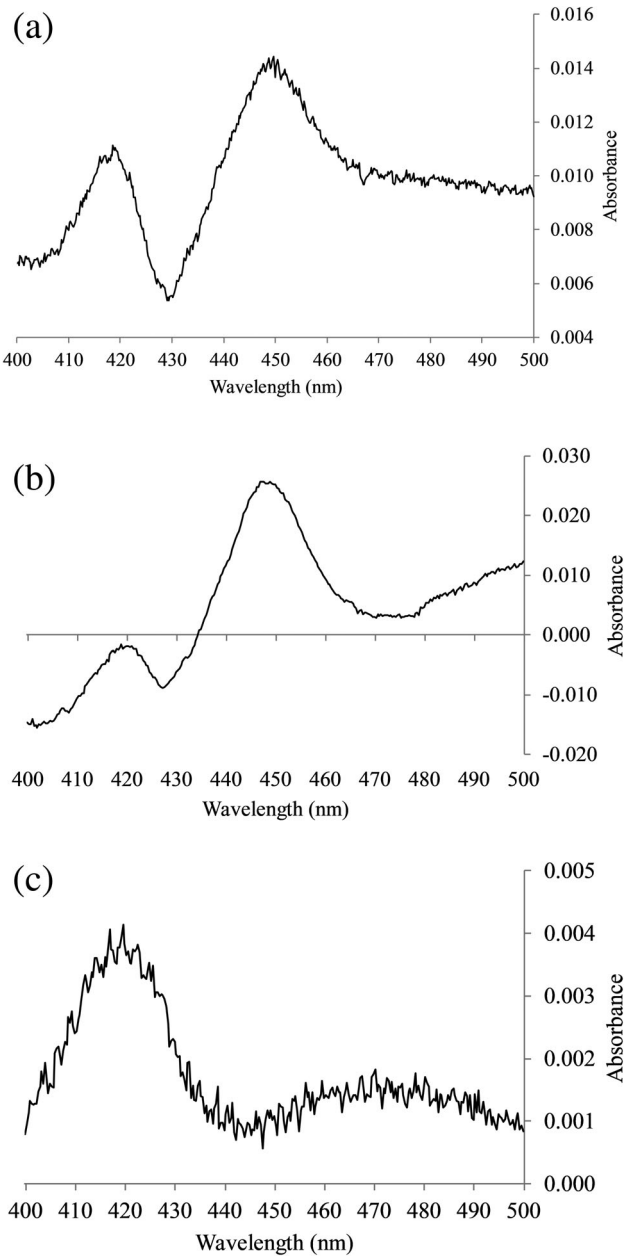

Figure 1. Carbon monoxide difference spectra of *E. coli*-expressed corn CYP81A9 from B73, Cr2 and Cr1.

