Genetic studies on the target-site resistance to sulfonylurea herbicides in *Schoenoplectus juncoides*

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CONTENTS

GENERAL INTRODUCTION

Schoenoplectus juncoides [Roxb.] Palla (also known as *Scirpus juncoides* Roxb. var. *ohwianus* T. Koyama) is a perennial Cyperaceae species, and one of the most noxious weeds in rice paddies in Japan referred to as *'Inu-hotarui'* in Japanese. A sulfonylurea (SU) herbicide, bensulfuron-methyl was registered to control *S. juncoides* in Japan in 1987. Subsequently, some other SUs such as pyrazosulfuron-ethyl and imazosulfuron were introduced.

SU herbicides are used in almost all the rice planted area (Takeshita 2004) due to their excellent efficacy on weeds and reduced risks on rice and mammals. SU was the sole chemical class of acetolactate synthase (ALS)-inhibiting herbicides registered in Japan to control *S. juncoides* in paddy rice for over 20 years. After an intensive use of SU herbicides in rice paddy fields, many SU-resistant weeds have been found from rice paddies in Japan. Nowadays, 19 SU-resistant paddy weeds in Japan are documented (Japan Herbicide Resistance Working Group 2014). SU-resistant *S. juncoides* was first found in Hokkaido, Japan in 1997 (Kohara *et al*. 1999). Today, SU-resistant *S. juncoides* is a nationwide problem (Uchino *et al.* 2006).

ALS is the first common enzyme in the biosynthetic pathways of branched-chain amino acids, such as valine, leucine and isoleucine. In addition to SU, imidazolinones (IMI), pyrimidinylcarboxylates (PC), triazolopyrimidines (TP), and sulfonylaminocarbonyltriazolinones (SCT) inhibit ALS (Duggleby and Pang 2000).

In many other weed species, biotypes showing resistance to ALS-inhibiting herbicides have been globally reported. In most cases, the target-site mutations of the ALS genes are found and the altered ALS causes insensitivity to the herbicides. The major mutation sites in ALS enzymes involved in resistance are Ala_{122}, Pro_{197}, Ala_{205}, Asp_{376}, Trp_{574} and Ser_{653} (the amino acid numbers are standardized to the *Arabidopsis thaliana* sequence), among which Pro_{197}, Asp_{376}, and Trp_{574} are frequently mutated in SU resistant weeds. In the ALS genes of field-selected SU-resistant weeds, the following amino acid substitutions are reported for the three sites: (i) Pro_{197}Ser (P197S), Pro_{197}Leu (P197L), Pro_{197}Ala (P197A), Pro_{197}Arg (P197R), Pro_{197}His (P197H), Pro_{197}Thr (P197T), Pro_{197}Gln (P197Q), Pro_{197}Asn (P197N) and Pro_{197}Ile (P197I); (ii) Asp_{376}Glu (D376E); and (iii) Trp_{574}Leu (W574L) and Trp_{574}Gly (W574G) (for whole the paragraph, Heap 2014).

All the amino acid substitutions are caused by a single-nucleotide substitution in the ALS genes, except for the P197I substitution in the ALS of *Sysimbrium orientale* (Boutsalis *et al*. 1999) and for the P197N substitution in the ALS of *Apera spica-venti* (Massa *et al.* 2011), both of which resulted from an adjacent double-nucleotide substitution.

Uchino *et al.* (2007) reported that *S. juncoides* has two ALS genes, *ALS1* and *ALS2*, and that SU-resistant *S. juncoides* plants have one of the known amino acid substitutions in either the ALS1 or ALS2 enzyme. The amino acid substitutions of P197S, P197H, P197A, P197L and W574L were

identified in ALS1, and so were in ALS2 separately (Yoshida *et al*. 2004; Uchino *et al*. 2006; Uchino *et al.* 2007; Okawa *et al*. 2013).

Such SU resistant weeds in Japan including *S. juncoides* have been managed by the mixture herbicides of a conventional SU and other herbicides with different modes of action, such as bromobutide, pyraclonil and benzobicyclon, or by a newly introduced ALS-inhibiting herbicides such as propyrisulfuron (Ikeda *et al*. 2011) and pyrimisulfan (Asakura *et al.* 2012). Nowadays, the total area treated with such SU-resistance-controlling herbicides is thought to be close to 100% of the planted area.

Researches on SU-resistant *S. juncoides* so far focused on 'weed management', especially on effective herbicides. On the other hand, research efforts from the 'weed biology' viewpoint have not been intensively made after Uchino *et al.* (2007). Since 2007, in terms of weed biology, except for the author's publications, there are only two original publications about SU-resistant *S. juncoides* (Yamada *et al.* 2013; Imaizumi *et al.* 2013), both of which are on genetic structure of populations.

The fundamental information, such as, (a) diversity of mutations, (b) geographical distribution of each mutation, (c) frequencies of each mutation, (d) resistance profiles (cross resistance and resistance levels) of each mutation, and (e) difference of ALS1 and ALS2 in frequencies and resistance profiles, is still lacking. Rapid diagnostic method (f) for SU-resistance *S. juncoides* is not known, either. Such information is essentially needed to construct weed management tactics.

In Chapter 1, the author investigated the occurrences of SU-resistant *S. juncoides* in paddy rice in Japan for the lacking information of (a), (b), (c) and (e). In Chapter 2 and 3, resistance profiles of diverse mutations were investigated for whole plants and enzymes, respectively for the lacking information of (d) and (e). In Chapter 4, the author intended to reveal the resistance profile of a newly found mutation, D376E for the lacking information of (a) and (d), specifically on this mutation. Lastly, in Chapter 5, the author developed and proposed rapid diagnostic methods to detect known mutations causing SU-resistance on *S. juncoides* for the lacking information of (f).

Showing solutions to these questions, the author will i) discuss how quantitatively a target-site mutation confers whole-plant resistance, ii) review the occurrences of *S. juncoides* mutations as a general discussion, along with an insight to a possible change in occurrences of herbicide resistance in near future, and iii) show insights for *S. juncoides* management.

Chapter 1. Occurrences of diverse SU-resistant *S. juncoides* in Japan

1-1. Introduction

For the frequencies and geographical distributions of the mutations in the ALS of SU-resistant *Schoenoplectus juncoides*, Uchino *et al*. (2006) reported that the P197S substitution was found the most frequently and that each substitution was found in ALS1 and ALS2 with an almost equal frequency in the accessions collected from rice paddy fields mainly in northern Japan. However, there is still a lack on the detailed occurrences and diversity of mutations in the ALS of SU-resistant *S. juncoides*. For example, there is little information about western part of Japan from the nationwide scale. Furthermore, occurrences on the local-field scale are not available.

In this Chapter, SU-resistant *S. juncoides* plants were collected from rice paddy fields mainly from the western part of Japan and the sequences of their ALS genes were analyzed in order to investigate the occurrence of ALS mutations not only on a nationwide scale but also at a local field scale in two areas.

1-2. Materials and Methods

Plant materials

Nationwide surveys

In 2001 and 2003, the seeds of suspected SU-resistant *S. juncoides* plants were collected from rice paddy fields severely infested with the species at three collection sites (Fig. 1-1 and Table 1-1; site numbers 20–22). The SU resistance of these seed populations was confirmed in conventional pot tests using imazosulfuron, based on the method reported by Tanaka (2003).

In 2005 and 2006, suspected SU-resistant *S. juncoides* plants were collected from rice paddy fields severely infested with the species at other 19 collection sites in 14 prefectures (Fig. 1-1 and Table 1-1; site numbers 1–19) in Japan. One paddy field or two neighboring paddy fields were selected as the sources of the plant materials in each collection site. The SU resistance of the collected plants was confirmed by the rooting method (Hamamura *et al*. 2003).

All the plants confirmed to be SU-resistant by the rooting method in the collections in 2005 and 2006 were used for the genetic analysis. For the three lines obtained in 2001 and 2003, the germinated seeds were cultivated and the resultant three-to-five green plants for each collection site were used for the genetic analysis. The seeds of a SU-susceptible biotype were obtained from a self-pollinated line derived from a plant collected in Kyoto City before SU herbicides were commercialized in Japan (Iwasaki and Ueki 1979). Then, the germinated seeds were cultivated and the resultant three green plants were used for the genetic analysis.

Local field-scale surveys

Nine paddy fields located close together (within a range of approximately 500m-radius) in Kasai in Hyogo Prefecture (fields A–I in Fig. 1-2a) and 14 paddy fields located close together in Miki in Hyogo Prefecture (fields J–W in Fig. 1-2b) were investigated in 2006 (Fig. 1-1 and Table 1-1; site numbers 23 and 24, respectively). *S. juncoides* plants were collected from the fields regardless of the infestation level. Two to six plants were collected from each of all fields, except for three fields where only one plant was collected because of very low infestation. The collected plants were directly used for the genetic analysis. The plants were kept in water until the results of the genetic analysis were obtained in order to be optionally checked for their SU resistance by the rooting method.

SU-resistance check by the rooting method

Each of the test plants was divided into two parts at the bottom. The existing roots were cut to 2 cm long. The plant materials were disinfected by dipping them into water solutions of benomyl (1000 mg L^{-1}) for 1 h. For each of the test plants, an aliquot was incubated in water containing imazosulfuron at 0.18 mg L^{-1} in glass tubes for 10 days while another aliquot was incubated in deionized water as an untreated reference. After the incubation, the newly generated roots were counted. The plants with new roots coming out in both conditions were judged to be SU-resistant, while the plants with new roots coming out only in the deionized water were judged to be SU-susceptible.

Sequencing of the ALS genes

Genomic DNA was extracted from the green, mature stems by using an extraction kit (Isoplant; Nippongene, Tokyo, Japan). The genomic DNA fragments of *ALS1* and *ALS2* were amplified separately by polymerase chain reaction (PCR) by using isogene-specific primer sets 1 and 2, respectively (Table 1-2) for 40 cycles of 95°C for 30 s, 55°C for 1 min and 72°C for 1 min. In case that the first PCR did not produce a strong band in the agarose gel electrophoresis, a nested PCR was subsequently carried out with the primer set 3 (Table 1-2). The PCR products were directly sequenced using the BigDye Terminator ver. 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) after being purified with a purification kit (ExoSAP-IT; USB, Cleveland, OH, USA).

1-3. Results

Nationwide surveys

The results of the genomic DNA sequencing are summarized in Table 1-3. The sequence of the ALS genes of the SU-susceptible line collected from Kyoto City showed that Pro_{197}, Asp_{376} and

Trp_{574} were conserved in both ALS1 and ALS2. In contrast, in each of all the SU-resistant plants (so-confirmed in advance by the rooting method or pot tests), a single-nucleotide substitution causing an amino acid substitution was observed at Pro_{197}, Asp_{376} or Trp_{574} in either ALS1 or ALS2. The P197S substitution was observed in ALS1 of the plants from Kuriyama, Ichihasama, Minoo, Matsue and Seiyo and in ALS2 of the plants from Mitsuke, Yokkaichi, Sasayama, Yamaguchi and Matsuyama. The P197L substitution was observed in ALS1 of the plants from Matsuyama, Tsu and Kakogawa and in ALS2 of the plants from Kato, Ono and Mihara. The P197H substitution was observed in ALS1 of the plant from Ajisu. The P197A substitution was observed in ALS1 of one of the plants from Mitsuke. The P197R substitution was observed in ALS1 of the plants from Ishikoshi and Sugagawa. The P197T substitution was observed in ALS1 of one of the plants from Ritto and in ALS2 of the plants from Tokushima. The D376E substitution was observed in ALS2 of the plants from Kurashiki. The W574L substitution was observed in ALS2 of the plants from Chiyokawa and Ritto.

All the SU-resistant plants investigated were homozygous in the substituted ALS genes, except for a plant from Mitsuke and a plant from Yamaguchi. In the collection sites where several plants were tested, the 'substitution type' (which amino acid site is substituted by what amino acid in which ALS gene, regardless heterozygous or homozygous) was uniform, except for Ritto, Mitsuke and Matsuyama, where two substitution types were observed in the same site.

Local field-scale surveys

The results of the genomic DNA sequencing of the plants from closely located fields in Kasai and Miki are shown in Table 1-4. Commonly in both collection sites, some *S. juncoides* plants showed single-nucleotide substitutions causing amino acid substitutions at Pro_{197} in either ALS1 or ALS2, while the other plants showed conserved Pro_{197}, Asp_{376} and Trp_{574} in ALS1 and ALS2. These unmutated plants were subsequently tested by the rooting method and were judged to be SU-susceptible without exception (Table 1-4). As a reference, some selected plants with Pro_{197} substitutions were tested simultaneously by the rooting method and all were found to be SU-resistant (Table 1-4).

In Kasai, the P197R substitution in ALS2 was observed in 15 plants from six fields (A, B, D, E, G and H), the P197L substitution in ALS2 was observed in two plants from two fields (B and F) and the P197S substitution in ALS2 (a heterozygote) was observed in a plant from Field E. Seven SU-susceptible plants were found in three fields (C, H and I). Fields B and E had two substitution types within each field and Field H had both SU-resistant and SU-susceptible plants within a single field. In Field I, only SU-susceptible plants were found.

In Miki, the P197S substitution in ALS2 was observed in 25 plants from 10 fields (J-R and W) and the P197L substitution in ALS1 was observed in 11 plants from six fields (J, K, O and R-T).

Seven SU-susceptible plants were found in four fields (K, R, U andV). Fields J and O had the two substitution types within each field. Fields K and R had a SU-susceptible plant in addition to the two substitution types within each field. In fields U and V, only SU-susceptible plants were found.

1-4. Discussion

Occurrence of SU-resistant mutants in the nationwide surveys

The number of sites in which each amino acid substitution of ALS was found in the collected *S. juncoides* plants is summarized in Table 1-5. Each of the two collection sites in the local field-scale surveys (Kasai and Miki) is counted as one site and included.

The frequently found amino acid substitutions were P197S and P197L, which were found in 12 and eight collection sites, respectively (the number of sites was expressed as the total number of ALS1 and ALS2 mutations).

The frequencies of the P197S substitution in ALS1 and that in ALS2 were similar to each other (five and seven sites, respectively) and this was the case with the P197L substitution, as well (both four sites). Other amino acid substitutions were found in one-to-three collection sites.

Uchino *et al*. (2006) already suggested that the P197S substitution was the most frequently found in SU-resistant *S. juncoides* populations and that *ALS1* and *ALS2* were equivalent in terms of frequencies of mutations. The main differences from Uchino *et al*. (2006) in this study are the higher frequency of the P197L substitution and the lower frequency of the P197H substitution. These might be related to a regional bias because the plants were collected mainly from the western part of Japan, where the P197L substitution was frequently found (Fig. 1-1 and Table 1-1), while Uchino *et al*. (2006) mainly collected plants from the northern part of Japan, where the P197H substitution was frequently found.

In order to reveal the factors responsible for the different frequencies and different distribution of the substitutions among regions, further studies are required that investigate various aspects, such as mutability at the molecular level, fitness and cross-resistance patterns to several SU and other ALS-inhibiting herbicides, as well as genetic drifts.

In addition, it is not clarified yet whether or not a common substitution type found in different collection sites is “identical by descent” (namely, plants from a single origin or multiple origins). It would be natural to think that a frequently found substitution type is derived from multiple origins on a nationwide scale, but that the common substitution type from neighboring cities or towns might be derived from the same origin in some cases. A further study, such as a fingerprinting analysis, is needed for the clarification of such genetic structures.

Local field-scale surveys

In each of the two collection sites, Kasai and Miki, the number of substitution types was

limited to three or less (Table 1-4). In Kasai, three substitution types were found; one substitution type, the P197R substitution in ALS2, dominated. In Miki, two substitution types were found; one substitution type, the P197S substitution in ALS2, dominated. In this study, the two collection sites shared one common substitution type (P197S in ALS2), so that four substitution types (P197S, P197R, P197L in ALS2 and P197L in ALS1) were found in the two collection sites.

The substitution types found in each site were less diverse than those found in the nationwide surveys. Although a fingerprinting analysis is not made, the limited number of substitution types in the local field-scale surveys suggests that colonization of SU-resistant *S. juncoides* would be caused by seeds or pollen scattering among closely located fields.

The P197R substitution in ALS2 in Kasai, in particular, is a unique substitution type that has not been found at any other collection site in this study or in previous studies (Yoshida *et al*. 2004; Uchino *et al*. 2004; 2006; 2007). It is assumed that the dominance of this rare substitution type in Kasai was caused mainly by the diffusion of the same plant rather than by multiple occurrences of this substitution type in every field of the collection site. In fact, recently Yamada *et al.* (2013) investigated genetic structures of SU-resistant *S. juncoides* populations based on microsatellite variability among the populations from neighboring fields and indicated the possibility of a gene flow.

In contrast, in both the local sites, two or more substitution types were found. In the nationwide surveys, three sites (Ritto, Mitsuke and Matsuyama) gave mixed substitution types (Table 1-3). Yoshida *et al*. (2004) also reported that they found more than one substitution type from a single field, as well as from closely located fields within 200 m, by analyzing SU-resistant *S. juncoides* populations.

This previous study, along with the present data, suggest that SU-resistant *S. juncoides* populations at a local field scale sometimes come from multiple origins. The monitoring of SU-resistant *S. juncoides* in paddy fields so far usually has been conducted by bioassays with herbicidal chemicals to distinguish the SU-susceptible and the SU-resistant biotypes. Nevertheless, a genetic analysis is required to establish an effective way to control SU-resistant *S. juncoides* because the susceptibility of SU-resistant weeds to ALS-inhibiting herbicides in some cases is different, depending on the amino acid substitution type in the ALS genes (Tranel and Wright 2002; Uchino *et al*. 2007; Chapter 2). This study has suggested that a large number of plant samples have to be monitored in order to determine the occurrence and diversity of SU-resistant weeds, even in a local area, where multiple types of mutation might exist. For such an ideal type of monitoring, the development of a simple and rapid diagnostic method to analyze the genetics of SU-resistant *S. juncoides* was greatly desired, so such methods were developed and proposed in Chapter 5.

In some paddy fields in Kasai or Miki, SU-susceptible plants existed along with SU-resistant biotypes (Table 1-4). This suggests that SU-susceptible plants occasionally exist and grow without

extinction even in the areas severely infested with SU-resistant biotypes, where SU herbicides could have been used for several years. Further studies on the herbicide usage history in the collection fields and fitness costs of SU-resistant *S. juncoides* would clarify the mechanism of survival of SU-susceptible plants. At any rate, such SU-susceptible plants in SU-resistant populations are expected to be a source of SU-susceptible genes, which would help the population to recover susceptibility to SU herbicides, especially when ALS-inhibiting herbicides are not applied (such as in fallow or in the rotational use of herbicides).

