Molecular mechanism of resistance in a multiple-herbicide resistant *Echinochloa phyllopogon*

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Echinochloa phyllopogon is an allotetraploid grass species and one of the most serious weeds in flooded rice fields. A herbicide-resistant (R) population of *E. phyllopogon* has been found in the Sacramento Valley of California, USA, with plants showing multiple resistance to herbicides with different modes of action and from different chemical groups such as acetolactate synthase (ALS) inhibiting herbicides and acetyl-CoA carboxyase (ACCase) inhibiting herbicides. Previous studies suggested that the multiple resistance is caused by non-target-site resistance mediated mainly by enhanced herbicide detoxification by cytochrome P450s (P450s), not by target-site resistance as observed in most cases of resistance to ALS and ACCase inhibitors in other weed species.

The first focus of this study was the analysis of genes encoding herbicide target-sites (Chapter I). I confirmed that multiple resistance was not caused by a target-site resistance mechanism and also discussed the influence of copy number of genes encoding target-sites on the evolution of herbicide resistance in polyploid species. Then, I analyzed P450 genes belonging to the CYP81A subfamily and examined their involvement in the resistance to three ALS inhibitors: bensulfuron-methyl (BSM), penoxsulam (PX) and bispyribac-sodium (BS) (Chapter II). The results suggested that two CYP81A genes are involved in BSM and PX resistances but are not involved in BS resistance. For BS resistance, I analyzed additional P450 genes of *E. phyllopogon* and identified candidate genes related to BS resistance (Chapter III).

Chapter I Isolation and expression of genes for acetolactate synthase and acetyl-CoA carboxylase

Target-site resistance is the major cause of herbicide resistance to ALS- and ACCase-inhibiting herbicides in arable weeds, whereas non-target-site resistance is rarely reported. The resistance mechanism of *E. phyllopogon* R plants is presumed not to be due to resistant ALS or ACCase

because enzyme assays revealed that the enzymes' sensitivity to ALS inhibitors or an ACCase inhibitor did not differ between the R and susceptible (S) plants, although verification of no genetic changes has yet to be conducted. To confirm that there are no genetic changes within the target-site encoding genes and to explore why target-site resistance had not occurred, I isolated the target-site genes for these herbicides from *E phyllopogon* and determined their expression levels in R plants. Two complete ALS genes and the carboxyltransferase domain of four ACCase genes were isolated and characterized. The nucleotide sequences were completely identical between the R and S plants, indicating that the resistance of *E. phyllopogon* to ALS and ACCase inhibitors is not due to target-site resistance. All copies of the ALS and ACCase genes except for *ACC4* were expressed with slight differences found in the expression patterns of the tested organs. The existence of three active ACCase genes and the difference in their relative expression levels could influence the occurrence of target-site resistance to ACCase inhibitors in *E. phyllopogon*.

Chapter II Identification of cytochrome P450 genes involved in different classes of ALS inhibitor resistances

Section I Establishment of herbicide susceptibility assay

The R line exhibits resistance to three different chemical groups of ALS inhibitors, BSM (sulfonylurea group), PX (triazolopyridine group) and BS (pyrimidinyl carboxy group). In this study, the herbicide susceptibilities on solid medium supplemented with each herbicide were evaluated because considerable variation in the herbicide responses of the R and S lines were observed when evaluated via a spray treatment method, as conducted in previous reports. In the solid medium method, the R and S plants had reproducible responses between experiments, and the R/S resistance index determined by the 50% growth inhibition rate in the presence of BSM, PX or BS was calculated to be 1100, 6.2 and 1.8, respectively. The values for BSM and PX resistances were

generally similar to previous reports. On the other hand, the value for BS resistance was much smaller than previously reported. Accordingly, the solid medium method can be used to evaluate BSM and PX susceptibilities. Further work is required to develop a method for evaluating BS susceptibility .

Section II Involvement of two cytochrome P450s, CYP81A12 and CYP81A21, in bensulfuron-methyl resistance

The resistance mechanism for BSM is assumed to be due to enhanced activities of herbicide-metabolizing P450s. To elucidate the molecular basis for the BSM resistance, I isolated and analyzed several P450 genes that are members of the CYP81A subfamily in *E. phyllopogon* because a rice P450 from this group was reported to be involved in BSM tolerance. In a comparison of gene expression between the R and S plants, *CYP81A12* and *CYP81A21* were more actively transcribed in the R plants under both the BSM-treated and -untreated conditions. The two genes conferred BSM resistance to Arabidopsis; transgenic Arabidopsis plants survived in media containing BSM at a level in which wild-type completely stopped growing. Heterologous expression of the *CYP81A12* or *CYP81A21* genes in yeast metabolized BSM through O-demethylation at the 4-position of the pyrimidine ring. Segregation of resistance in the F_2 generation from a cross of the R and S plants indicated that a single genetic element regulates BSM resistance. In F_6 recombinant inbred lines, BSM resistance co-segregated perfectly with higher transcript levels of both *CYP81A12* and *CYP81A21*. These results strongly suggest that overexpression of the two P450 genes conferred BSM resistance in *E. phyllopogon*. Overexpression of the two genes could be regulated simultaneously by a single *trans*-acting element in the R line of *E. phyllopogon*.

Section III Involvement of two cytochrome P450s in penoxsulam resistance

Unexpected resistance is the most serious aspect of P450-mediate herbicide resistance, although it remains unclear if multiple-herbicide resistance in weeds is caused by incremental activity of a single P450 isozyme. I examined whether *CYP81A12* and *CYP81A21* are also involved in the resistance to the other two ALS inhibitors, PX and BS. Transgenic Arabidopsis highly expressing either *CYP81A12* or *CYP81A21* exhibited significant resistance to PX. In contrast, the susceptibility of transformed Arabidopsis to BS was not significantly different from wild-type Arabidopsis. From a cross of *E. phyllopogon* R and S plants, PX resistance segregated with a ratio of 3:1 (resistant+intermediate:susceptible) in the F_2 generation. In F_6 recombinant inbred lines, PX resistance co-segregated perfectly with higher transcript levels of both *CYP81A12* and *CYP81A21*. Accordingly, the same P450s are strongly associated with BSM and PX resistance in *E. phyllopogon*, and the resistance mechanism for BSM and PX appeared to be identical. Conversely, different P450 isozymes seemed to be involved in BS resistance.

Chapter III Cytochrome P450 genes induced by bispyribac-sodium treatment

A previous study reported that the BS-metabolizing P450 activity was induced by BS treatment both in the R and S lines of *E. phyllopogon*, with activity being significantly higher in the R line. The results indicate that P450s induced by BS are involved in BS resistance. I used a PCR-based cloning strategy and isolated 32 putative P450 genes in addition to the previously isolated CYP81A genes (Chapter II) from the R line of *E. phyllopogon*. Expression analysis by real-time reverse transcription PCR revealed that seven of the isolated genes were up-regulated in response to BS treatment of seedlings at the three-leaf stage. The transcript levels and protein sequences of the seven genes were compared between the R and S lines. *CYP71AK2* and *CYP72A254* were transcribed prominently in the R line. Amino acid polymorphisms were found in three genes including *CYP72A254*.

Up-regulated expression of these genes is consistent with the inducible herbicide-metabolizing P450 activity under BS stress reported in the previous study.

Conclusions

In this study, I showed 1) the resistance to ALS and ACCase inhibitors are non-target-site based, 2) up-regulation of two P450s are associated with BSM and PX resistances, and 3) the possibility that other P450s are involved in BS resistance. This study will contribute to elucidating the mechanism of non-target-site resistance in arable weeds.