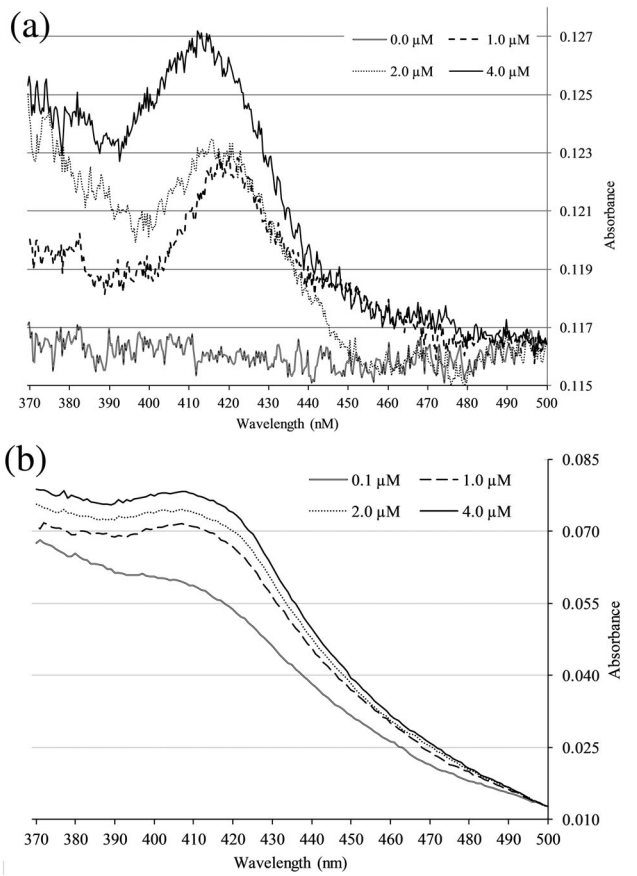

Figure 2. Substrate binding assay results by addition of different concentrations of nicosulfuron to 0.5 μM of CYP81A9$_{B73}$ and 0.4 μM of CYP81A9$_{Cr2}$.

injury, hereafter called nicosulfuron sensitive, resulting in death of most plants (Table 2). Variation in nicosulfuron sensitivity was observed across all seed sources. Phenotypic responses of inbred lines were consistent over runs ($p = 0.25$).

Comparing band sizes of PCR products in agarose gel showed that none of the sweet corn inbred lines had the 392 bp insertion in *Nsf1* coding region described by Williams *et al*. (2006). Meanwhile 28 field corn inbred lines with >50% injury to either mesotrione (one of P450-metabolized herbicide) or nicosulfuron had the 392 bp insertion in *Nsf1* (data not shown). The result led to sequencing of *Nsf1* to identify possible amino acid changes in sensitive sweet corn inbreds.

By comparing Nsf1 amino acid sequence of Cr1 (CYP81A9$_{Cr1}$) and Cr2 (CYP81A9$_{Cr2}$) to sequences of B73 (CYP81A9$_{B73}$), 9 amino acid changes were identified (Fig. 3). One amino acid change at 263 was ignored since it did not occur on all sweet corn CYP81A9s except CYP81A9$_{Cr1}$ and CYP81A9$_{Cr2}$. After sequencing 106 sweet corn inbred lines, 4 additional amino acid changes were shared among the inbred lines (Table 3). No frame shift in amino acid sequence was observed in any of the sweet corn inbreds.

Since some sweet corn lines have the same amino acid change patterns in their P450 sequences, 106 sweet corn lines were grouped into six groups sharing the same patterns of amino acid changes (Table 3). Thirty-three sweet corn lines had CYP81A9$_{B73}$ sequence whereas 11 lines had CYP81A9$_{Cr1}$ sequence. Ten lines had the same sequence as CYP81A9$_{B73}$ except at 150 where glycine changes to alanine. Two lines had mostly the same sequence as CYP81A9$_{B73}$ except at 28 and 31. Six lines had the same as CYP81A9$_{B73}$ at seven positions and showed amino acid changes at five positions. Forty-four lines had the same as CYP81A9$_{B73}$ at six positions and showed amino acid changes at six positions.

When sweet corn inbreds were grouped with the same sequence pattern at 12 amino acid positions, nicosulfuron injury of sweet corn inbreds within the same group showed similar responses (Table 3). Sweet corn inbreds with CYP81A9$_{B73}$ sequence, which represent most tolerant lines (33 out of 49 lines), showed mean nicosulfuron injury of 3%. Some of the tolerant lines (12 out of 49 lines) had either one amino acid change at 150 (10 out of 49 lines with mean injury of 3%) or two amino acid changes at 28 and 31 (2 out of 49 lines with mean injury of 3%). Sweet corn inbreds with CYP81A9$_{Cr1}$ sequence showed mean

Table 2. Number of sweet corn inbred lines exhibiting nicosulfuron injury in the greenhouse experiment

	No. of lines			
Injury (%)	Company A	Company B	Company C	Total
0 to <20	12	29	8	49
20 to <40	3	2	0	5
40 to <60	16	7	1	24
60 to <80	12	3	3	18
80 to 100	7	2	1	10
Total	50	43	13	106

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CYP81A9_CR1       MDKAYIAALSAAALFLLHYLLGRRAGGSGK--AKGSRRRLPPSPPAIPFLGHLHLVKAPF    58
CYP81A9_B73       MDKAYIAALSAAALFLLHYLLGRRAGGEGKAKAKGSRRRLPPSPPAIPFLGHLHLVKAPF    60
CYP81A9_CR2       MDKAYIAALSAAALFLLHYLLGRRAGGEGKAKAKGSRRRLPPSPPAIPFLGHLHLVKAPF    60
                  ***************************.**  ****************************