Discovery of new mutations and a broad variety of SU-resistant *S. juncoides* biotypes

S. juncoides with the substitution of P197R, P197T or D376E in ALS is reported for the first time in this study. For the Pro_{197} substitutions, all of the six possible amino acid substitutions caused by a single-nucleotide substitution (i.e. P197S, P197L, P197H, P197A, P197R and P197T) have been found in this study, along with previous studies (Yoshida *et al*. 2004; Uchino *et al*. 2006; 2007). In addition, *S. juncoides* is the first weed species in which the above six possible Pro_{197} substitutions, as well as the D376E and W574L substitutions, all have been found to confer SU resistance. *S. juncoides* could be a model weed for research into SU resistance, as the wide range of mutations can be compared within a species at the same time and in the same condition in relation to various aspects, such as resistance profiles (cross resistance and resistance levels) to be discussed in Chapter 2 and 3, and physiological and ecological characteristics of each substitution type. The set of mutation types also can be used for the establishment and validation of comprehensive diagnostic methods of SU resistance in *S. juncoides* as discussed in Chapter 5.

Recently, the D376E substitution in ALS of field-selected SU-resistant weeds also has been reported intensively (Park *et al*. 2003; Kaku *et al*. 2006; Imaizumi *et al*. 2008a; Ashigh *et al.* 2009; Whaley *et al*. 2007; Warwick *et al*. 2008; Zheng *et al*. 2011; Schaedler *et al.* 2012). As this mutation is relatively new, little information is available on the characteristics of SU-resistant weeds with this mutation. The accession having this mutation is profiled in Chapter 4.

The W574L substitution in ALS already has been reported in *S. juncoides* and proved to be resistant to a wide range of ALS-inhibiting herbicides (Uchino *et al*. 2007), just as is the case with other weed species (Tranel and Wright 2002). This amino acid substitution was found in plants collected in the northern part of Japan (Uchino *et al*. 2006), as well as in the western and the eastern parts of Japan (Fig. 1-1 and Table 1-1; collection sites 8 and 21). The ratio of this amino acid substitution seemed to be <10% of all SU-resistant *S. juncoides* plants, judging from the number of collection sites in this study and from the possessed accession numbers in Uchino *et al*. (2006). However, it is suggested that SU-resistant *S. juncoides* plants with this specific mutation already have spread in a geographically wide region of Japan, although they are not dominating over other mutants.

1-5. Summary

Suspected SU-resistant *S. juncoides* plants were collected from rice paddy fields at 24 sites in Japan in order to discover the occurrences of target-site substitutions on a nationwide scale and at a local field scale. A genetic analysis of the two ALS genes, *ALS1* and *ALS2*, of the collected plants confirmed that a single-nucleotide mutation at the Pro_{197}, Asp_{376} or Trp_{574} site of either *ALS1* or *ALS2* existed in each suspected SU-resistant plant. On a nationwide scale, it was shown that (i) the *ALS1* mutations and the *ALS2* mutations occurred at a similar frequency, that (ii) the P197S and the P197L substitutions were found most frequently among all the substitutions, and that (iii) the W574L substitutions (known as global resistance to any ALS-inhibiting herbicide) were found at a relatively low frequency but in a geographically wide range. In the local field-scale survey, which was conducted at two sites in Hyogo Prefecture, it was shown that (i) the substitutions were less diverse compared to on a nationwide scale suggesting that dispersion is one of factors to infest multiple fields, and that (ii) several substitutions and a susceptible biotype were found in single fields suggesting that a number of collections is required in order to understand the local SU-resistant status of *S. juncoides*. In addition, this study reported new findings, those of the P197R, P197T and D376E substitutions in *S. juncoides*. This set of diverse substitutions in a weed species can be used for further research purposes.

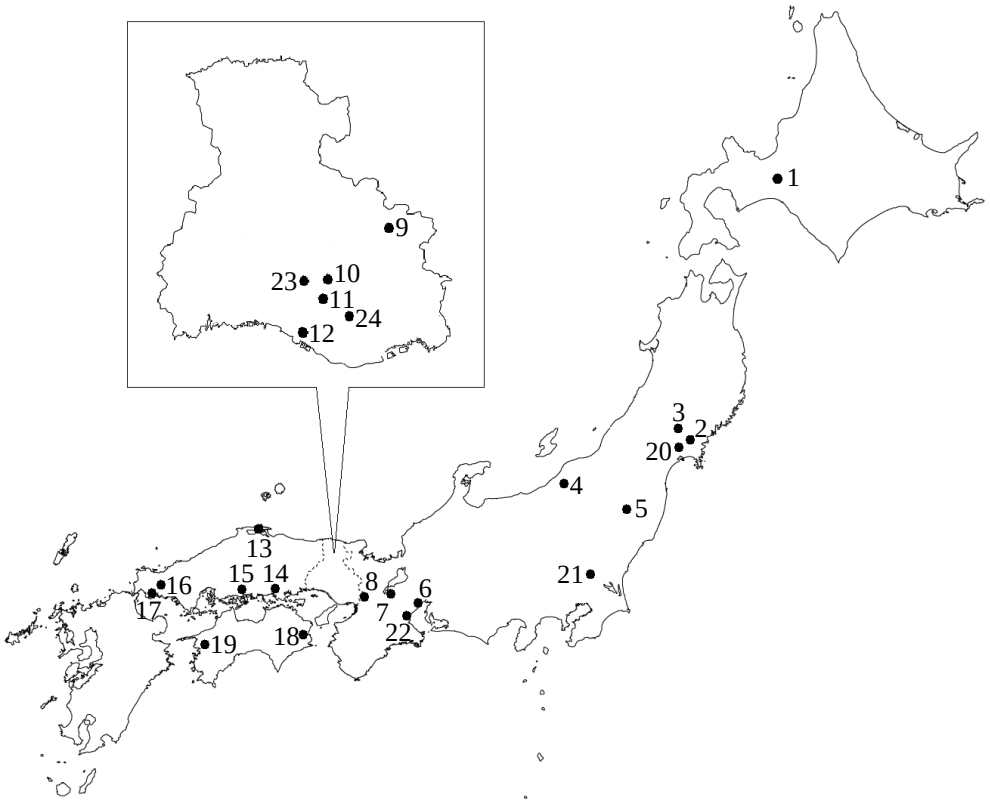

Fig. 1-1. Map of Japan showing the collection sites in this study. The enlarged prefecture is Hyogo. Detailed descriptions of each collection site are shown in Table 1-1.

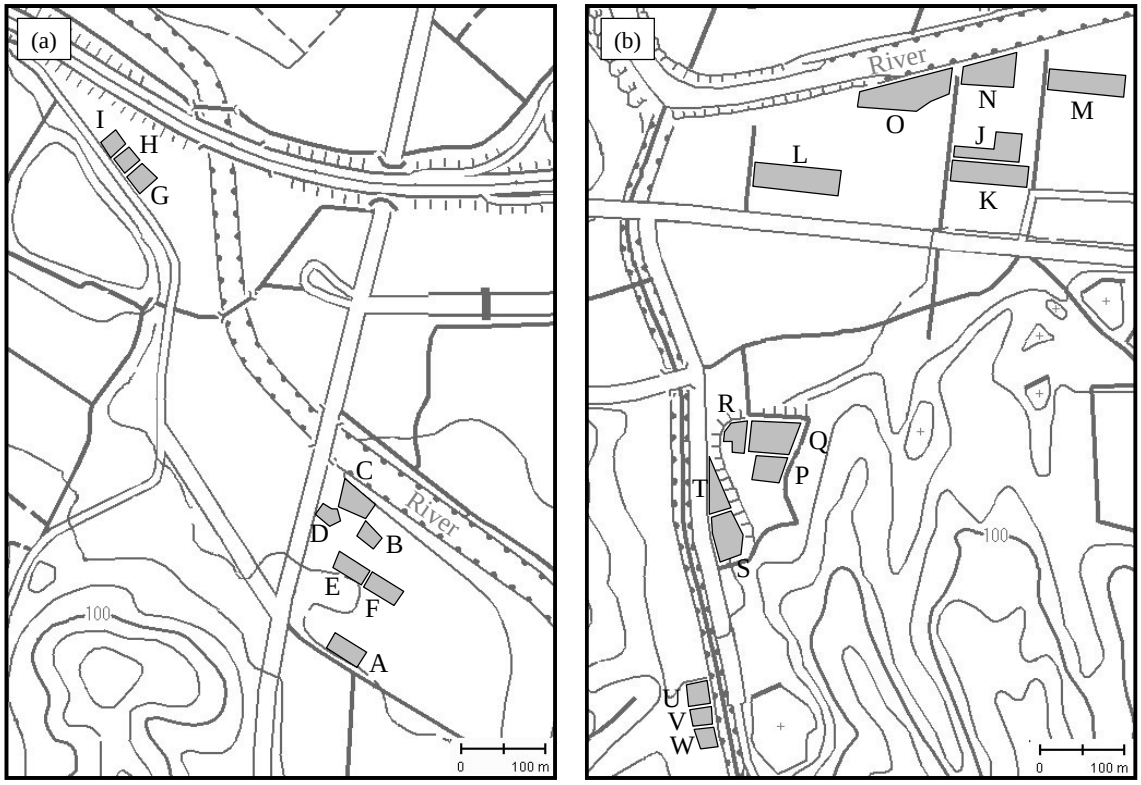

Fig. 1-2. Maps showing the rice paddy fields where the plant materials were collected in the local field-scale surveys. (a) Kasai, Hyogo Prefecture (23 in Fig. 1-1): 34°55'35.867"N, 134°51'50.906'E (center of the map) and (b) Miki, Hyogo Prefecture (24 in Fig. 1-1): 34°47'16.325"N, 135°1'41.276" E (center of the map). The closed curves with "+" are water reservoirs.

Table 1-1. Collection sites of *S. juncoides* in this study

Site number	Location		Year of collection	number of plants investigated‡
	City/Town†	Prefecture		
1	Kuriyama	Hokkaido	2005	1
2	Ishikoshi	Miyagi	2005	3
3	Ichihasama	Miyagi	2005	2
4	Mitsuke	Niigata	2005	2
5	Sugagawa	Fukushima	2005	5
6	Yokkaichi	Mie	2005	2
7	Ritto	Shiga	2005	4
8	Minoo	Osaka	2006	6
9	Sasayama	Hyogo	2006	2
10	Kato	Hyogo	2006	3
11	Ono	Hyogo	2006	4
12	Kakogawa	Hyogo	2006	5
13	Matsue	Shimane	2006	2
14	Kurashiki	Okayama	2005	5
15	Mihara	Hiroshima	2005	2
16	Yamaguchi	Yamaguchi	2005	2
17	Ajisu	Yamaguchi	2005	1
18	Tokushima	Tokushima	2005	3
19	Seiyo	Ehime	2005	1
20	Matsuyama	Miyagi	2001	4
21	Chiyokawa	Ibaragi	2003	3
22	Tsu	Mie	2003	5
23	Kasai	Hyogo	2006	25
24	Miki	Hyogo	2006	43

†Name at the time of the collection; ‡for sites 1 and 20–22, the investigated plants were grown from the seed populations. For sites 2–19, 23 and 24, field-grown plants were directly investigated.

Table 1-2. PCR primers used in the amplifications of the ALS genes of *S. juncoides*

Primer[1]	DNA sequence	Annealing sites in genomic DNA	
		ALS1 (AB257441)†	*ALS2* (AB257443)†
Primer set 1			
Forward	TCTTTGCTCCCGTTCGACT	100-118	—
Reverse	CAAACGACAAATTCGCATTA	2276-2295	—
Primer set 2			
Forward	TCTTTCGATCGCTGTCC	—	60-76
Reverse	TTGTCAAGTGATCCCTTCCC	—	2211-2230
Primer set 3			
Forward	ATGGCTTCCTCTCTCTCCA	148-156	87-105
Reverse	GCACACAGTACATCAGCATTACA	2232-2254	2167-2189

†GenBank accession number. —, the non-existence of annealing sites in the isogene.

Table 1-3. Results of the DNA sequencing at the Pro_{197}, Asp_{376} and Trp_{574} sites in the ALS genes of *S. juncoides* in the nationwide surveys and the deduced amino acid mutations (in parentheses)

Site number	Collection site	Number of plants	*ALS1*			*ALS2*		
			Pro_{197}	Asp_{376}	Trp_{574}	Pro_{197}	Asp_{376}	Trp_{574}
-	Kyoto	3	CCT (Pro)	GAT (Asp)	TGG (Trp)	CCT (Pro)	GAT (Asp)	TGG (Trp)
1	Kuriyama	1	TCT (Ser)	†	†	†	†	†
2	Ishikoshi	3	CGT (Arg)	†	†	†	†	†
3	Ichihasama	2	TCT (Ser)	†	†	†	†	†
4	Mitsuke	1	GCT (Ala)‡	†	†	†	†	†
4	Mitsuke	1	†	†	†	TCT (Ser)	†	†
5	Sugagawa	5	CGT (Arg)	†	†	†	†	†
6	Yokkaichi	2	†	†	†	TCT (Ser)	†	†
7	Ritto	3	†	†	†	†	†	TTG (Leu)
7	Ritto	1	ACT (Thr)	†	†	†	†	†
8	Minoo	6	TCT (Ser)	†	†	†	†	†
9	Sasayama	2	†	†	†	TCT (Ser)	†	†
10	Kato	3	†	†	†	CTT (Leu)	†	†
11	Ono	4	†	†	†	CTT (Leu)	†	†
12	Kakogawa	5	CTT (Leu)	†	†	†	†	†
13	Matsue	2	TCT (Ser)	†	†	†	†	†
14	Kurashiki	5	†	†	†	†	GAA (Glu)	†
15	Mihara	2	†	†	†	CTT (Leu)	†	†
16	Yamaguchi	1	†	†	†	TCT (Ser)	†	†
16	Yamaguchi	1	†	†	†	TCT (Ser)‡	†	†
17	Ajisu	1	CAT (His)	†	†	†	†	†
18	Tokushima	3	†	†	†	ACT (Thr)	†	†
19	Seiyo	1	TCT (Ser)	†	†	†	†	†
20	Matsuyama	3	CTT (Leu)	†	†	†	†	†
20	Matsuyama	1	†	†	†	TCT (Ser)	†	†
21	Chiyokawa	3	†	†	†	†	†	TTG (Leu)
22	Tsu	5	CTT (Leu)	†	†	†	†	†

†Identical to the SU-susceptible accessions; ‡a heterozygote at this locus.

Table 1-4. Results of the DNA sequencing of the ALS genes of *S. juncoides* in the local field-scale surveys and the mutations are shown in the deduced amino acid substitutions

Collection site	Field	Number of plants	Detected deduced mutations for resistance†	Results of the rooting method
Kasai				
	A	3	P197R in ALS2	R‡
	B	1	P197R in ALS2	
	B	1	P197L in ALS2	
	C	2	Not found	S
	D	6	P197R in ALS2	
	E	2	P197R in ALS2	
	E	1	P197S in ALS2§	
	F	1	P197L in ALS2	R
	G	2	P197R in ALS2	
	H	1	P197R in ALS2	R
	H	2	Not found	S
	I	3	Not found	S
Miki				
	J	3	P197S in ALS2	
	J	1	P197L in ALS1	
	K	1	P197S in ALS2	
	K	3	P197L in ALS1	
	K	1	Not found	S
	L	2	P197S in ALS2	
	M	1	P197S in ALS2	
	N	4	P197S in ALS2	
	O	2	P197S in ALS2	
	O	3	P197L in ALS1	
	P	5	P197S in ALS2	
	Q	3	P197S in ALS2	
	R	2	P197S in ALS2	
	R	1	P197L in ALS1	
	R	1	Not found	S
	S	2	P197L in ALS1	
	T	1	P197L in ALS1	
	U	2	Not found	S
	V	3	Not found	S
	W	2	P197S in ALS2	R

†All the mutations that were found were caused by a single-nucleotide substitution; ‡one of the three plants was investigated; § a heterozygote at this locus. R, SU-resistant; S, SU-susceptible.

Table 1-5. Number of sites† in which each substitution type was found

Deduced Amino acid substitution	ALS1	ALS2	Total
P197S	5	7	12
P197L	4	4	8
P197H	1	0	1
P197A	1	0	1
P197R	2	1	3
P197T	1	1	2
D376E	0	1	1
W574L	0	2	2
Total	14	16	30

†Kasai and Miki in Table 1-4 were regarded as one site, respectively.

Chapter 2. Whole plant resistance profile of SU-resistant *S. juncoides*

2-1. Introduction

Pro_{197} is the most frequently mutated site in ALS of field-selected SU-resistant *S. juncoides*, from which all the 12 possible Pro_{197} mutation patterns caused by a single-nucleotide substitution (six amino acid substitutions x two ALS genes) were found (Uchino *et al*. 2006; 2007 and Chapter 1).

The whole-plant resistance level (referred as R/S ratio, expressed by a resistance/susceptibility ratio of ED_{50} or ED_{90}, the effective doses of herbicides causing a 50% or 90% growth reduction, respectively) is essential information for herbicide-resistant weeds because it directly relates to the effectiveness of the herbicides applied to weeds. Therefore, whole-plant dose–response analyses have been carried out on many weeds that have become resistant to ALS-inhibiting herbicides as a result of Pro_{197} mutations (Boutsalis *et al*. 1999; Sibony *et al*. 2001; Sibony and Rubin 2003; Yu *et al*. 2003; Park and Mallory-Smith 2004; Roux *et al*. 2005; Intanon *et al*. 2011; Kaloumenos *et al*. 2011; Zheng *et al*. 2011).

Among the *S. juncoides* plants with a substitution at Pro_{197} in ALS (Uchino *et al*. 2007), the dose-responses of the crude ALS enzymes with a substitution of P197S, P197L or P197H to thifensulfuron-methyl were investigated to calculate the R/S ratios based on the I_{50} (the 50% inhibitory concentration of herbicides to enzymatic activities *in vitro*). However, a whole-plant dose-response was not determined, though single-dose tests were reported for the dry matter reductions. Enzymatic resistance levels could show a physiological basis of target-site resistance. However, in order to quantify the practical effectiveness of SU or other ALS-inhibiting herbicides on SU-resistant *S. juncoides*, whole-plant dose–response tests are essential because the enzymatic inhibitory tests could not take into account other potential resistance basis, if any, such as the expression levels of the ALS enzymes at exposure to the herbicides and detoxification (absorption, translocation or degradation) of the herbicides in whole plants.

Several other reports discussed the whole-plant dose-responses of SU-resistant *S. juncoides*, based on dry matter reductions (Yoshida *et al*. 1999; Tanaka 2003), plant mortality (Kohara *et al*. 1999) or bolting rate reductions (Yoshida *et al*. 1999; Ohdan *et al*. 2004). However, these reports do not have genetic information even though some of the SU-resistant *S. juncoides* accessions in these reports might be the same as the accessions that have been genetically analyzed in separate studies reported later (Uchino *et al*. 2004; 2006; 2007). Thus, the whole-plant dose-responses of SU-resistant *S. juncoides* accessions, for which genetic information is well known, have not been reported so far.