CYP81A9_CR1       HGALARLAARHGPVFSMRLGTRRAVVVSSPDCARECFTEHDVNFANRPLFPSMRLASFDG    118
CYP81A9_B73       HGALARLAARHGPVFSMRLGTRRAVVVSSPDCARECFTEHDVNFANRPLFPSMRLASFDG    120
CYP81A9_CR2       HGALARLAARHGPVFSMRLGTRRAVVVSSPDCARECFTEHDVNFANRPLFPSMRLASFDG    120
                  ************************************************************

CYP81A9_CR1       AMLSVSSYGPYWRNLRRVAAVQLLSAHRVGCMAPAIEAQVRAMVRRMDRAAAAGGGGVAR    178
CYP81A9_B73       AMLSVSSYGPYWRNLRRVAAVQLLSAHRVGCMAPAIEAQVRAMVRRMDRAAAAGGGGVAR    180
CYP81A9_CR2       AMLSVSSYGPYWRNLRRVAAVQLLSAHRVGCMAPAIEAQVRAMVRRMDRAAAAGGGGVAR    180
                  ************************************************************

CYP81A9_CR1       VQLKRRLFELSLSVLMETIAHTKTSRA----DSDMSTEAHEFKQIVDELVPYIGTANRWD    234
CYP81A9_B73       VQLKRRLFELSLSVLMETIAHTKTSRAEADADSDMSTEAHEFKQIVDELVPYIGTANRWD    240
CYP81A9_CR2       VQLKRRLFELSLSVLMETIAHTKTSRAEADADSDMSTEAHEFKQIVDELVPYIGTANRWD    240
                  ***************************    ****************************

CYP81A9_CR1       YLPVLRWFDVFGVRNKILDAVGRRDAFLRRLIDGERRRLDAGDDSESKSMIAVLLTLQKS    294
CYP81A9_B73       YLPVLRWFDVFGVRNKILDAVGTRDAFLGRLIDGERRRLDAGDESESKSMIAVLLTLQKS    300
CYP81A9_CR2       YLPVLRWFDVFGVRNKILDAVGRRDAFLGRLIDGERRRLDAGDESESKSMIAVLLTLQKS    300
                  ********************* ***** **************:****************

CYP81A9_CR1       EPEVYTDTVITALCANLFGAGTETTSTTTEWAMSLLLNHREALKKAQAEIDAAVGTSRLV    354
CYP81A9_B73       EPEVYTDTVITALCANLFGAGTETTSTTTEWAMSLLLNHREALKKAQAEIDAAVGTSRLV    360
CYP81A9_CR2       EPEVYTDTVITALCANLFGAGTETTSTTTEWAMSLLLNHREALKKAQAEIDAAVGTSRLV    360
                  ************************************************************

CYP81A9_CR1       TADDVPHLTYLQCILDETLRLHPAAPLLLPHESAADCTVGGYDVPRGTMLLVNVHAVHRD    414
CYP81A9_B73       TADDVPHLTYLQCIVDETLRLHPAAPLLLPHESAADCTVGGYDVPRGTMLLVNVHAVHRD    420
CYP81A9_CR2       TADDVPHLTYLQCIVDETLRLHPAAPLLLPHESAADCTVGGYDVPRGTMLLVNVHAVHRD    420
                  **************:*********************************************

CYP81A9_CR1       PAVWEDPDRFVPERFEGAGGKAEGRLLMPFGMGRRKCPGETLALRTVGLVLGTLLQCLCF    474
CYP81A9_B73       PAVWEDPDRFVPERFEGAGGKAEGRLLMPFGMGRRKCPGETLALRTVGLVLATLLQ--CF    478
CYP81A9_CR2       PAVWEDPDRFVPERFEGAGGKAEGRLLMPFGMGRRKCPGETLALRTVGLVLATLLQ--CF    478
                  ************************************************.****  **

CYP81A9_CR1       DWDTVDGAQVDMKASGGLTMPRAVPLEAMCRPRTAMRGVLKRL   517
CYP81A9_B73       DWDTVDGAQVDMKASGGLTMPRAVPLEAMCRPRTAMRGVLKRL   521
CYP81A9_CR2       DWDTVDGAQVDMKASGGLTMPRAVPLEAMCRPRTAMRGVLKRL   521
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Figure 3. Amino acid alignment of CYP81A9$_{Cr1}$, CYP81A9$_{B73}$, and CYP81A9$_{Cr2}$.

nicosulfuron injury of 91%, which represent all 10 sensitive lines and one line with intermediate response of 63%. A majority (44 out of 47) of sweet corn inbreds with intermediate injury had six amino acid changes with mean injury of 57%. The rest of the sweet corn inbreds (four tolerant lines and two intermediate lines), with mean injury of 14%, had five amino acid changes. The only difference between these two groups consisted of lines with intermediate and tolerant responses and was one amino acid change at 62 from glycine to alanine.

4 DISCUSSION

Isolation of Nsf1 variants, comparison of their spectra absorbance, and substrate binding results provide evidence that CYP81A9$_{Cr1}$

Table 3. Types of amino acid changes that occurred in sweet corn CYP81A9 sequences with their mean nicosulfuron injury and standard error

Variant	Position												N	Mean injury	Standard error
	28	31	269	284	375	477	208	472	62	150	425	438			
$CYP81A9_{B73 \text{ and } Cr2}$	E	AK	G	E	V	—	EADA	A	G	G	E	AG	33	3	1
	E	AK	G	E	V	—	EADA	A	G	A	E	AG	10	3	1
	S	—	G	E	V	–	EADA	A	G	G	E	AG	2	3	1
	E	AK	G	E	V	–	—	G	G	A	D	—	6	14	4
	E	AK	G	E	V	—	—	G	A	A	D	—	44	57	2
$CYP81A9_{Cr1}$	S	—	R	D	L	CL	—	G	G	G	E	AG	11	91	3

differs from $CYP81A9_{B73}$ and $CYP81A9_{Cr2}$ and lacks binding with nicosulfuron. The absorbance peak of $CYP81A9_{Cr1}$ at 420 nm shows that $CYP81A9_{Cr1}$ is an inactive form of P450, and possibly an improperly folded protein, while $CYP81A9_{B73}$ and $CYP81A9_{Cr2}$ are active P450s and have an absorbance peak at 450 nm. Failure to be expressed in its correct form is likely the reason that $CYP81A9_{Cr1}$ displayed no change in absorbance pattern with addition of nicosulfuron in substrate binding assays. With the addition of nicosulfuron, $CYP81A9_{B73}$ and $CYP81A9_{Cr2}$ showed reverse type I substrate binding pattern, creating a weak bonding of functional group to the heme iron and promoting low-spin state stabilized by water in the resting state. Due to the low concentration of CYP81A9s used for binding assays, absorbance difference from 420 nm to 390 nm is relatively small. Yet, the consistent absorbance patterns and similar results across the replicated trials support nicosulfuron affinity to $CYP81A9_{B73}$ and $CYP81A9_{Cr2}$.

The general reaction of P450 metabolizing herbicides can be explained by P450 with cytochrome P450 reductase transferring electrons from NADPH to substrate ($S\text{-}H + O_2 + NADPH + H^+ \rightarrow S\text{-}OH + H_2O + NADP^+$). Typical reaction of P450 results in ring hydroxylation, alkyl hydroxylation, and N/O demethylations of the herbicide. Previous studies showed detoxification of different types of ALS-inhibiting herbicides such as overexpressed CYP81A12 and CYP81A21 in *Echinochloa phyllopogon* metabolizing bensulfuron-methyl through O-demethylation.[22] Also, Moreland *et al*. showed that tolerant corn P450 sufficiently metabolizes nicosulfuron into hydroxypyrimidine.[34] Successful binding of nicosulfuron with $CYP81A9_{B73}$ and $CYP81A9_{Cr2}$ provides evidence that nicosulfuron is metabolized by CYP81A9, resulting in hydroxylation of the chemical that is non-toxic to the plant.