Resistance levels conferred by the substitution of P197A, P197R or P197T for SU-resistant *S. juncoides* have not been reported yet at any scale (whole-plant or enzyme) or dose model

(single-dose or dose–response). In addition, differences between ALS1 mutations and ALS2 mutations in resistance levels have not been reported for any of the Pro_{197} mutations. It also is unknown yet whether or not a resistance level is consistent among accessions with the same mutation, even if they originate from different sites. Thus, knowledge of the resistance levels of SU-resistant *S. juncoides*, caused by various Pro_{197} mutations in ALS1 and ALS2, is fragmented and imperfect.

In this Chapter, the whole-plant dose-responses of SU-resistant *S. juncoides* to imazosulfuron (ISF) and bensulfuron-methyl (BSM) (both of which are SU herbicides used widely for rice in Japan), as well as metsulfuron-methyl (MSM) and imazaquin (IMQ) (either of which is not used for rice in Japan), are investigated in order to compare the resistance levels: (i) between accessions with the same Pro_{197} mutation in ALS1 but which originated from independent mutation events, as suggested by their distantly located origins; (ii) among all the known six Pro_{197} mutations in ALS1; and (iii) between ALS1-mutated accessions and ALS2-mutated accessions with the same substituting amino acid at Pro_{197}. MSM and IMQ also were tested for another purpose: in several weed species, some Pro_{197} mutations have been reported to confer low resistance to certain SU herbicides, such as MSM (Yu *et al*. 2003; Roux *et al*. 2005), and to imidazolinone (IMI) herbicides, such as IMQ (Tranel and Wright 2002; Roux *et al*. 2005; Uchino *et al*. 2007). This research attempted to verify these commonly held assumptions by analyzing the dose-responses of the whole set of Pro_{197} mutants within a species, compared at the one time, under the same condition.

2-2. Materials and Methods

Plant materials

The plant materials used in this study are shown in Table 2-1, which shows one accession of SU-susceptible *S. juncoides* and 13 accessions of SU-resistant *S. juncoides* with a Pro_{197} mutation in ALS1 or ALS2. The accessions are named *Kyo* (susceptible), *Ich* (mutation: P197S in ALS1 hereafter referred to as P197S1), *Kur* (P197S1), *Mat* (P197L1), *Tsu* (P197L1), *Rit* (P197T1), *Ish* (P197R1), *Sug* (P197R1), *Iwa* (P197H1), *Aji* (P197H1), *Mit* (P197A1), *Sas* (P197S2), *Mih* (P197L2) and *Tok* (P197T2). The seed populations of all the accessions, except for *Iwa*, were self-pollinated progenies of the homozygous plants that had been genetically analyzed and reported in Chapter 1 originated from, in the same sequence, Kyoto, Ichihasama, Kuriyama, Matsuyama, Tsu, Ritto, Ishikoshi, Sugagawa, Ajisu, Mitsuke, Sasayama, Mihara, and Tokushima, respectively. The accession, *Iwa*, had been supplied by Dr H. Kohara (Tanaka 2003) and was genetically analyzed before this study, using the method described in Chapter 1 to confirm that it has P197H1, as previously reported for the presumably same-source accession (Uchino *et al*. 2004).

Whole-plant tests

The seeds of *S. juncoides* were incubated in water in Petri dishes at 35°C for 2 days to induce germination. Polystyrene pots (33 cm^2 area x 8 cm depth) were filled with light clay soil and water and the soil was puddled and leveled with a spatula. Four germinated seeds were sown per pot at a depth of 5 mm at 1 day after the soil puddling. The plants were grown in a flooded condition (3 cm water depth) in a greenhouse in Takarazuka, Hyogo Prefecture, Japan. The required amount of water dilution of imazosulfuron (ISF, 40% AI (active ingredient) suspension concentrate), bensulfuron-methyl (BSM, 50% AI water-dispersible granule), metsulfuron-methyl (MSM, 50% AI water-dispersible granule) or imazaquin (IMQ, 20% AI soluble liquid) was applied at four elevated doses (see Fig. 2-1) by dripping the solution into the surface water when the plants reached the two-leaf stage. Each treatment was replicated by three pots. The whole plants were harvested at 60 days after the herbicide treatment and subsequently air-dried for 48 h at 80°C.

Data analysis

A non-linear regression was made by Equation (1), using NLRAna software (Kawamata 2014), with 12 data points (three data points at four dose rates) for each of the data series, except for one data series (*Mat* response to MSM), for which 11 data points were regressed due to one lost pot:

$$Y = \ln\{c + [100 - c] / [1 + 9 \times \exp(b \times [X - a])]\}, \quad (1)$$

where Y is the natural log of the relative dry weights (percentage of the untreated control), X is the natural log of the dose rates, a is the natural log of ED_{90}, b is proportional to the slopes around the dose of ED_{90} and c is the lower limit of the relative dry weights at very large doses. As the tested dose ranges did not always produce efficacies around the 50% reductions, the ED_{90} values were calculated. When c was not significantly different from zero ($p > 0.05$) as a result of the regression, such data series again were regressed by the formula, with the substitution of $c = 0$. As 95% confidence intervals of a are available in the form of "plus/minus a constant" from NLRAna software, the confidence intervals for ED_{90} (which is an exponential of the value a) were calculated as confidential ratios in the form of "multiplied/divided by a constant".

2-3. Results and Discussion

Overall results

The *S. juncoides* accessions in Table 2-1 were treated at the two-leaf stage with ISF, BSM, MSM and IMQ, separately. All the dose–response curves are shown grouped by accession in Figure 2-1. The ED_{90} values and the R/S ratios, based on ED_{90}, are shown in Table 2-2. For three out of all (56) of the data series, c was not significantly different from zero. Such data series were reanalyzed by Equation (1), with the substitution of c = 0. The reanalyzed data series were the responses of three accessions with a P197S mutation in ALS1 or ALS2 (i.e. *Kur*, *Ich* and *Sas*) to ISF.

Generally, all the data series were well-fitted by the regression with Equation (1) (Fig. 2-1), showing high (>0.9) R^2 values in almost all (54 out of 56) of the data series (Table 2-2). The ED_{90} values of all the data series, except for one accession, *Rit* treated with BSM, were within the dose ranges tested. As a calculated extrapolative ED_{90} value of BSM to *Rit* was 23 g AI ha^{-1}, which was much less than the lowest dose tested (75 g AI ha^{-1}), the ED_{90} value for this data series is regarded as "<75 g AI ha^{-1}" in discussions, hereafter. The resistance levels of the SU-resistant accessions were calculated as the R/S ratios based on the ED_{90} values (Table 2-2).

All the accessions with a Pro_{197} mutation showed high resistance to ISF (the R/S ratios were in the range of 20 to 2000) and BSM (the R/S ratios were in the range of 20 to 1000), except for *Rit*, which was without a determined ED_{90} of BSM. For MSM, the R/S ratios of all the accessions were in the range of 3 to 16, which were lower than those of ISF and BSM. For IMQ, the R/S ratios of all the accessions were in the range of 0.6 to 2, which means that the ED_{90} values of IMQ for all the Pro_{197}-mutated accessions did not differ from those of the SU-susceptible accession by more than twofold. Thus, cross-resistance of the SU-resistant accessions with IMQ was not substantial. Therefore, the following discussion about the comparison of resistance levels among the Pro_{197}-mutated accessions is made for the other three SU herbicides tested.

Comparisons between the two distantly originated accessions with the same mutation

The SU-resistant *S. juncoides* accessions with the ALS1 mutations of P197S (*Ich vs Kur*), P197L (*Mat vs Tsu*), P197R (*Ish vs Sug*) and P197H (*Iwa vs Aji*) were compared in terms of their ED_{90} values. The places where the plant materials were collected for each of the four compared pairs of accessions were located 100–1000 km apart (Fig. 1-1). For each of the 12 compared pairs (four mutations x three SU herbicides), the two corresponding ED_{90} values were very close to each other (Table 2-2). For instance, the difference in the ED_{90} values between the compared pairs was 1.28-fold, on average, and 1.86-fold at the largest value as a ratio of the higher to the lower (the difference figures are not shown in the tables), while different amino acids conferred much more different ED_{90} values (e.g. ~50-fold at the maximum value in the case of ISF and BSM). This tendency for a similarity between the compared pairs also was visually observed in the dose–response curves' lateral positional relationships (Fig. 2-1; [b] *vs* [c], [d] *vs* [e], [g] *vs* [h] and [i] *vs* [j]). Thus, it was concluded that SU-resistant *S. juncoides* plants with the same mutation at the Pro_{197} site of ALS1 respond similarly to SU herbicides, regardless of where the plants originated.

Similar investigations were rarely reported in the past, except for Yu *et al*. (2003). They showed that two P197S-mutated *Raphanus raphanistrum* populations collected from different sites (estimated to be 80 km apart) were similarly resistant to chlorsulfuron, in which 162-fold and 127-fold resistance were found in relation to ALS enzyme inhibition (I_{50}), while other mutations, such as P197T and P197H, conferred much lower resistance.

Comparisons among the six different Pro_{197} mutations

The order of resistance levels of the SU-resistant accessions with a different substituting amino acid at the Pro_{197} site of ALS1 was nominally determined, based on the point estimations of the ED_{90} values (Table 2-2). For ISF, the highest resistance was conferred by P197S, followed by P197A, followed by the group of P197R and P197H (the nominal range, defined as 'a range between two point estimations of ED_{90}', of *Ish* and *Sug* (P197R1) overlapped that of *Iwa* and *Aji* (P197H1)), followed by P197T and then followed by P197L. For BSM, the highest resistance was conferred by P197A, followed by the group of P197R, P197S and P197H (the nominal range of *Ish* and *Sug* (P197R1) fully covered that of *Ich* and *Kur* (P197S1) and that of *Iwa* and *Aji* (P197H1)), followed by P197L and then finally by P197T (the ED_{90} value was undetermined, but was lower than any of the others as it was calculated as <75 g AI ha^{-1}). For MSM, the highest resistance was conferred by P197H, followed by P197R, then by P197T and finally followed by the group of P197S, P197L and P197A (the nominal range of *Ich* and *Kur* (P197S1) overlapped that of *Mat* and *Tsu* (P197L1), in which *Mit* (P197A1) was included). The order of resistance levels to MSM was rather gradual because of the narrow range of resistance levels among the total number of accessions compared.

The order of resistance levels of the accessions tested varied among the three SU herbicides. For instance, the amino acid substitution conferring the nominal highest resistance to ISF, BSM and MSM was P197S, P197A and P197H, respectively.

The degree of resistance of the Pro_{197}-mutated *S. juncoides* depended both on the substituting amino acid at the Pro_{197} site of ALS1 and on the structure of the SU herbicides. The results suggest that the interactions between the altered ALS1 with a Pro_{197} mutation and SU molecules are slightly different depending on the combinations, even within SU family.

Comparisons between the ALS1 mutants and the ALS2 mutants

The ED_{90} values were compared between the ALS1-mutated accessions and the ALS2-mutated accessions with the same substituting amino acid at Pro_{197} (Table 2-2). The ED_{90} values of the three SU herbicides for *Sas* (P197S2) were similarly lower (~0.3–0.5-fold) than the respective values for *Kur* and *Ich* (P197S1). Those for *Tok* (P197T2) were similarly higher (approximately twofold) than the respective values for *Rit* (P197T1), with the consideration that, as for BSM, the ED_{90} values for *Tok* were calculated as at least 1.7-fold higher than those for *Rit*. Those for *Mih* (P197L2) were similar to the respective values for *Mat* and *Tsu* (P197L1). Thus, the three different ALS2-mutated accessions showed the resistance levels in three different ways: negatively deviated, positively deviated and undeviated from the corresponding ALS1-mutated accessions. The magnitude and the direction of the deviation were generally parallel among the three SU herbicides and the same deviation directions were observed in the ED_{90} values of IMQ. Such parallel shifts

were visually observed at the dose–response curves (Fig. 2-1; [l] *vs* [b] and [n] *vs* [f]). As a result of these deviations, when the ALS1-mutated accessions and the ALS2-mutated accessions are considered together, the nominal order of resistance levels of the Pro_{197} mutants became unaccountable by the substituting amino acid at Pro_{197}, unlike the orders obtained by the ALS1-mutated accessions only.

Although the reasons as to why the ALS2 mutants showed such complicated deviations are yet to be investigated, the author raise the hypothesis that the abundance of ALS2 was not as stable as that of ALS1 in the test conditions (in the exposure to ALS-inhibiting herbicides). Further research is needed, such as dose–response analyses using a large number of ALS2-mutated accessions (this time, only one accession for each amino acid substitution), enzymological comparisons between ALS1 and ALS2 and gene expression comparisons between *ALS1* and *ALS2*. Scarabel *et al.* (2010) reported that *Schoenoplectus mucronatus* (a closely related weed species to *S. juncoides*) has three ALS genes (*ALS1*, *ALS2* and *ALS3*) and that *ALS1* is more abundantly expressed than *ALS2* and *ALS3*. Wang and Tominaga (2012) reported that *Monochoria korsakowii* expresses *ALS1* and *ALS3* steadily, but its *ALS2* expression increases by being exposed to a SU herbicide. Thus, a gene expression study for *S. juncoides*, with and without exposure to SU herbicides, is especially desirable to verify the above-said hypothesis.

Estimation of the substituting amino acid at Pro_{197} by the resistance level

All the ED_{90} values of ISF and BSM for the Pro_{197}-mutated accessions were plotted on a two-dimensional graph with 95% confidence intervals (Fig. 2-2). Using those of MSM or IMQ, in addition or in substitution, was not effective in estimating the substituting amino acids because of the narrower ranges of their resistance levels (graphs not shown). The accessions sharing the same amino acid substitution could be viewed as distinct clusters. The clusters are circled in the figure. The exception to the pattern was that the accessions with P197R (*Ish* and *Sug*) and P197H (*Iwa* and *Aji*) were clustered in close proximity and were not distinguishable as separate groups. Therefore, they are enclosed in a single circle in the figure.

The severability of each cluster can be described as follows, based on the 95% confidence intervals. The three accessions with P197S were located exclusively in between (significantly deviated from) the two lines of 100:1 and 10:1. The three accessions with P197L were exclusively located around the line of 1:1. The two accessions with P197T were located slightly over (but significantly deviated from) the line of 10:1 and were distinguishable from those with P197S, in terms of the lower resistance levels to ISF (*Rit* was estimated to be located more on the left of the plotted position of Figure 2-2). The two accessions with P197R and the other two accessions with P197H were located around the line of 10:1 and the two amino acid substitutions were indistinguishable (also see the text in *Comparisons among the six different Pro_{197} mutations* of this

Results and Discussion). The one accession with a P197A substitution was located exclusively in between (significantly deviated from) the two lines of 10:1 and 1:1 and was distinctive by its highest resistance to BSM.

Thus, it appears that, for any accession of SU-resistant *S. juncoides*, the substituting amino acid at Pro_{197} (of either ALS1 or ALS2) could be determined with some confidence by determining the ED_{90} values of ISF and BSM, with the exception of the indistinguishable two amino acid substitutions, P197H and P197R. This estimation was workable in spite of the fact that the order of resistance levels among the amino acid substitutions for each SU herbicide became obscure with the ALS1-mutated accessions and the ALS2-mutated accessions considered together. This is partly because the deviations of the ALS2-mutated accessions from the ALS1-mutated ones were parallel between ISF and BSM (such shifts occurred in parallel to the 1:1 line) and because the deviations were nonetheless much smaller than the differences in the resistance levels among the different amino acid substitutions at Pro_{197}.

Further investigations with a large number of accessions, including the accessions with W574L mutations and the accessions with D376E mutations (Uchino *et al*. 2007; Chapter 1), would make the estimation more reliable and useful.

Resistance levels to metsulfuron-methyl and imazaquin

In this study, all the accessions with a Pro_{197} mutation showed 3–16-fold whole-plant resistance to MSM (Table 2-2). The R/S ratios of MSM are generally much lower than those of ISF and BSM. Roux *et al*. (2005) showed that *A. thaliana*, with a P197S substitution in ALS, was twofold resistant to MSM and Yu *et al*. (2003) showed that ALS enzymes extracted from *R. raphanistrum* with a P197S, P197H or P197T substitution were three-to-six-fold resistant to MSM. Although these previous reports focused on limited Pro_{197} mutants, this study demonstrated the lower resistance levels to MSM of the whole set of Pro_{197} mutants. Though the values of the R/S ratio in this study were not identical to those of previous reports, when compared between the common amino acid substitutions, this could be explained by species differences and/or methodological differences.

Although 10-fold resistance is usually referred to as high resistance (Tranel and Wright 2002), the R/S ratios observed with MSM suggest that there is no significant meaning in setting a criterion of 10-fold resistance. For example, *Ish* and *Sug* (P197R1), which showed eightfold and 13-fold resistance, respectively, were regarded as showing a similar resistance level, compared to lower-resistance groups that showed three-to-five-fold resistance.

The ED_{90} value of MSM for the SU-susceptible accession (*Kyo*) was much lower than those of ISF and BSM. As a result, the ED_{90} value of MSM for *Aji* (P197H1) (6.2 g AI ha^{-1}), with 16-fold resistance, was still at a reasonable level for practical weed control, compared to the ED_{90} value of

ISF for *Kyo* (7.7 g AI ha^{-1}). Though MSM is not used for rice in Japan, this suggests that the possibility still exists for some other SU herbicides to control SU-resistant *S. juncoides* with various Pro_{197} mutations if they have acceptable rice selectivity at effective doses. Based on these results, a new SU herbicides propyrisulfuron has actually been developed and commercialized (Ikeda *et al.* 2011).

The results of this study also confirmed the common theory that weeds with Pro_{197}-mutated ALS are, in general, susceptible or not highly resistant to IMI herbicides (Tranel and Wright 2002). This theory has been supported by a set of many studies, each of which investigated a part of the possible six Pro_{197} mutants by dose–response tests (Boutsalis *et al*. 1999; Yu *et al*. 2003; Intanon *et al*. 2011; Kaloumenos *et al*. 2011; Zheng *et al*. 2011) or even by single-dose tests (Uchino *et al*. 2007; Yu *et al*. 2008). Therefore, this is the first report that demonstrates the susceptibility of weeds with Pro_{197}-mutated ALS to an IMI herbicide by analyzing the dose-responses of the whole set of Pro_{197} mutants, caused by a single-nucleotide substitution within a species.

However, as the three SU herbicides tested showed different resistance profiles (cross-resistance and resistance levels) in this study, some IMI herbicides might show different resistance profiles to other IMI herbicides. Further studies with several IMI herbicides are desired in order to achieve a comprehensive perspective on cross-resistance to IMI herbicides by the SU-resistant *S. juncoides* with Pro_{197}-mutated ALS. In addition, it is desirable to investigate the responses of the whole set of Pro_{197} mutants of SU-resistant *S. juncoides* to other classes of ALS-inhibiting herbicides, such as TP and PC, which have been used recently to control *S. juncoides* in Japan.