Inactivity of $CYP81A9_{Cr1}$ and its inability to bind to nicosulfuron can be explained by amino acid changes within the protein compared to $CYP81A9_{B73}$. This is supported by our survey result that found sensitive sweet corn inbreds with the same amino acid changes with $CYP81A9_{Cr1}$. Furthermore, variation in CYP81A9 sequences were found with moderately sensitive sweet corn inbreds. Amino acid changes in sensitive sweet corn with CYP81A9s did not follow other known amino acid changes in P450s or happen at known conserved domains or substrate binding sites. Nicosulfuron sensitive field corn with the 392 bp insertion made a truncated protein that resulted in missing substrate binding sites and heme binding sites.[19] While all sensitive field corn lines we tested had this truncated CYP81A9, sensitive sweet corn inbreds we tested did not have this truncated CYP81A9. Instead, they had various amino acid changes in CYP81A9 that may indicate sweet corn inbreds have been bred with relatively more diverse sources of P450 than field corn. Cytochrome P450s belong to a superfamily with highly diverse sequences while maintaining overall conserved tridimensional structure. When these conserved sequences were compared to $CYP81A9_{B73}$ and $CYP81A9_{Cr1}$, none of the amino acid changes occurred at the known conserved sequences such as F-x-x-G-x-R-x-C-x-G (heme binding site), P–P-x-P, W-x-x-R, E-x-x-R or P-E/D-R/H-F/W (Fig. 4).[35,36] Also, when all sequences were compared to the known substrate recognition sites (SRS) of *Arabidopsis* CYP81D1, none of the amino acid changes occurred at SRS (Fig. 4).[37,38] Amino acid changes in P450s were not linked to direct interference of protein function, but rather, may be related to more complicated structural modification that lead to variation in nicosulfuron injury.

Investigation of amino acid changes among sweet corn CYPs and association of these changes with nicosulfuron injury identified possible amino acid changes that are linked to the variation in nicosulfuron sensitivity. Sweet corn inbreds with a sequence the same as $CYP81A9_{Cr1}$ had nicosulfuron injury above 90%, indicating the changes in this sequence relate to complete loss of protein function (Table 3). Eight amino acid changes occurred in $CYP81A9_{Cr1}$ compared to $CYP81A9_{B73}$. However, changes at 28 and 31 close to the N-terminus are likely unrelated to nicosulfuron sensitivity since they also occurred in tolerant CYP81A9s. Changes at 269, 284, 375, and 477 only occurred in sensitive CYP81A9s, indicating these amino acid changes relate to complete loss of P450 function. Changes at 208 and 472 may relate to variation in sensitivity since they occurred in both CYP81A9s with intermediate and complete nicosulfuron injury.

Other amino acid changes also related to variation in nicosulfuron injury. Amino acid changes at 425 and 438 may relate to variation in sensitivity since they occurred in sweet corn lines with intermediate injury (Table 3). Two groups of CYP81A9 sequence with intermediate injury had the same sequence except at 62. A change at 62 with alanine only occurred in sweet corn lines with relatively higher mean sensitivity than lines with glycine at 62. Collectively, these amino acid changes likely determine how the enzyme forms or binds with nicosulfuron. Further investigation of protein modeling and metabolic analysis will shed light on how the amino acid changes affect protein stability or interaction.

Isolation and purification of CYP81A9 in corn is a critical first step to expand direct understanding of P450-metabolized herbicide detoxification. The interaction of CYP81A9 with other P450-metabolized herbicides, such as HPPD-inhibitors, PPO-inhibitor (carfentrazone), PSII-inhibitor (bentazon) and other ALS-inhibitors, should be further investigated as previous studies showed that herbicides with different sites of action were metabolized by a single P450.[39,40] Our results on CYP81A9s with nicosulfuron shows that P450 function in sweet corn depends on their diverse amino acid sequence variations. Our results also show that despite single gene inheritance, plant response to nicosulfuron

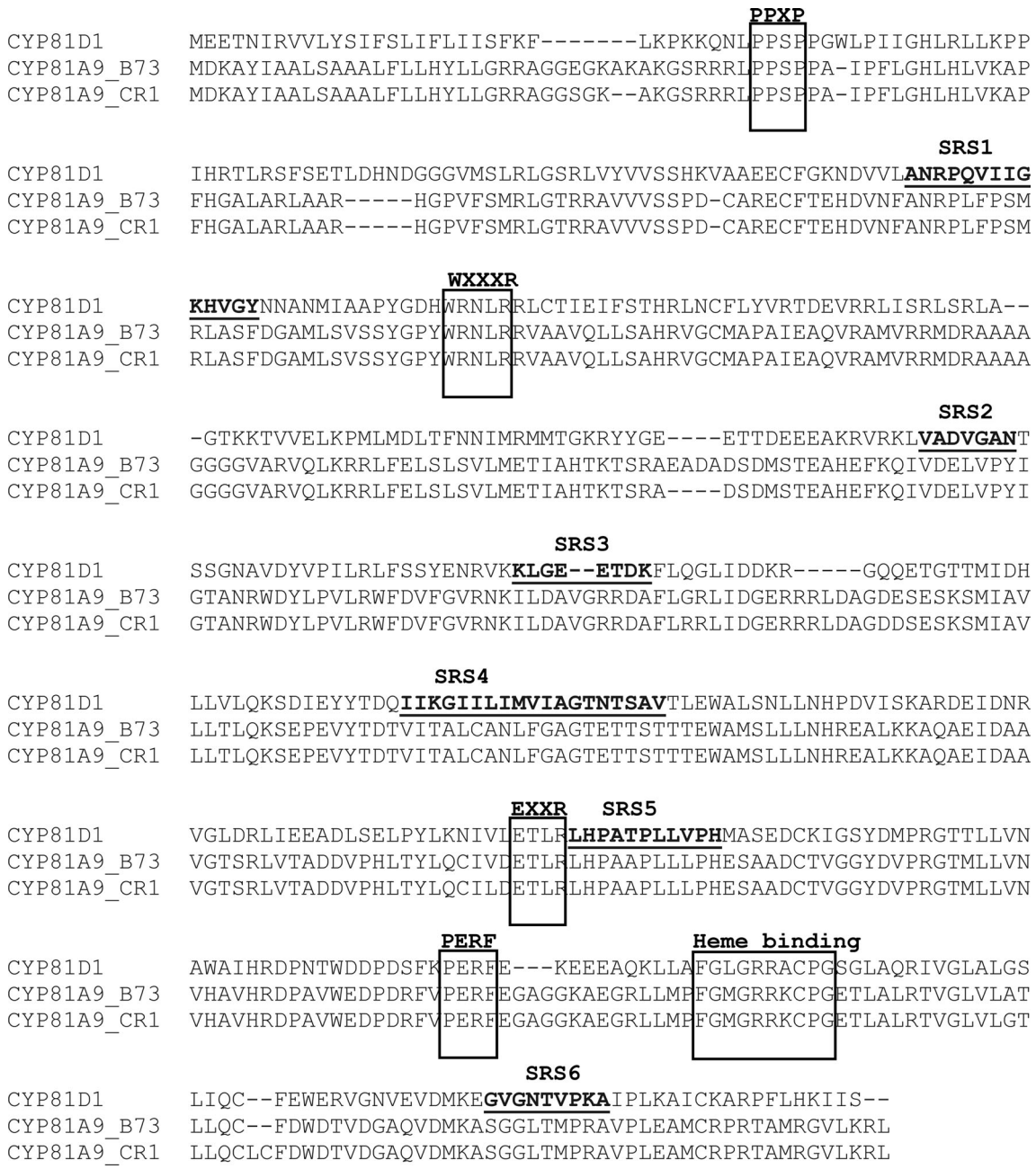

Figure 4. Protein sequence alignment of corn CYP81A9s with *Arabidopsis* CYP81D1 to identify possible changes on known conserved sites in squares and substrate recognition sites (SRS1-6) in bold and underlined.

varies depending on *Nsf1* alleles due to P450 sequence variation. In corn, especially sweet corn, sensitivity to nicosulfuron and other P450-metabolized herbicides is still an on-going problem for private and public breeding programs. Therefore, a careful investigation of *Nsf1* alleles and selection of tolerant alleles will improve P450-metabolized herbicide tolerance.

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SUPPORTING INFORMATION

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

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