2-4. Summary

An investigation, using herbicidal pot tests in a greenhouse condition, was conducted to determine the whole-plant dose-responses to several ALS-inhibiting herbicides of SU-resistant *S. juncoides* with various Pro_{197} mutations in ALS collected from rice paddy fields in Japan. All the tested SU-resistant accessions with a Pro_{197} mutation were highly resistant to two commonly used SU herbicides (ISF and BSM), but were much less resistant to another SU herbicide, MSM, and were substantially not resistant to IMQ. These cross-resistance patterns have been known previously in fragments of *S. juncoides* and other weed species and were comprehensively confirmed in this study with a whole set of Pro_{197} mutations. The analyses of resistance levels, based on ED_{90} values, newly showed that different accessions with a common amino acid substitution in ALS1 showed similar responses to these herbicides (confirmed with four amino acid substitutions), that the rankings of resistance levels conferred by various Pro_{197} mutations in ALS1 differed among the SU herbicides and that the resistance levels of the ALS2-mutated accessions were higher than, lower than or similar to those of the corresponding ALS1-mutated accessions, depending on the compared

pair, but the deviation patterns were generally similar among the SU herbicides in each compared pair. The final finding might suggest that the abundance of ALS2 is not as stable as that of ALS1. In addition, as a result of these new findings, together with expected further research, a suggested possibility is that substituting amino acids at Pro_{197} generally could be estimated by plotting each accession's ED_{90} values of ISF and BSM in a two-dimensional graph.

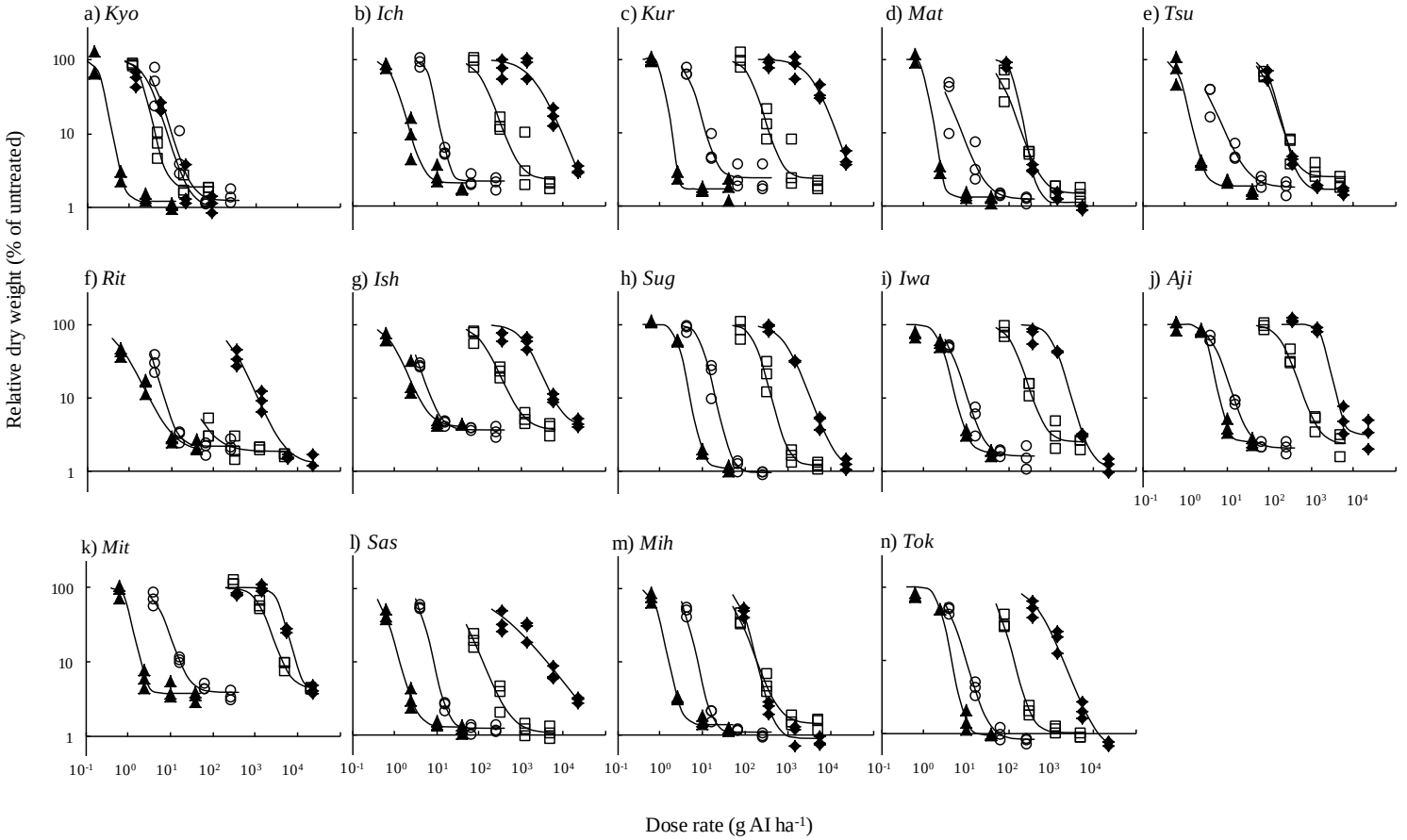

Fig. 2-1. Dose–response relationships of the *S. juncoides* accessions to the four ALS-inhibiting herbicides. The tested dose rates of each herbicide are four-times/0.25-times series of the standard rate for each herbicide. The standard rates are defined as 90 g AI ha^{-1} for imazosulfuron (♦), 75 g AI ha^{-1} for bensulfuron-methyl (□),10 g AI ha^{-1} for metsulfuron-methyl (▲) and 250 g AI ha^{-1} for imazaquin-ammonium (○).

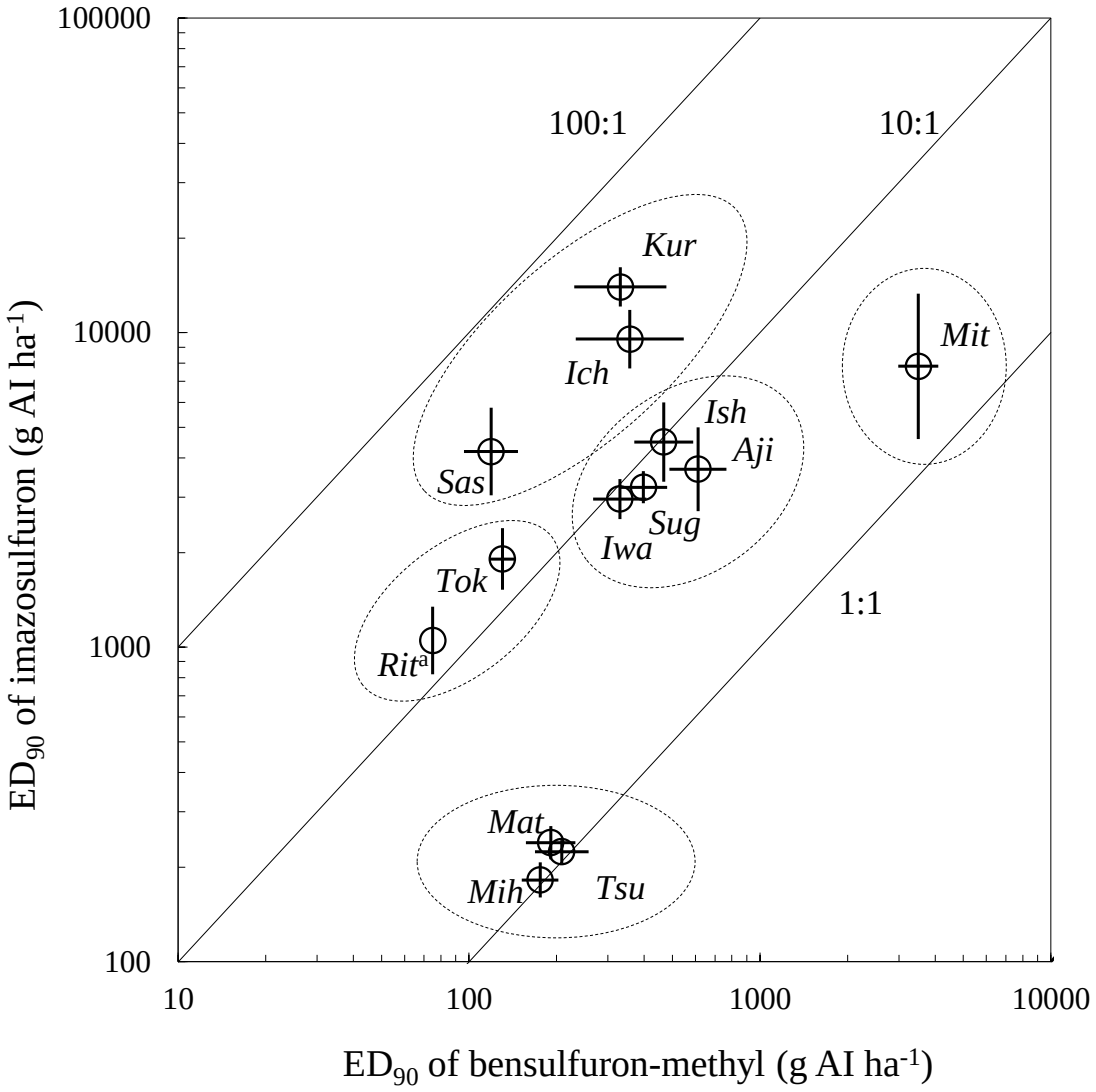

Fig. 2-2. Distribution of ED_{90} values of imazosulfuron and bensulfuron-methyl to SU-resistant *S. juncoides* accessions. *Rit*[a] is plotted on the position of 75 g AI ha^{-1} of bensulfuron-methyl (Fig. 2-1, Table 2-2). Each cluster circle (dotted line) contains the accessions with a common amino acid substitution at the Pro_{197} site, whether ALS1 or ALS2, except for the center cluster circle, which contains the accessions with Arg or His in ALS1. The error bars are the ±95% confidence intervals.

Table 2-1. *S. juncoides* plant materials and their mutations in ALS

Accession	Collection site		Mutation in ALS at the Pro_{197} site	
	City/Town†	Prefecture	Putative amino acid substitution	Enzyme with the substitution
Kyo	Kyoto	Kyoto	-	-
Ich	Ichihasama	Miyagi	Ser	ALS1
Kur	Kuriyama	Hokkaido	Ser	ALS1
Mat	Matsuyama	Miyagi	Leu	ALS1
Tsu	Tsu	Mie	Leu	ALS1
Rit	Ritto	Shiga	Thr	ALS1
Ish	Ishikoshi	Miyagi	Arg	ALS1
Sug	Sugagawa	Fukushima	Arg	ALS1
Iwa	Iwamizawa	Hokkaido	His	ALS1
Aji	Ajisu	Yamaguchi	His	ALS1
Mit	Mitsuke	Niigata	Ala	ALS1
Sas	Sasayama	Hyogo	Ser	ALS2
Mih	Mihara	Hiroshima	Leu	ALS2
Tok	Tokushima	Tokushima	Thr	ALS2

† Name at the timing of collection

Table 2-2. ED_{90} for *S. juncoides* accessions of the four ALS inhibiting herbicides and R/S ratios

Accession													
	Mutation in ALS		Imazosulfuron			Bensulfuron-methyl			Metsulfuron-methyl			Imazaquin-ammonium		
	X of P197X	n of ALSn	ED_{90} (g AI ha^{-1})	95% interval†	R/S ratio‡	ED_{90} (g AI ha^{-1})	95% interval	R/S ratio	ED_{90} (g AI ha^{-1})	95% interval	R/S ratio	ED_{90} (g AI ha^{-1})	95% interval	R/S ratio
Kyo	-	-	7.7	1.35	-	3.9	1.17	-	0.39	1.19	-	9.8	1.37	-
Ich	S	1	9545	1.24	1242	357	1.53	92	2.2	1.28	6	12.1	1.16	1.2
Kur	S	1	13973	1.15	1818	331	1.44	86	1.8	3.69	5	10.8	1.28	1.1
Mat	L	1	239	1.13	31	191	1.22	49	1.8	1.29	5	7.5	1.46	0.8
Tsu	L	1	224	1.09	29	208	1.24	54	1.5	1.19	4	8.4	1.31	0.9
Rit	T	1	1050	1.28	137	<75	-	<19	2.6	1.29	7	6.3	1.19	0.6
Ish	R	1	4492	1.34	584	467	1.26	121	2.9	1.36	8	6.2	1.13	0.6
Sug	R	1	3224	1.12	419	397	1.21	103	4.9	1.05	13	19.5	1.18	2.0
Iwa	H	1	2961	1.16	385	330	1.23	85	5.3	1.15	14	10.0	1.22	1.0
Aji	H	1	3681	1.36	479	612	1.25	159	6.2	1.13	16	13.3	1.09	1.4
Mit	A	1	7817	1.70	1017	3502	1.17	908	1.7	1.29	4	13.8	1.15	1.4
Sas	S	2	4189	1.38	545	119	1.24	31	1.3	1.12	3	8.3	1.07	0.8
Mih	L	2	182	1.14	24	176	1.16	46	1.5	1.11	4	7.4	1.10	0.8
Tok	T	2	1907	1.25	248	130	1.10	34	4.5	1.14	11	9.7	1.10	1.0

† 95% confidence interval is shown as a ratio, which gives the lower limit and the upper limit by dividing and multiplying, respectively, the ED90 value by the confidence interval value. ‡ R/S ratio, a ratio of ED_{90} of a SU-resistant accession per that of *Kyo*.

Chapter 3. Enzymatic resistance profile of SU-resistant *S. juncoides*

3-1. Introduction

Uchino *et al*. (2007) reported the enzymatic dose-responses of *S. juncoides* and concluded that the substitutions at Pro_{197} or Trp_{574} are the basis of the resistance to the SU herbicides, bensulfuron-methyl and thifensulfuron-methyl, in several accessions of *S. juncoides*. Tanaka (2003) also reported a dose-response to imazosulfuron of a SU-resistant *S. juncoides* accession with an unknown mutation that has been subsequently revealed to have P197H1 mutation, as *Iwa* in Chapter 2.

However, as in the whole-plant dose responses, it was not clarified yet whether different accession sharing a common identical mutation showed a consistent enzymatic response or not, or weather ALS1-mutated and ALS2-mutated ones shows different enzymatic dose-responses or not. It is not fully understood yet about the difference among various amino acid substitutions, either.

In this Chapter, ALS enzyme inhibition by imazosulfuron was examined in order to characterize ALS from 10 accessions of SU-resistant *S. juncoides* collected from paddy fields in Japan.

3-2. Materials and Methods

Plant materials

One SU-susceptible *S. juncoides* accession *Kyo* and ten SU-resistant *S. juncoides* accession, *Ich* (P197S1), *Sei* (P197S1), *Tai* (P197S2), *Yok* (P197S2), *Rit* (P197T1), *Tok* (P197T2), *Tsu* (P197L1), *Mih* (P197L2), *Chi* (W574L2) and *Hon* (W574L2), were used for this study. *Kyo*, *Ich*, *Rit*, *Tok*, *Tsu*, and *Mih* are the same ones described in Chapter 2. *Sei*, *Yok*, and *Chi* are self-pollinated progenies of the homozygous plants that had been genetically analyzed in Chapter 1, originated from Seiyo, Yokkaichi, and Chiyokawa, respectively. *Tai* and *Hon* had been supplied by Dr. S. Yoshida and Dr. A. Uchino, respectively and were genetically analyzed before this study, using the method described in Chapter 1.

Whole-plant assay

In order to supplementary support the understandings of the enzymatic profiles, the whole-plant growth inhibition of five accessions by imazosulfuron was examined by greenhouse pot tests. Though the author already reported comprehensive dose-responses of whole plants in Chapter 2 including imazosulfuron, this assay was made again because i) whole-plant responses at the enzyme extraction stage that was mainly a stem-elongation stage in accordance with Tanaka (2003) are considered useful and ii) plant materials in this Chapter are not always same as in Chapter 2 due to some material availability at the timing of the study.

The accessions *Kyo*, *Ich*, *Tai*, *Tsu* and *Mih* were grown in 100 cm^2 plastic pots (15 cm high) filled with paddy soil (light clay, pH = 5.5, organic matter content = 1.9%) in a greenhouse in Takarazuka, Japan. The temperature in the greenhouse ranged from 15°C to 30°C. Imazosulfuron (technical grade; Sumitomo Chemical Company, Tokyo, Japan), dissolved in aqueous acetone solution, was added to flooding water in a pot at the two-leaf stage (2–3 cm high) or the stem-elongation stage (17–18 cm high) of the plants (four plants per pot). The untreated plants were applied with the same amount of aqueous acetone solution. The application rates of imazosulfuron were 9, 90 and 900 g AI ha^{-1} and 9, 90 and 900 kg AI ha^{-1}. The flooding water was maintained at a depth of ~4 cm after the application. The shoots were harvested 4 weeks after the application and the dry weights were measured. The experimental layout was a randomized complete block with three replications.

ALS extraction and enzyme activity assay

ALS was extracted and assayed by following the procedure of Nandula and Messersmith (2000), with slight modifications. The *S. juncoides* shoots at the stem-elongation stage (15–20 cm high) or at the two-to-three-leaf stage (2–4 cm high) grown in a greenhouse were harvested, frozen in liquid nitrogen and stored at -80°C before the ALS extraction.

The enzyme extraction was carried out at 5°C. The frozen shoots were homogenized by a blender in liquid nitrogen. After the addition of three volumes of buffer A (100 mM potassium phosphate, pH = 7.5, 1 mM sodium pyruvate, 0.5 mM $MgCl_2$, 0.5 mM thiamine pyrophosphate [TPP], 10 μM flavine adenine dinucleotide [FAD], 1 mM dithiothreitol and 10% glycerol), the homogenate was filtered through four-layered gauze. The filtrate was centrifuged at 27,000 *g* for 20 min. The enzyme was precipitated from the supernatant with 50% saturated ammonium sulfate and was centrifuged at 17,000 *g* for 30 min. The pellet was suspended in buffer B (100 mM potassium phosphate, pH = 7.5, 20 mM sodium pyruvate and 0.5 mM $MgCl_2$) and was desalted by a desalting column (PD-10; GE Healthcare Bio-Sciences, Uppsala, Sweden) equilibrated with buffer B. After the addition of 10% glycerol, the desalted enzyme was frozen in liquid nitrogen and stored at -80°C until the assay.

The reaction mixture (0.5 mL) consisted of 20 mM potassium phosphate (pH = 7.0), 20 mM sodium pyruvate, 0.5 mM $MgCl_2$, 0.5 mM TPP, 10 μM FAD, the crude enzyme preparation and the herbicide (0–100 μM). The herbicide (technical grade), dissolved in dimethyl sulfoxide (DMSO), was added to the reaction mixture. The final concentration of DMSO in the reaction mixture was 1% (v/v). In order to adjust the enzyme activity (~100 μM product h^{-1}), the enzyme preparation was diluted with buffer B. The reaction mixture was incubated for 60 min at 30°C and the reaction was stopped by the addition of 6 N H_2SO_4 (50 μL). The acidified reaction mixture was heated for 15 min at 60°C. Then, 0.5% creatine (0.5 mL), followed by 5% α-naphtol (0.5 mL) dissolved in 2.5 N

NaOH, was added to the reaction mixture and the mixture was heated for 15 min at 60°C. The absorbance was measured at 525 nm. A standard curve was constructed using acetoin. The protein concentration was determined by the method of Bradford (1976), with bovine serum albumin as a standard. The test was implemented with four (stem-elongation stage) or three (two-to-three-leaf stage) replications.

Regression analysis

The dose–response curves of the SU-resistant accessions were recognized visually as double-sigmoid curves. Therefore, instead of applying an ordinary single-sigmoid equation, the following equation (2) was applied as a regression equation:

$y = p \times f(x) + (1 - p) \times g(x)$, (2)

where

$f(x) = 100 \times (1 + 10^{(a-x)})$, and

$g(x) = 100 \times (1 + 10^{(b-x)})$.

For equation (2), a synthetic function of two different single-sigmoid curves composed in a certain ratio was used (Lipovetsky 2010), based on the assumption that a double-sigmoid dose–response curve suggests the coexistence of two inhibition targets with different sensitivities (Tsuneki *et al*. 2004). In equation (2), y is the percentage inhibition of the extracted ALS activity (untreated: 0); x is the log of the herbicide concentration; a is the log of the I_{50} of one sigmoid curve (referred to as $f(x)$); b is the log of the I_{50} of the other sigmoid curve (referred as $g(x)$), located in higher-concentration regions than $f(x)$ ($b > a$); and p is a synthetic ratio of $f(x)$ and $g(x)$.

Regression analyses were carried out using equation (2) for the SU-resistant accessions with NLRAna software (Kawamata 2014), with 28 data points (four data points at seven concentrations) for the stem-elongation stage and 21 data points (three data points at seven concentrations) for the two-to-three-leaf stage for each data series. The I_{50} values of the extracted ALS were obtained by a back-calculation of x with the substitution of $y = 50$ in equation (2).

For the SU-susceptible accession, from which single-sigmoid curves were obtained for both of the stem-elongation and the two-to-three-leaf stages, the same regression procedures were carried out, except that single-sigmoid regression analyses were done with the equation $f(x)$.

3-3. Results

Whole-plant growth inhibition

The whole-plant response of five accessions (*Kyo*, *Ich*, *Tai*, *Tsu* and *Mih*) to imazosulfuron was examined in greenhouse pot tests. The growth stage of *S. juncoides* at the application of imazosulfuron was the two-leaf stage or the stem-elongation stage. At the standard application rate (90 g AI ha^{-1}) of imazosulfuron, the accessions *Ich*, *Tai*, *Tsu* and *Mih* survived and the accession *Kyo*

died, (Fig. 3-1a). *Tsu* (P197L1) and *Mih* (P197L2) were controlled by 900 g AI ha^{-1} imazosulfuron, but *Ich* (P197S1) and *Tai* (P197S2) were not controlled at the same rate. Thus, the P197S mutation conferred higher resistance than the P197L mutation. The dose-responses of ALS1-mutated and ALS2-mutated accessions were similar to each other in the two comparing pairs (*Tsu vs Mih*, and *Ich vs Tai*) at the two-leaf stage (Fig. 3-1a) and at the stem-elongation stage (Fig. 3-1b).

Inhibition of ALS enzyme activity

Overall observation

The effect of imazosulfuron on the activity of the ALS enzyme extracted from *S. juncoides* was examined. The enzyme preparations partially purified from 11 accessions at the stem-elongation stage were used for the assay (Figs 3-2 to 3-6). The ALS of all the resistant accessions was less sensitive to imazosulfuron than that of the susceptible accession (*Kyo*). These results suggest that the resistance is related to the altered ALS enzyme, which is less sensitive to SUs. These results are in agreement with previous articles about *S. juncoides* (Tanaka 2003; Uchino *et al*. 2007) and other plants (Tranel and Wright 2002).

The dose–response inhibition of ALS activity in *Ich* (P197S1), *Sei* (P197S1), *Tai* (P197S2) and *Yok* (P197S2) exhibited a double-sigmoid curve (Fig. 3-2). In addition, the dose–response inhibition of ALS activity by imazosulfuron in the resistant accessions was remarkably different depending on whether ALS1 or ALS2 has a mutation.

In the accessions with P197S1 (*Ich* and *Sei*), there were plateau regions on the double-sigmoid curves of between 25% and 35% inhibition at concentrations between 0.1 μM and 10 μM of imazosulfuron. In contrast, in the accessions with P197S2 (*Tai* and *Yok*), there were plateau regions of between 65% and 80% inhibition at concentrations between 0.1 μM and 100 μM of imazosulfuron.

The ALS inhibition of the accessions *Ich* (P197S1) and *Sei* (P197S1) showed a similar dose–response, although the collection sites of these accessions were different. The ALS inhibition of *Tai* (P197S2) and *Yok* (P197S2) also showed a similar response.

The dose–response inhibition of *Rit* (P197T1) and *Tok* (P197T2) exhibited a double-sigmoid curve, too (Fig 3-3). There was a plateau region on the curve of *Rit* of a ~30% inhibition at concentrations between 0.1 μM and 1 μM of imazosulfuron. In contrast, there was a plateau region on the curve of *Tok* of between 70% and 80% inhibition at concentrations between 0.1 μM and 10 μM of imazosulfuron.

The dose–response inhibition of *Tsu* (P197L1) and *Mih* (P197L2) did not show a distinct double-sigmoid curve (Fig. 3-4), while they showed distinctively different curves from each other as seen in the other pairs of ALS1-mutated and ALS2-mutated accessions.

The enzyme inhibition of both *Chi* (W574L2) and *Hon* (W574L2) showed a double-sigmoid

curve, with plateau regions of 60–70% inhibition at concentrations between 0.1 μM and 10 μM of imazosulfuron (Fig. 3-5).

Finally, the effect of imazosulfuron on the activity of the ALS enzyme extracted from the two-to-three leaf stage of *Ich* (P197S1) and *Tai* (P197S2) was examined (Fig. 3-6) for supplementary purpose to the mainly conducted stem-elongation stage assays. The dose–responses of these accessions showed the double-sigmoid curve. The dose–response inhibition of the ALS activity in these accessions at the two-to-three-leaf stage and stem-elongation stage was similar (Fig. 3-2).

Regression analysis

The results of the regression analysis are shown in Table 3-1. The data were well fitted by this regression, as shown in the high determination coefficients (0.986–0.998). The above-mentioned remarkable differences in the curve positions between the ALS1-mutated and the ALS2-mutated accessions were described by the I_{50} values of the extracted ALS (partially purified ALS enzyme preparations). Those of the ALS1-mutated accessions ranged from 0.96 to 164.9 μM, while those of the ALS2-mutated ones ranged from 0.0196 to 0.0519 μM (Table 3-1). Compared with that of *Kyo* (0.0148 or 0.0166 μM), all the ALS1-mutated accessions showed high (>65) R/S ratios, the ratios of those of SU-resistant ones to that of *Kyo*), while the ALS2-mutated ones showed small (<4) R/S ratios, in terms of the I_{50} values of the extracted ALS.

The $f(x)$ equations of the SU-resistant accessions calculated by the regressions were similar to each other and the regression curves were located very close to the regression curve of the *Kyo* accession (Figs 3-2 to 3-6) as the R/S ratios ranged from 0.766 to 1.200 (Table 3-1).

The I_{50} values of the $g(x)$ were 429.0–777.2 μM in the accessions with a P197S mutation, 86.2–139.8 μM in the accessions with a P197T mutation, 3.2–17.3 μM in the accessions with a P197L mutation and 196.7–380.8 μM in the accessions with a W574L mutation. The R/S ratios ranged from 213 to 52,405.

The *p*-values, that are the mixture ratios of $f(x)$ and $g(x)$, ranged from 0.308 to 0.399 for ALS1-mutated accessions, and 0.649 and 0.847 for ALS2-mutated accessions.

3-4. Discussion

In the whole-plant growth inhibition, the fact that P197S conferred higher resistance than P197L to imazosulfuron is consistent to the results of Chapter 2, though *Tai* (P197S2) in this Chapter is a different accession from the one used in Chapter 2 having the same mutations (*Sas*). In this sense, this observation is considered more general and repeatable one. For the comparison between ALS1-mutated and ALS2-mutated accessions sharing a common amino acid substitution, newly tested *Tai* (P197S2) showed very similar dose-response to *Ich*, though *Sas* (another P197S2)

had shown slightly lower (ca. 1/2-fold) resistance than *Ich* in Chapter 2. As already mentioned as 'not stable' for ALS2-mutated accessions, this suggests that the observation in Chapter 2 (P197S2 < P197S1) is not considered a general rule since the phenomenon of similar resistance levels between ALS1-mutated and ALS2-mutated was also observed in P197S (*Tai* vs *Ich*). In addition, the similarity of *Tsu* (P197L1) and *Mih* (P197L2) were repeated in this re-examination. Thus, it is strengthened that ALS1-mutation and ALS-2 mutation overall conferred similar resistance level on whole plants if the amino acid substitution is common.

On the other hand, it is surprising that extracted ALS consistently (through various accessions and growth stages) showed a very different sensitivity (based on I_{50}) to imazosulfuron between ALS1-mutated and ALS2- mutated ones, even if the amino acid substitution is common.

From the three facts of i) double-sigmoid dose-responses, ii) $f(x)$ curves very similar to that of *Kyo*, and iii) $g(x)$ positions that suggest high resistance, of all the SU-resistant accessions, it is considered that the SU-resistant accessions have a mixed ALS enzyme, consisting of wild-type and mutated ones, and that the $f(x)$ in the synthetic regression equation (2) is a putative response of the wild-type ALS existing in the extracted ALS. In this consideration, the $g(x)$ in the synthetic equation is a putative response of the mutated ALS existing in the extracted ALS and the p-value is considered to be a putative proportion of the wild-type ALS existing in the extracted ALS.

In this case, a height level of the plateau of the double-sigmoid curves is an indicator of the proportion of wild-type ALS existing in the extracted ALS because the first saturation level of the curve drawn by equation (2) is expressed as 100 x p (Lipovetsky 2010). From the p-values, the proportion of wild-type ALS existing in the extracted ALS was estimated as 31–40% in the ALS1-mutated SU-resistant accessions and 65–85% in the ALS2-mutated SU-resistant accessions. Considering the result of both the ALS1- and ALS2-mutated SU-resistant accessions, it was estimated that the proportion of ALS1 was 60–85% (71% on average) and that of ALS2 was 15–40% (29% on average). Thus, the ratio of abundance of ALS1 and ALS2 was estimated as ~70:30%, with a range of ±15%.

There is a possibility that the specific activity of ALS1 is much higher than that of ALS2 with the same abundance. However, it is unlikely that the specific activity between ALS1 and ALS2 is different because of the high identity between the two genes (98.0% identity).

Scarabel *et al*. (2010) reported that *Schoenoplectus mucronatus* has multiple ALS genes (referred to as *ALS1*, *ALS2* and *ALS3*). They also reported that the transcript of *ALS1* was detected more frequently than those of *ALS2* and *ALS3* in the leaves of *S. mucronatus*. Though correspondence between *S. mucronatus* ALS and *S. juncoides* ALS is unclear, this kind of biased expression may be a common phenomenon of *Schoenoplectus* genus.

It is unclear why these consistent observations (double-sigmoid curves and not-so-high R/S ratios of ALS2-mutated accessions based on I_{50}) were not mentioned in the past publications that did

dose-response inhibitions on extracted ALS of SU-resistant *S. juncoides* (Uchino *et al.* 2007; Tanaka 2003). Anyway, taking into account the fact that a positional difference of the response curves of the ALS2-mutated SU-resistant accessions from that of a SU-susceptible accession was more distinctive in the higher inhibitory regions >50%, the I_{50} values of the extracted ALS could not be representative values of a double-sigmoid curve.

These I_{50} values of *g(x)* suggest that the order of the putative resistance levels of the mutated ALS existing in the extracted ALS to imazosulfuron was: P197S group > W574L group > P197T group > P197L group, without nominal overlapping (Table 3-1). Unlike very similar I_{50} values of all *f*(*x*), *g*(*x*) contains some ranges in the I_{50} values (ca. 2- to 5-fold) within the group. These ranges may be contributed by artificial regression errors because the double-sigmoid curves of ALS2-mutated accessions are close to the curve of susceptible one (*Kyo*) away from the putative *g*(*x*) to construct, and because the double-sigmoid curves of ALS1-mutated accessions are generally difficult to reach closely 100% inhibition.

Anyway, this rough sequence of the order of resistance levels based on the *g*(*x*), is much more consistent to that of whole-plants than I_{50} of extracted ALS, because P197S conferred higher resistance than P197L on whole-plants in Chapter 2 and 3, and because P197T conferred an in-between resistance level of P197S and P197L on whole plants in Chapter 2 (same accessions used: *Rit* and *Tok*). So, as a methodology, the author propose to use a double-sigmoid curve regression when dose-responses are investigated on SU-resistant *S. juncoides* ALS, to construct *g*(*x*). Otherwise (if with a single-sigmoid regression), the researcher might conclude that a target-site mutation is not a basis of resistance looking at very low I_{50} of ALS2-mutated extracted enzyme (Table 3-1). Another emphasis of the methodology is that this study shows a possibility to know whether ALS1 or ALS2 has a mutation in SU-resistant *S. juncoides*, by regressing the dose-response curves of extracted ALS without doing genetic analysis on DNA.

As described above, the sensitivities of extracted ALS are not consistent to whole-plant sensitivity, when compared between ALS1-mutated and ALS2-mutated accessions sharing a common amino acid substitution. The mechanism to make whole plant sensitivities unbiased is unclear. Possible hypothetical explanations would be that *ALS2* expression could be enhanced when exposed to herbicide if *ALS2* has a resistant mutation. This hypothesis may be related to the fact that ALS2-mutated accessions' whole-plant responses among the ones sharing the same mutations are not stable as those of ALS1-mutated accessions (Chapter 2, and *Tai*'s dose-response in this Chapter). Further studies are desired to clarify the relationship between ALS inhibition and the whole-plant response.

3-5. Summary

Ten accessions of SU-resistant *S. juncoides* were collected from paddy fields in Japan. In

order to characterize ALS from SU-resistant *S. juncoides*, whole-plant growth inhibition and ALS enzyme inhibition were examined. *S. juncoides* has two ALS genes (*ALS1* and *ALS2*). The tested SU-resistant accessions harbored amino acid substitutions at Pro_{197} or Trp_{574} in either ALS1 or ALS2. The whole plants of all the SU-resistant accessions showed resistance to imazosulfuron. The resistance level depended on the altered amino acid residues in ALS, but did not depend on which ALS gene is mutated. The ALS enzyme partially purified from all the SU-resistant accessions was less sensitive to imazosulfuron, compared to the susceptible accession, suggesting that the resistance is related to the altered ALS enzyme. In addition, the dose–response inhibition of ALS activity by imazosulfuron in the SU-resistant accessions was remarkably different with the presence of an amino acid substitution in either ALS1 or ALS2. Furthermore, the dose–response inhibition of ALS activity in the SU-resistant accessions with a P197S, P197T or W574L mutation showed a double-sigmoid curve. The regression analysis of enzyme inhibition suggested that the abundance ratio of ALS1 to ALS2 enzymes was approximately 70:30%, with a range of ±15%. Taken together, these results suggest that the resistance of SU-resistant accessions of *S. juncoides* is related to ALS in either ALS1 or ALS2, although the abundance of the altered ALS in the plants was not quantitatively related to whole-plant responses.

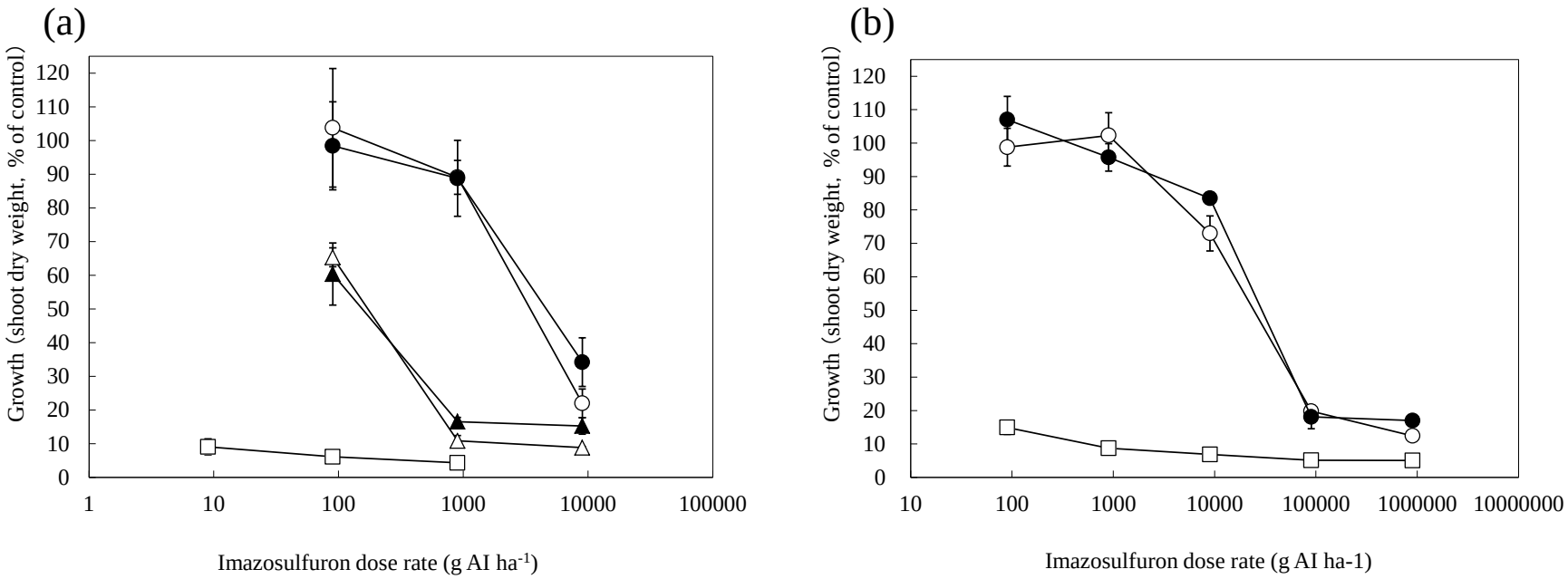

Fig. 3-1. Herbicidal activity of imazosulfuron on five accessions of *S. juncoides* at the (a) two-leaf stage and (b) stem-elongation stage. The standard application rate is 90 g AI ha^{-1}. The data are the mean value (n = 3) ±standard error. (□), *Kyo*; (○), *Ich*; (●),*Tai*; (△),*Tsu*; (▲), *Mih*.

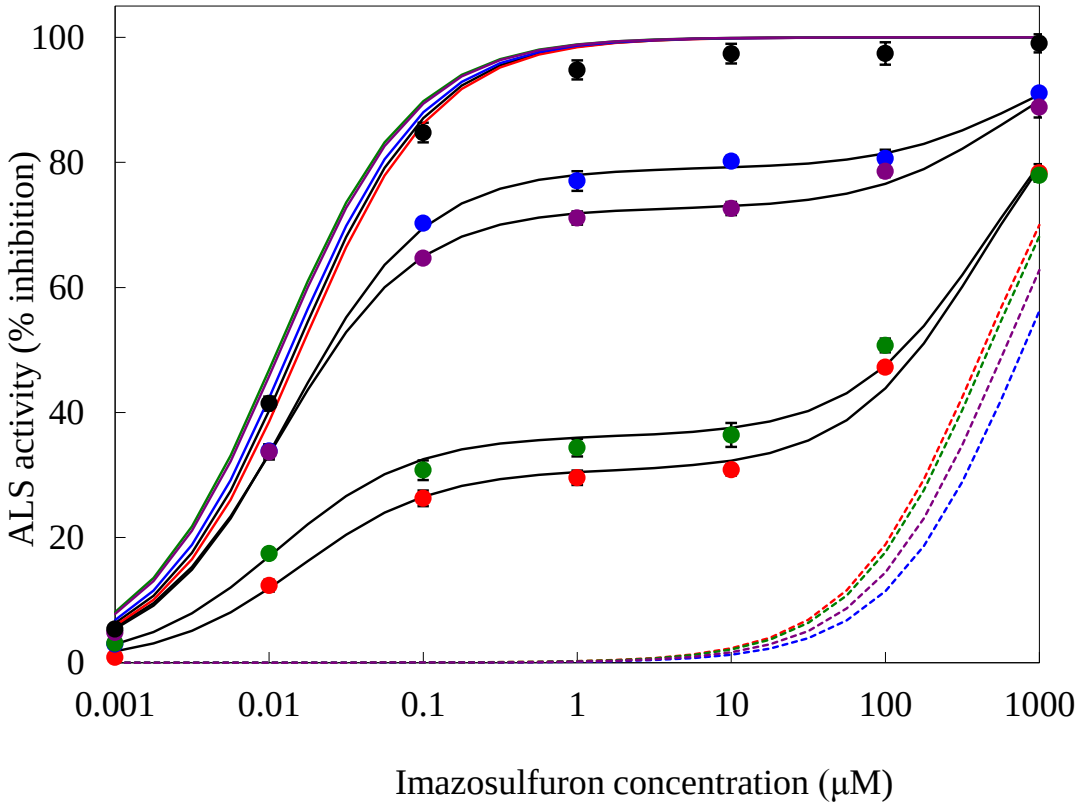

Fig. 3-2. Effect of imazosulfuron on the ALS activity of the SU-resistant accessions with a P197S mutation and the wild-type accession at the stem-elongation stage. Accessions: black, *Kyo*; blue, *Tai*; purple, *Yok*; green, *Sei*; red, *Ich*; symbols, the mean value ± standard error; black solid lines, the regression line calculated from the data points; colored solid lines, the sigmoid curve of $f(x)$ (the putative response of the wild-type ALS in the SU-resistant accession); colored dotted lines, the sigmoid curve of $g(x)$ (the putative response of the mutated ALS in the SU-resistant accession).

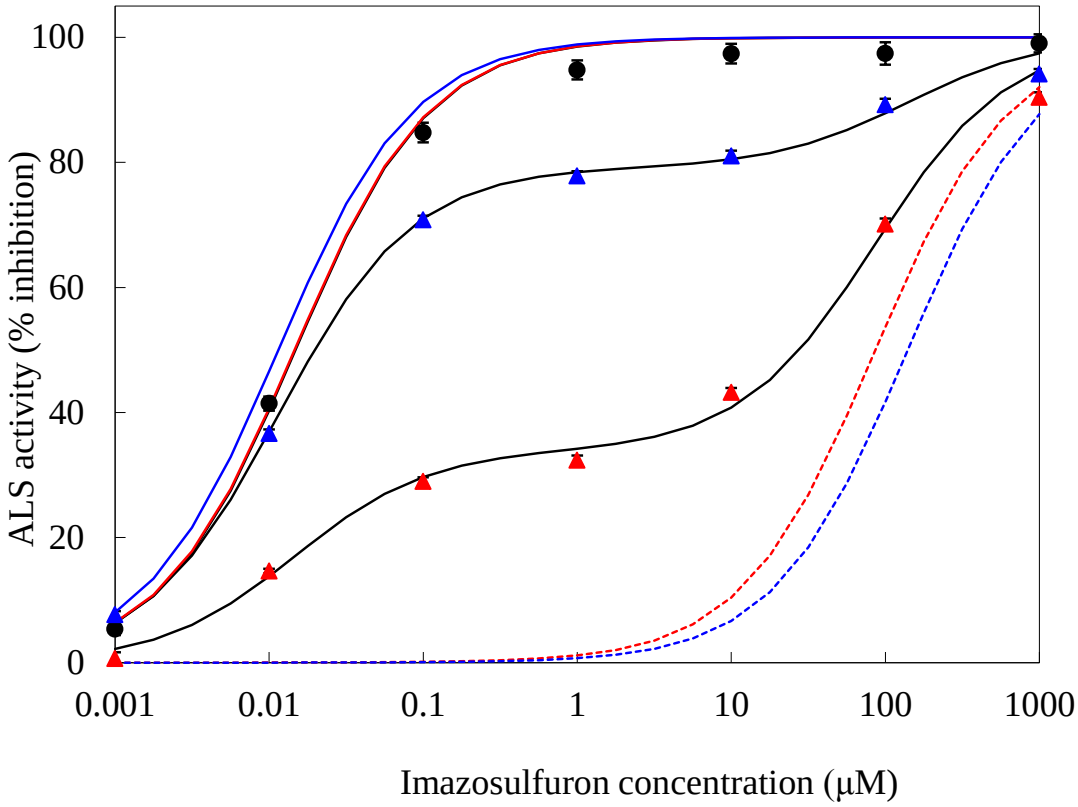

Fig. 3-3. Effect of imazosulfuron on the ALS activity of the SU-resistant accessions with a P197T mutation and the wild-type accession at the stem-elongation stage. Accessions: black, *Kyo*; blue, *Tok*; red, *Rit*; symbols, the mean value ± standard error; black solid lines, the regression line calculated from the data points; colored solid lines, the sigmoid curve of $f(x)$ (the putative response of the wild-type ALS in the SU-resistant accession); colored dotted lines, the sigmoid curve of $g(x)$ (the putative response of the mutated ALS in the SU-resistant accession).

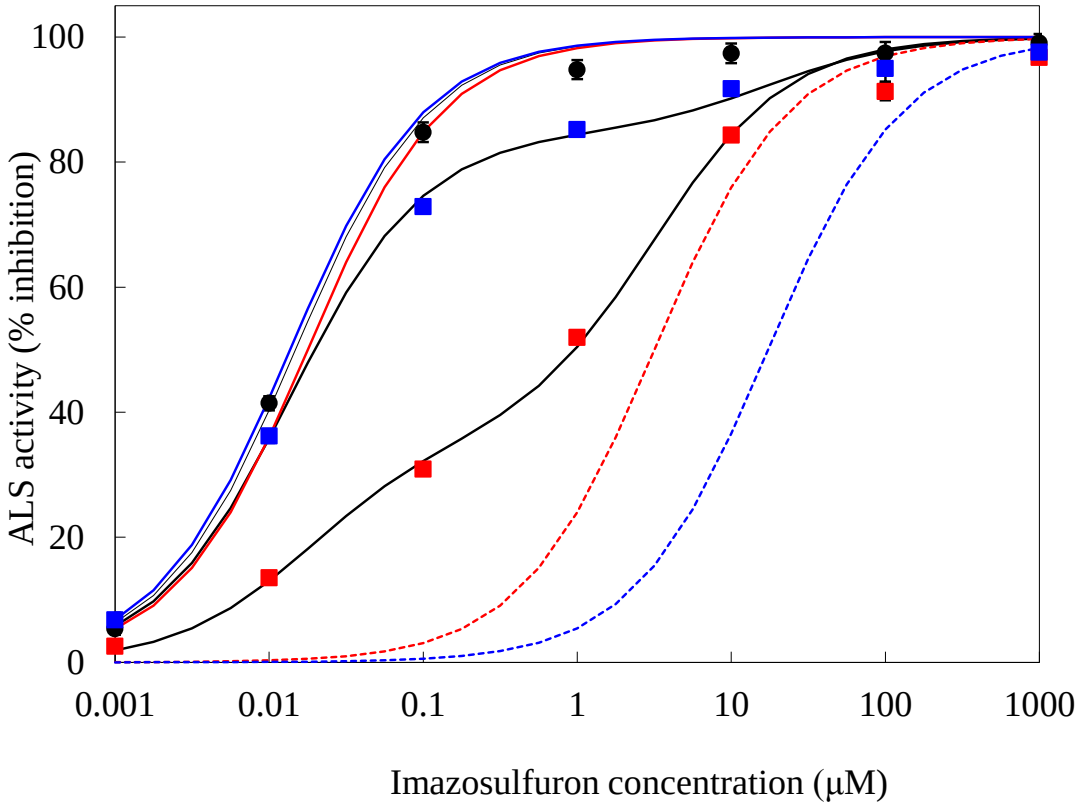

Fig. 3-4. Effect of imazosulfuron on the ALS activity of the SU-resistant accessions with a P197L mutation and the wild-type accession at the stem-elongation stage. Accessions: black, *Kyo*; blue, *Mih*; red, *Tsu*; symbols, the mean value ± standard error; black solid lines, the regression line calculated from the data points; colored solid lines, the sigmoid curve of $f(x)$ (the putative response of the wild-type ALS in the SU-resistant accession); colored dotted lines, the sigmoid curve of $g(x)$ (the putative response of the mutated ALS in the SU-resistant accession).

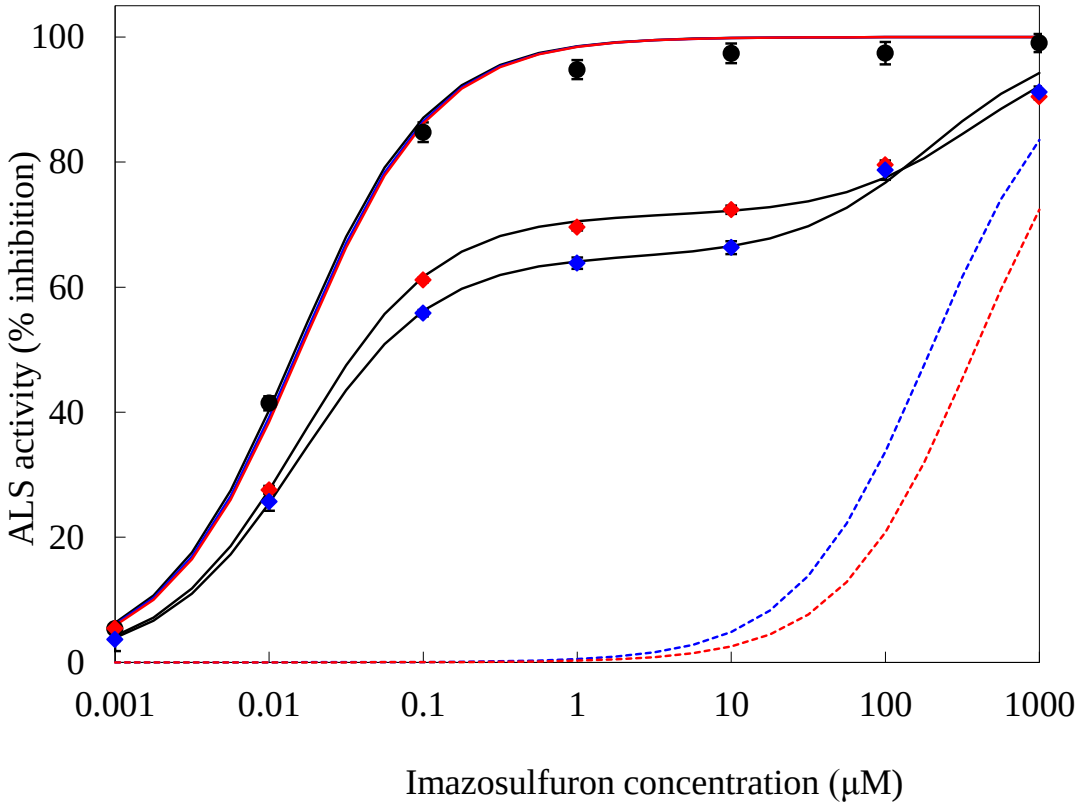

Fig. 3-5. Effect of imazosulfuron on the ALS activity of the SU-resistant accessions with a W574L mutation and the wild-type accession at the stem-elongation stage. Accessions: black, *Kyo*; blue, *Hon*; red, *Chi*; symbols, the mean value ± standard error; black solid lines, the regression line calculated from the data points; colored solid line, the sigmoid curve of $f(x)$ (the putative response of the wild-type ALS in the SU-resistant accession); colored dotted lines, the sigmoid curve of $g(x)$ (the putative response of the mutated ALS in the SU-R accession).

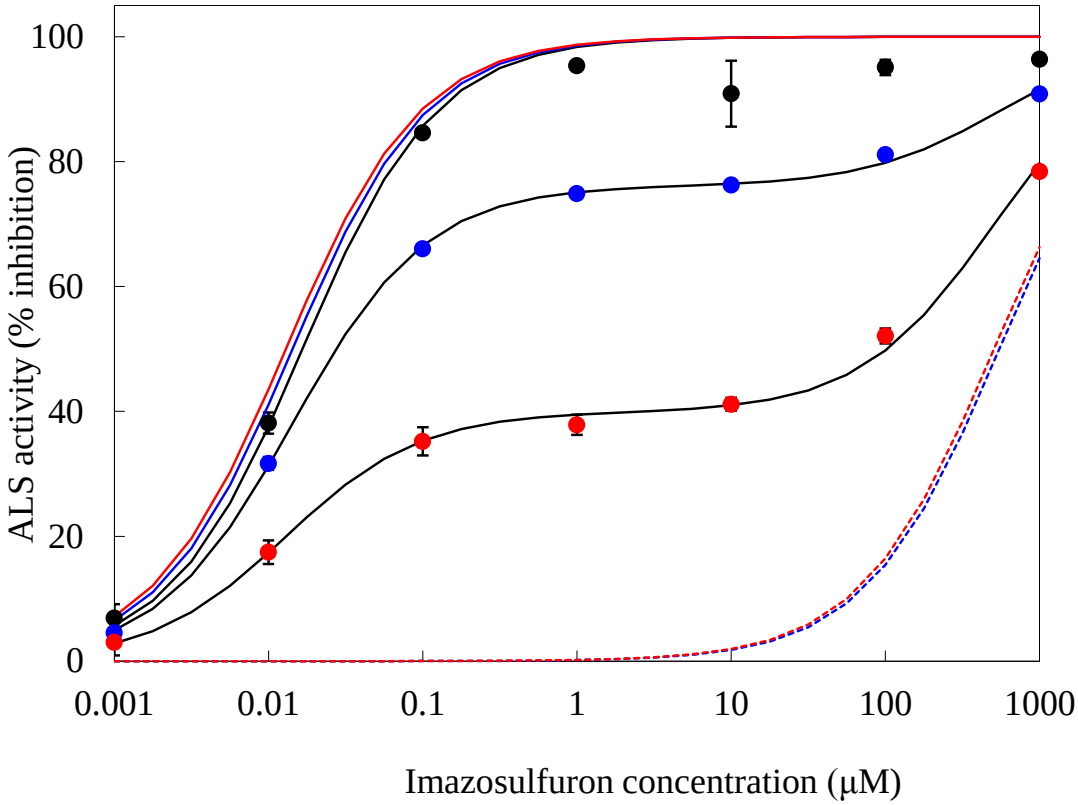

Fig. 3-6. Effect of imazosulfuron on the ALS activity of the SU-resistant accessions with a P197S mutation and the wild-type accession at the two-to-three-leaf stage. Accessions: black, *Kyo*; blue, *Tai*; red, *Ich*; symbols, the mean value ± standard error; black solid lines, the regression line calculated from the data points; colored solid lines, the sigmoid curve of $f(x)$ (the putative response of the wild-type ALS in the SU-R accession); colored dotted lines, the sigmoid curve of $g(x)$ (the putative response of the mutated ALS in the SU-resistant accession).

Table 3-1. Regression analysis of the inhibition of ALS extracted from *S. juncoides* by imazosulfuron

	Stem-elongation stage											Two-to-three leaf stage		
	Kyo	*Ich*	*Tai*	*Sei*	*Yok*	*Rit*	*Tok*	*Tsu*	*Mih*	*Hon*	*Chi*	*Kyo*	*Ich*	*Tai*
	-	P197S1	P197S2	P197S1	P197S2	P197T1	P197T2	P197L1	P197L2	W574L2	W574L2	-	P197S1	P197S2
I_{50}(μM) of extracted ALS	0.0148	164.9	0.0235	128.5	0.0261	27.7	0.0196	0.96	0.0197	0.0519	0.0371	0.0166	102.9	0.0274
R/S ratio of extracted ALS	-	11122	1.582	8667	1.761	1868	1.324	65	1.327	3.501	2.499	-	6186	1.649
I_{50}(μM) of $f(x)$ (10^a)	-	0.0159	0.0136	0.0114	0.0118	0.0146	0.0115	0.0178	0.0137	0.0155	0.0160	-	0.0130	0.0143
R/S ratio of $f(x)$	-	1.075	0.919	0.766	0.798	0.986	0.774	1.200	0.922	1.047	1.080	-	0.873	0.967
I_{50}(μM) of $g(x)$ (10^b)	-	429.0	777.2	466.8	592.8	86.2	139.8	3.2	17.3	196.7	380.8	-	507.8	549.1
R/S ratio of $g(x)$	-	28928	52405	31478	39972	5812	9428	213	1169	13265	25676	-	34237	37027
R^2	0.993	0.990	0.994	0.986	0.993	0.994	0.996	0.995	0.995	0.992	0.997	0.986	0.986	0.998
p	-	0.308	0.791	0.362	0.727	0.340	0.792	0.356	0.847	0.649	0.716	-	0.399	0.761
±95% confidence interval	-	0.015	0.016	0.017	0.015	0.021	0.015	0.061	0.022	0.020	0.011	-	0.022	0.010
a	-1.829	-1.797	-1.865	-1.945	-1.927	-1.835	-1.940	-1.750	-1.864	-1.809	-1.796	-1.779	-1.888	-1.843
±95% confidence interval	0.0002	0.1425	0.0578	0.1275	0.0603	0.1486	0.0489	0.2478	0.0001	0.0796	0.0413	0.0004	0.1494	0.0367
b	-	2.632	2.891	2.669	2.773	1.935	2.146	0.500	1.239	2.294	2.581	-	2.706	2.740
±95% confidence interval	-	0.0002	0.2248	0.0893	0.1622	0.0776	0.1888	0.1452	0.3485	0.1523	0.1095	-	0.1052	0.1214

R/S ratio, I_{50} value of the SU-resistant accession/I_{50} value of *Kyo* accession.

Chateper 4. Characterization of *S. juncoides* with $Asp_{376}Glu$ mutation in ALS

4-1. Introduction

The D376E mutations in ALS have been reported intensively for many weed species in the last decade. Park *et al.* (2003) firstly reported *Monochoria korsakowii* having this mutation although they could not conclude this is a basis of SU-resistance. Thereafter, this mutation was found from *Amaranthus hybridus* (Whaley *et al.* 2004) and *Amaranthus powellii* (Corbett 2004), both of which were reported in detail afterwards respectively (Whaley *et al.* 2007; Ashigh *et al.* 2009). Then, this mutation was reported from *Monochoria vaginalis* (Kaku *et al*. 2006; Imaizumi *et al*. 2008a), *Elatine triandra* (Kaku *et al*. 2006), *Kochia scoparia* (Warwick *et al.* 2008), *Conyza canadensis* (Zheng *et al.* 2011), *Galium spurium* (Beckie *et al.* 2012), *Raphanus raphanistrum* (Yu *et al.* 2012), *Fimbristylis miliacea* (Shaedler *et al.* 2012) and *S. juncoides* (Chapter 1).

The D376E mutation is believed to confer resistance to broad classes of ALS-inhibiting herbicide, based on the whole-plant dose–response (Zheng *et al.* 2011) on the dose responses of the crude extracted ALS and whole plants (Ashigh *et al.* 2009; Yu *et al.* 2012), and on the whole-plant dose–responses of the weed species and transgenic *A. thaliana* (Whaley *et al.* 2007). The belief, however, is supported by indirect evidence within a limited number of plant species; direct evidence with resistant-weed-originated, heterologously-expressed ALS having the mutation is of importance to verify the belief.

In this Chapter, the author investigated the dose–response of the SU-resistant *S. juncoides* accession with the D376E mutation in ALS2 at *in vivo* whole-plant level as well as at *in vitro* enzymatic level, to three SU herbicides (imazosulfuron, bensulfuron-methyl and metsulfuron-methyl), a PC herbicide (bispyribac-sodium), and an IMI herbicide (imazaquin). Then, the author discuss differences and similarities of the resistant levels between the *in vivo* and *in vitro* dose–response assays.

4-2. Materials and Methods

Plant materials

Seeds of the SU-resistant *S. juncoides* accession were obtained from self-pollination of a plant having a homozygous D376E mutation in ALS2, collected from Kurashiki city, Japan (Chapter 1). Seeds of SU-susceptible *S. juncoides* were obtained from *Kyo*.

The genomic DNA sequences of the full exon regions of ALS1 and ALS2 were determined by the DNA sequencing method described in Chapter 1. The SU-susceptible accession was confirmed to conserve the putative amino acid sequences in ALS1 and ALS2 identically to those available as BAE97675 and BAE97677 (GenBank accession numbers), respectively. The SU-resistant accession

was confirmed to have the putative amino acid sequences in ALS1 and ALS2 identically to those of the SU-susceptible accession except for the only one amino acid difference of D376E in ALS2.

Whole-plant dose–response tests

Seeds of the SU-resistant and SU-susceptible *S. juncoides* were separately incubated in water in Petri dishes at 35 ºC for 2 days to induce germination. Polystyrene pots (33 cm^2 area x 8 cm depth) were filled with a light clay soil and water, and the soil was puddled and leveled with a spatula. Four germinated seeds were sown per pot at a depth of 5 mm at 1 day after the soil puddling. Plants were grown in a flooded condition (3 cm water depth) in a greenhouse at Takarazuka-city, Japan. The required amount of water-diluted imazosulfuron (ISF, 40% AI suspension concentrate), bensulfuron-methyl (BSM, 50% AI water dispersible granule), metsulfuron-methyl (MSM, 50% AI water dispersible granule), bispyribac-sodium (BPS, 2% AI soluble liquid) or imazaquin (IMQ, 20% AI soluble liquid) was applied at five elevated doses, respectively (see the legend of Fig. 4-2), by dripping into the surface water when plants reached 2-leaf stage. After a preparatory whole-plant test to scout the appropriate dose ranges, the definitive dose–response test was implemented once. Each treatment was replicated by three pots. Whole plants were harvested at 60 days after the herbicide treatments, and subsequently air-dried for 48 h at 80 ºC.

Cloning and enzyme preparation

The following total procedures were made for both of the SU-resistant and the SU-susceptible accessions in accordance with the manufacturer's instructions. From the step of transformation, the procedures were made additionally for a non-inserted expression vector as a control.

Total RNA was extracted from young green stems of *S. juncoides* plants with RNeasy Plant Mini Kit (Qiagen, Chatsworth, CA, USA), and first strand cDNA was synthesized with a reverse transcriptase, SuperScript RT III (Invitrogen, Carlsbad, CA, USA). ALS2 was amplified from the cDNA with the following primer set using a high-fidelity DNA polymerase, KOD Plus (Toyobo, Osaka, Japan). The sense primer introduces an *Nde*I recognition site (underlined) at the start codon of ALS2 while the antisense primer introduces an *Eco*RI recognition site (underlined) at the downstream of the stop codon of ALS2, as shown below.

Sense: 5' -TACATATGGCTTCCTCTCTCTCCACC-3'.

Antisense: 5'-TAGAATTCTTGTCAAGTGATCCCTTCCC-3'.

The amplified PCR product was sub-cloned into the pCR-Blunt-II-TOPO plasmid (Invitrogen), and the inserted fragment was sequenced and confirmed to have the appropriate sequences without PCR errors. The constructed plasmid was double-digested with *Nde*I and *Eco*RI, and the fragment was purified by gel extraction. The purified fragment was inserted into the similarly double-digested pCold II vector plasmid (Takara Bio, Otsu, Japan) using DNA Ligation Kit (Takara Bio). The

constructed expression vector was transformed into *Escherichia coli* competent cell (BL21 strain). Thus, a full coding region of the ALS2 was designed to express in *E. coli* though it is regarded to contain a plastid transit peptide region. This construction was prepared because (i) a cleavage site is unknown for *S. juncoides* (ALS2 has no homology to *A. thaliana* ALS around Thr_{86} and its downstream that was estimated the N-terminal of the cleaved ALS based on the homology among dicotyledonous plants (Duggleby and Pang 2000)), and (ii) a full coding region of *A. thaliana* ALS expressed in *E. coli* still keeps a relatively high specific activity (Chang and Duggleby 1997). The transformed competent cells were inoculated into 100 mL LB media to have an OD600 at 0.05, and cultured for approximately 2.5 h at 37 ºC in rotation until the OD600 became 0.5. Thereafter, the cells were given a cold shock for 1 h at 15 ºC, then added with IPTG to be 0.5 mM at the final concentration, and cultured in rotation for another 1 h at 15 ºC.

BugBuster MasterMix (Merck, Darmstadt, Germany) containing 5 mM $MgCl_2$, 5 mM b-Me, 150 mM NaCl, 0.1 mM FAD (final concentration for all additions) was mixed with the harvested competent cell at 5 mL per g harvest. After suspended completely by pipetting, the suspension was left for 10 min at room temperature. The suspension was then centrifuged at 14,000 rpm for 30 min at 4 ºC. The supernatant was collected; added glycerol at final concentration of 10% (v/v); and the mixture was frozen by liquid nitrogen, and then stored at -80 ºC until the enzymatic tests. An aliquot of the supernatant was separately visualized by SDS–PAGE.

Enzymatic dose–response tests

Enzymatic dose–response tests on the heterologously-expressed ALS2 *in vitro* were carried out in general accordance with the previous report (Tanaka 2003). A reaction mixture consisted of 20 mM potassium phosphate (pH 7.0), 20 mM sodium pyruvate, 0.5 mM $MgCl_2$, 0.5 mM TPP, 20 μM FAD, the enzyme preparation and a herbicide (zero to 10 or 100 μM; See Fig. 4-3). The enzyme activity was adjusted at approximately 70 μM product h^{-1} without a herbicide. The herbicide was added as a dimethyl sulfoxide (DMSO) solution. The final concentration of DMSO in the reaction mixture was 1% (v/v). The reaction mixture (0.5 mL) was incubated for 60 min at 30 ºC and the reaction was stopped by the addition of 6 N H_2SO_4 (50 μL). The acidified reaction mixture was heated for 15 min at 60 ºC. Then 0.5% creatine (0.5 mL), followed by 5% α-naphtol (0.5 mL) dissolved in 2.5 N NaOH were added to the reaction mixture and the mixture was heated for 15 min at 60 ºC. Acetoin formed from acetolactate by acidification was quantified by measuring the absorbance at 525 nm. Protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as a standard. The dose–response test was implemented twice where three data points were obtained at each herbicidal concentration in each implementation.

Separately from the dose–response tests, the above-described enzymatic reactions with 1.25–20 mM sodium pyruvate and no herbicides were conducted with three replicates to calculate *Km* for pyruvate and *Vmax* according to Michaelis–Menten equation.

Data analysis

For dose–response analysis of the whole-plant tests, non-linear regression was made by the equation (3) with 15 data points (three data points at five dose rates) for each of all data series.

$Y = \ln\{c + [100 - c] / [1 + \exp(b \times [X - a])]\}$, (3)

In the equation (3), *Y* is the natural log of the relative dry weights (untreated: 100); *X* is the natural log of the dose rates of ALS-inhibiting herbicides; *a* is the natural log of GR_{50} (the herbicide dose required for 50% biomass reduction compared with the untreated); *b* is proportional to the slopes around the dose of GR_{50}; and *c* is the lower limit of relative dry weights at very large doses.

For dose–response analysis of the enzymatic tests, non-linear regression was made by the equation (4). The two test implementations were joined together into single regression.

$Y = 100 / (1 + \exp(b \times [X - a])$, (4)

In the equation (4), *Y* is the relative ALS activity (uninhibited: 100); *X* is the natural log of the concentrations of ALS-inhibiting herbicides; *a* is the natural log of I_{50} (the herbicide concentration required for 50% enzyme inhibition compared with the untreated); and *b* is proportional to the slopes around the dose of I_{50}.

Since the GR_{50} and I_{50} and their confidence intervals were calculated as the exponentials of 'calculated *a* plus/minus its confidence interval', the confidence intervals of GR_{50} and I_{50} were available in the form of 'multiplied/divided by a constant' as in Chapter 2. R/S ratios of the whole-plant or the enzyme were calculated by dividing the GR_{50} or the I_{50} of the resistant by the GR_{50} or the I_{50} of the susceptible, respectively.

4-3. Results

Enzyme preparation

In the SDS–PAGE, the preparations derived from the SU-resistant and the SU-susceptible *S. juncoides* showed a strong band that was estimated to have a comparable size to the putative ALS2 (72 kDa), while the reference preparation from non-inserted expression vector did not show such a strong band (Fig. 4-1).

The *Km* (pyruvate) values of the wild-type ALS2 and the mutated ALS2 were 6.37 ± 0.59 and 3.79 ± 0.43 mM, respectively. The specific activity at 20 mM pyruvate of the wild-type ALS2 and the mutated ALS2 were 0.38 ± 0.2 and 1.77 ± 0.6 μmol h^{-1} mg $protein^{-1}$, respectively. The specific activity of the reference preparation was 0.017 ± 0.004 μmol h^{-1} mg $protein^{-1}$. Each datum is shown as a mean of three replicates ± a standard error.

The enzyme preparations were used for enzymatic dose–response tests without further purification, because the ALS2 appeared to be the majority of the total protein in each enzyme preparation on the SDS–PAGE, and because the majority of the specific activity of the enzyme preparations (approximately 96% and 99% for the wild-type and mutated, respectively) appeared to be contributed by the expressed ALS2.

Dose–response tests

In the whole-plant dose–response tests, plants of the SU-susceptible and the SU-resistant accessions were treated at 2-leaf stage with ISF, BSM, MSM, BPS and IMQ, separately. The dose–responses were generally well-fitted by the regression model (Fig. 4-2). GR_{50}-based R/S ratios of the SU-resistant accession having the D376E mutation for ISF, BSM, MSM, BPS, and IMQ were 176, 40, 14, 5.2 and 1.5, respectively (Table 4-1). Thus, the SU-resistant accession showed high (>10) resistance to the three SU herbicides and moderate (<10) resistance to BPS, but did not show substantial resistance to IMQ.

In the enzymatic dose–response tests, the heterologously-expressed ALS2 was inhibited by these herbicides *in vitro*. The dose–responses were generally well-fitted by the regression model (Fig. 4-3). I_{50}-based R/S ratios of the mutated ALS2 having the D376E mutation for ISF, BSM, MSM, BPS, and IMQ were 3699, 2438, 322, 80, and 4.8, respectively (Table 4-1). Thus, the mutation conferred high resistance to the three SU herbicides and BPS, and moderate resistance to IMQ on *S. juncoides* ALS2.

The R/S ratios of whole plants are highly correlated with those of the enzyme in the two dose–response tests. Pearson's product-moment correlation coefficient (at log–log conversions) and Spearman's rank correlation coefficient were 0.96 and 1.00, respectively, both of which were statistically significant ($p < 0.01$). The correlation is also visually-noticeable by the similarity of the positional relationships of the dose–response curves between whole plants and enzymes (Fig. 4-2 vs. Fig. 4-3). On the other hand, the R/S ratios obtained in the enzymatic tests were much higher than those obtained in the whole-plant tests.

4-4. Discussion

The D376E mutation was firstly reported by Falco *et al.* (1989) on the ALS of a spontaneous SU-resistant *Saccharomyces* mutant. Thereafter by using purified *Saccharomyces* ALS expressed in *E. coli*, Pang *et al.* (2003) suggested that the Asp_{376} is one of the interaction sites when a SU herbicide binds to ALS. For higher plants, Le *et al.* (2005) reported that a purified tobacco ALS having this mutation by a site-directed mutagenesis expressed in *E. coli* showed cross-resistance to SU, TP and IMI herbicides. The crude ALS extracted from the field-selected resistant weeds having the mutation showed resistance to ALS-inhibiting herbicides *in vitro* (Ashigh *et al.* 2009; Yu *et al.*

2012); the whole plants of transgenic *A. thaliana* transformed to have an *A. hybridus* ALS showed herbicide resistance (Whaley *et al.* 2007). Thus, the mutation is believed to confer resistance on ALS and consequently on whole plants.

In this study, as a different approach to investigate *in vitro* sensitivity of a heterologously-expressed mutated ALS, the D376E mutation was confirmed to confer resistance on the ALS enzyme. The high correlation between the responses of the whole plants and the enzymes strongly suggests that this mutation is a molecular basis to make whole-plant *S. juncoides* resistant to the ALS-inhibiting herbicides though the other possible mechanisms such as detoxification are not excluded in this study.

The author reported that the crude ALS extracted from ALS2-mutated SU-resistant *S. juncoides* shows a sensitivity fairly close to that from SU-susceptible plants without exceptions, probably because ALS2 in *S. juncoides* plants is consistently less abundant than ALS1 (Chapter 3). In contrast, ALS1-mutated plants and ALS2-mutated plants having a common amino acid substitution at Pro_{197} in ALS show similar whole-plant resistance levels although the reason has not been clarified (Chapter 2; Chapter 3). In these cases, the method using a heterologously-expressed ALS2 (free from wild-type ALS1) is considered as an effective tool to profile the responses of ALS2-mutated *S. juncoides* to ALS-inhibiting herbicides when the physiological basis of the whole-plant resistance is focused.

In this method, the ALS2 enzyme preparations could contain a small amount of the *E. coli* native ALS that is generally insensitive to herbicides (Duggleby and Pang 2000). However, the author is confident that the results reflected significantly the profiles of *S. juncoides* ALS2 because (i) SDS-PAGE showed the significant existence of the expressed ALS (Fig. 4-1) , (ii) the *E. coli* ALS constitutes only a minority of specific activities (that might be observed as the residual activities of the wild-type ALS2 at higher herbicide concentrations, forming a relatively flat bottom slightly over zero in Fig. 4-3A–D), and (iii) significant differences were observed in a presumable way between the wild-type and mutated ALS2 enzyme preparations that would have shared the *E. coli* ALS in common. The SU-resistant *S. juncoides* plants showed a slightly different cross-resistance pattern from the earlier results with other weeds, in which a D376E mutation confers resistance to broadly all the classes of ALS-inhibiting herbicides. The SU-resistant *S. juncoides* did not show high resistance to BPS and was not substantially resistant to IMQ. On the other hand, the heterologously-expressed mutated ALS2 showed resistance to broad chemical classes of ALS-inhibiting herbicides.

The whole-plant R/S ratios were correlated well to the enzymatic R/S ratios of ALS2. Quantitative relationships between the whole-plant R/S ratios and the enzymatic R/S ratios have been rarely discussed. Ashigh *et al.* (2009) showed the R/S ratios based on the GR_{50} of whole plants as well as that based on the I_{50} of crude ALS for *Amaranthus powelli* having the D376E mutation

across five ALS-inhibiting herbicides. However, a significant correlation was not observed. In addition, the whole-plant R/S ratios of *A. powelli* were generally less than the enzymatic R/S ratios, but the differences were smaller compared with the author's result.

The mechanism of making the whole-plant R/S ratios much lower than the enzymatic R/S ratios in *S. juncoides* has not been clarified yet. As one of possible explanations, the ALS2 would be less abundant in *S. juncoides* than the ALS in other weed species having a single functional ALS, assuming that the total amount of ALS1 plus ALS2 in *S. juncoides* is comparable to that of ALS in the weeds with a single ALS. In order to validate the hypothesis, it would be effective to analyze whole-plant dose–response of an accession having the same amino acid substitution at the same site both in ALS1 and ALS2. Though such an accession is unavailable yet, the hypothesis is possibly verified using mutants with amino acid substitutions at Pro_{197}, which mutated the most frequently in *S. juncoides* ALS (Uchino *et al.* 2007; Chapter 1).

The D376E substitution commonly conferred the least resistance to IMI herbicides among all ALS-inhibiting herbicides at whole-plant level and/or at enzymatic level (Whaley *et al.* 2007; Ashigh *et al.* 2009; Zheng *et al.* 2011; Yu *et al.* 2012; Schaedler *et al.* 2012, Le *et al.* 2005). While the author's results are consistent with this point, the R/S ratio of the mutated ALS2 of *S. juncoides* to IMQ was one of the smallest ever reported to IMI herbicides. This is probably due to weed species differences, or the difference of herbicidal molecules, since IMQ was not used in the previous studies. In fact, *R. raphanistrum* ALS having the mutation showed different R/S ratios to three IMI herbicides, imazamox, imazethapyr, and imazapyr (Yu *et al.* 2012). Further studies on several IMI herbicides, as well as several PC herbicides, with the SU-resistant *S. juncoides* would clarify the intra-chemical-class differences in R/S ratios of the D376E mutant.

The R/S ratios in the whole-plant tests and the enzymatic tests were different among the three SU herbicides. Notably, the R/S ratio of whole-plants to MSM was close to the level that is not regarded as highly resistant (10-fold). This was also observed on the Pro_{197} mutations (Chapter 2). In addition, the GR_{50} value of MSM on the SU-resistant plants (3.14 g AI ha^{-1}) is comparable to that of ISF, BPS and IMQ on the SU-susceptible ones (1.34, 9.23, and 4.16 g AI ha^{-1}, respectively). Though MSM is not used in rice fields in Japan, this fact suggests that some SU herbicides would be able to practically control 'SU-resistant' *S. juncoides* with the D376E mutation. Overall, the resistance conferred by a D376E mutation, depending on weed species, would not be as robust as the broad-scope high resistance conferred by a W574L mutation.

From an enzymological viewpoint, the mutated ALS2 derived from *S. juncoides* showed a lower *Km* (pyruvate) than the wild-type ALS2. This means that the affinity of pyruvate is higher with D376E-mutated one than with wild type. The tobacco ALS having this mutation also showed lower *Km* than the wild type (Le *et al.* 2005). The specific activities of the mutated ALS2 from *S. juncoides* were higher than the wild type, while the said tobacco's mutated ALS showed similar

activities to the wild type. As the lower *Km* and the higher specific activity suggest an improvement of enzymatic potency, further research on *S. juncoides* with the D376E mutation are of importance in order to investigate its ecological and physiological competitiveness, as well as its gene utilization.

The expressed ALS2 was estimated to keep totally or mostly the transit peptide region in the enzyme preparations. As the absence of the transit region improved the specific activities of wild-type *A. thaliana* ALS (Chang and Duggleby 1997), the above-said enzymological characteristics of *S. juncoides* ALS2 having this mutation can be further investigated in the absence of the region, after clarification of the cleavage site.

However, the author believe that the relationships among R/S ratios of ALS2 were effectively investigated with the method described in this Chapter, judging from the high correlation with those of whole plants.

In conclusions, the D376E mutation in ALS2 is considered to be responsible for whole-plant resistance of *S. juncoides*, and the *in vitro* and whole-plant cross resistance conferred by the mutation was not so broad as to cover all the ALS inhibiting herbicides.

4-5. Summary

S. juncoides plants having a D376E mutation in ALS2 were found from a paddy rice field in Japan, but their resistance profile has not been quantitatively investigated. In this Chapter, dose–response of the SU-resistant accession was compared with that of a SU-susceptible accession at *in vivo* whole-plant level as well as at *in vitro* enzymatic level.

In whole-plant tests, R/S ratios based on 50% growth reduction (GR_{50}) for imazosulfuron (ISF), bensulfuron-methyl (BSM), metsulfuron-methyl (MSM), bispyribac-sodium (BPS), and imazaquin (IMQ) were 176, 40, 14, 5.2 and 1.5, respectively. Thus, the accession having a D376E mutation in ALS2 was highly resistant to the three SU herbicides and moderately resistant to BPS, but was not substantially resistant to IMQ. This is slightly different from the earlier results reported from other weeds with a D376E mutation, in which the mutation confers resistance to broadly all the chemical classes of ALS-inhibiting herbicides.

In enzymatic tests, ALS2 of *S. juncoides* was expressed in *E. coli*; the resultant ALS2 was subjected to an *in vitro* assay. R/S ratios of the mutated ALS2 based on 50% enzymatic inhibition (I_{50}) for ISF, BSM, MSM, BPS, and IMQ were 3699, 2438, 322, 80, and 4.8, respectively. The R/S ratios of ALS2 were highly correlated with those of the whole-plant; this suggests that the D376E mutation in ALS2 is a molecular basis for the whole-plant resistance. The presence of two ALS genes in *S. juncoides* can at least partially explain why the whole-plant R/S ratios were less than those of the expressed ALS2 enzymes.

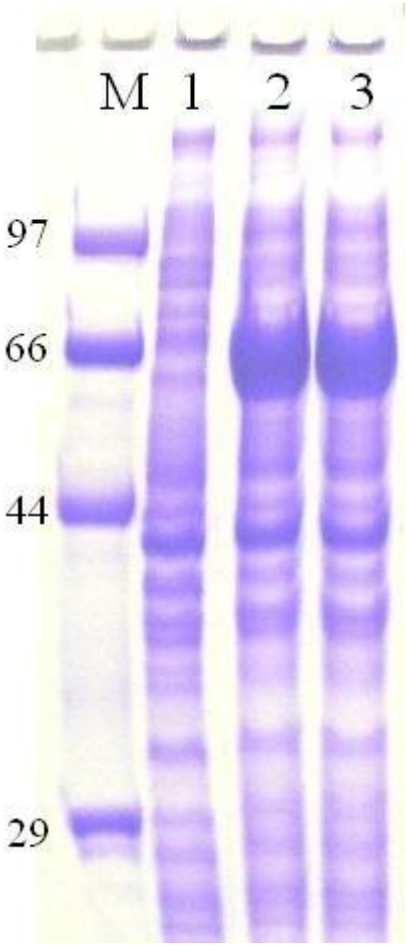

Fig. 4-1. SDS-PAGE of heterologously-expressed ALS2. Lane M: Size marker (kDa). Lane 1: non-inserted control, Lane2: SU-resistant ALS2 expression, and Lane 3: SU-susceptible ALS2 expression.

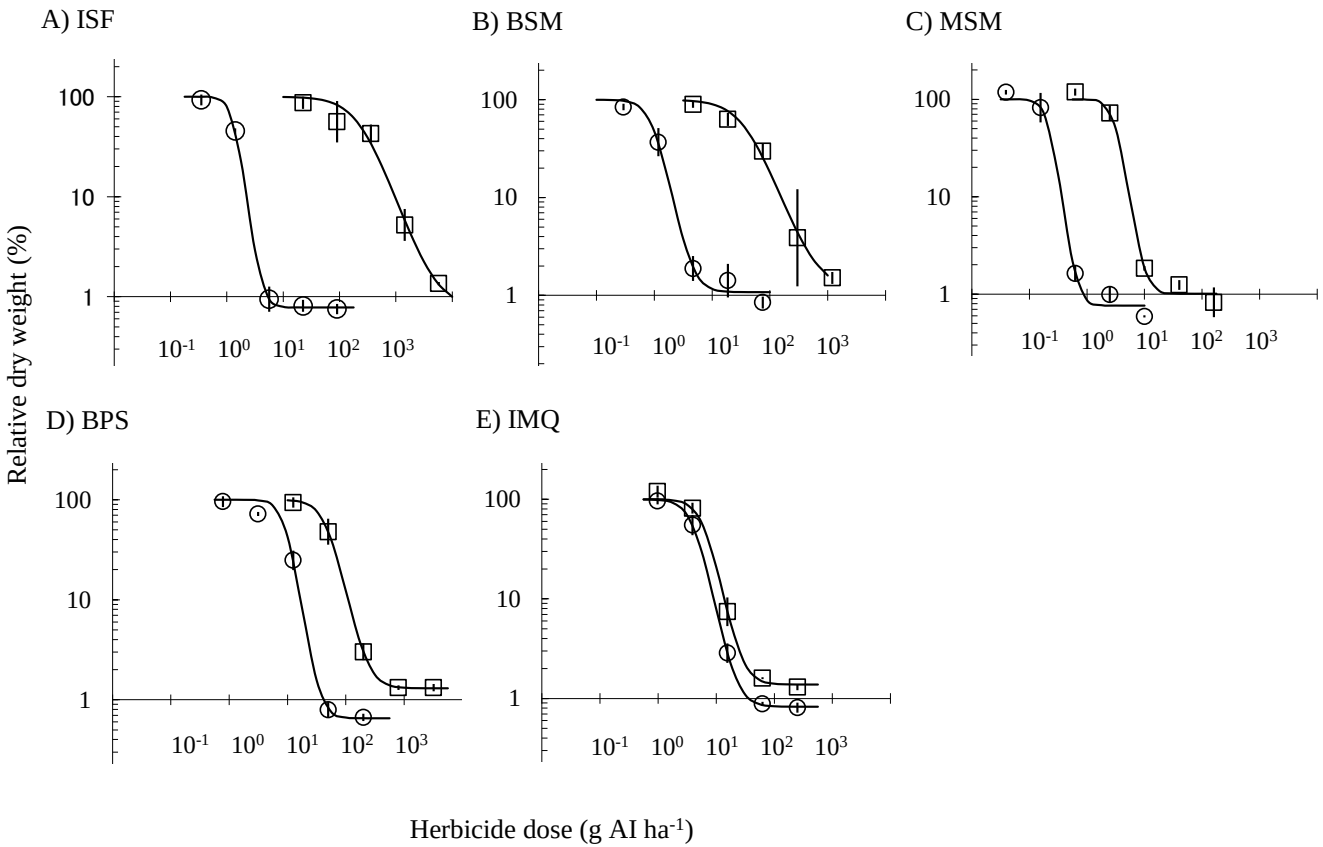

Fig. 4-2. Whole-plant dose–responses of the SU-resistant and the SU- susceptible *S. juncoides* accessions to ALS inhibiting herbicides. Tested dose rates of each herbicide are 4/0.25-times series of the standard rate for each herbicide. The standard rates are defined as 90 g AI ha^{-1} for imazosulfuron (ISF), 75 g AI ha^{-1} for bensulfuron-methyl (BSM), 10 g AI ha^{-1} for metsulfuron-methyl (MSM), 50 g AI ha^{-1} for bispyribac-sodium (BPS), and 250 g AI ha^{-1} for imazaquin (IMQ). Square for the SU-resistant, circle for the SU-susceptible accession. Each data point has ± a standard deviation.

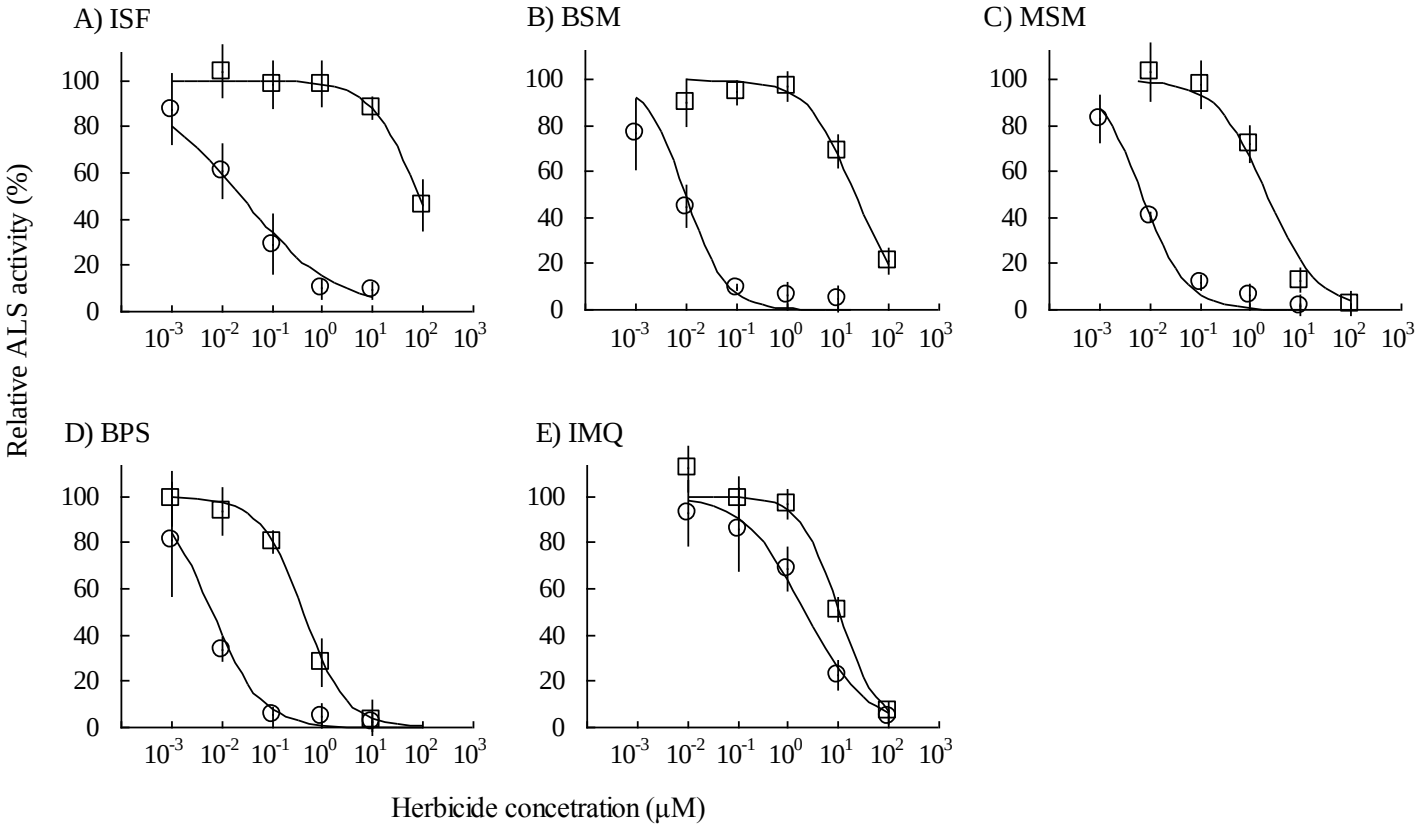

Fig. 4-3. Enzymatic dose-responses of the heterologously-expressed ALS2 of the SU-resistant and the SU- susceptible *S. juncoides* accessions to ALS inhibiting herbicides. ISF: imazosulfuron, BSM: bensulfuron-methyl, MSM: metsulfuron-methyl, BPS: bispyribac-sodium, IMQ imazaquin. Square for the SU-resistant, circle for the SU-susceptible accession. Each data point has ± a standard deviation.

Table 4-1. GR_{50} and I_{50} (ALS2) values of the SU-resistant and the SU-susceptible accessions.

Herbicide	Whole-plant test					Enzymatic test				
	GR_{50} (g AI ha^{-1})				R/S	I_{50} (μM)				R/S
	R ($Asp_{376}Glu$)		S		ratio	R ($Asp_{376}Glu$)		S		ratio
SU										
ISF	235.94	(1.64)	1.34	(1.09)	176	84.00	(1.78)	0.0227	(1.40)	3699
BSM	38.21	(1.73)	0.96	(1.28)	40	23.86	(1.33)	0.0098	(1.34)	2438
MSM	3.14	(1.25)	0.22	(1.21)	14	2.24	(1.25)	0.0070	(1.43)	322
PC										
BPS	47.97	(1.16)	9.23	(1.16)	5.2	0.39	(1.45)	0.0049	(1.35)	80
IMI										
IMQ	6.23	(1.40)	4.16	(1.14)	1.5	10.54	(1.56)	2.2083	(1.28)	4.8

R: SU-resistance, S: SU-susceptible. ISF: imazosulfuron, BSM: bensulfuron-methyl, MSM: metsulfuron-methyl, BPS: bispyribac-sodium, IMQ: imazaquin. Numbers in parentheses that follow GR_{50} and I_{50} values show 95% confidence intervals. The upper of each confidence interval can be calculated by multiplying the GR_{50} or I_{50} value by the following number in the parenthesis. The lower limit of each confidence interval can be calculated by dividing the GR_{50} or I_{50} value by the following number in the parenthesis.

Chapter 5. Rapid diagnosis of SU-resistance for *S. juncoides*

5-1. Introduction

SU-resistant *S. juncoides* is genetically diverse in target-site mutations and their cross-resistant patterns to ALS inhibitors vary depending on their amino acid substitutions in ALS (Uchino *et al*. 2007; Chapter 1, 2, 3). For example, the altered ALS having a substitution at Pro_{197} shows a different cross-resistance pattern, depending on the replacing amino acid at Pro_{197} (Chapter 2, 3). W574L (Uchino *et al.* 2007) and D376E (Chapter 4) separately showed unique cross-resistance patterns. Therefore, it is important to know not only the mutation sites in ALS genes but also the substituting amino acid in order to monitor SU-resistant *S. juncoides* and thus to determine which herbicide should or should not be used for the control of SU-resistant *S. juncoides*. In addition, in Chapter 1, based on the local-field scale showing mixed mutations in a small range, diagnostic methods having a high throughput are desired in order to understand the occurrence of local-field scale effectively.

For the diagnosis of SU resistance in *S. juncoides*, a rooting method (Hamamura *et al*. 2003), a shoot regeneration method (Ohno *et al*. 2004), an *in vivo* ALS assay (Uchino and Watanabe 2007), DNA sequencing (Uchino *et al*. 2007; Chapter 1), and Bio-photon detection (Inagaki *et al.* 2009) have been applied. Polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) is expected to be an effective diagnostic method, as it is more rapid and less expensive in determining the mutated sites than DNA sequencing (Corbett and Tardif 2006). However, the application of PCR–RFLP for SU-resistant *S. juncoides* has not been reported.

In contrast, for some other weeds, PCR–RFLP has been applied to detect mutations in the ALS genes. A restriction enzyme, *Nla*IV, was used to detect several Pro_{197} substitutions in *Raphanus raphanistrum* (Tan and Medd 2002) and *Monochoria vaginalis* (Inagaki *et al.* 2008). *Mbo*I was used for the D376E substitution in *Amaranthus hybridus* (Corbett 2004) and *M. vaginalis* (Imaizumi *et al.* 2008b), while *Mfe*I was used for the W574L substitution in *R. raphanistrum*, *Kochia scoparia*, *Amaranthus retroflexus* and *Amaranthus palmeri* (Foes *et al*. 1999; Tan and Medd 2002; McNaughton *et al*. 2005).

Although PCR–RFLP is an effective method for DNA analysis in general, it still will be a time-consuming process in genetic studies of SU-resistant weeds because several suspicious sites in the ALS gene have to be checked, while some paddy weed species have two or more ALS genes (Uchino and Watanabe 2002; Ohsako and Tominaga 2007*)*. In fact, for SU-resistant *R. raphanistrum* and *Alopecurus myosuroides*, PCR for the Pro_{197} and Trp_{574} sites was carried out separately in the PCR–RFLP analyses (Tan and Medd 2002; Délye and Boucansaud 2008). For *S. juncoides*, at least six sites (Pro_{197}, Asp_{376} and Trp_{574} sites in both *ALS1* and *ALS2*) have to be checked in order to detect all the known possible amino acid substitutions.

In this Chapter, PCR–RFLP was applied in order to detect a wide range of SU-resistant

mutants of *S. juncoides*. The restriction enzymes, *Bsp*LI (an isoschizomer of *Nla*IV), *Mbo*I and *Mun*I (an isoschizomer of *Mfe*I), were used to generate genomic DNA fragments for the detection of substitutions at Pro_{197}, Asp_{376} and Trp_{574}, respectively. At the same time, integrated PCR–RFLP analysis was developed, in which a single PCR product amplified from genomic DNA was commonly used for the detection of substitutions at the Pro_{197}, Asp_{376} and Trp_{574} sites. In addition, simplified direct sequencing for Pro_{197} was developed, where *ALS1* or *ALS2* is selectively sequenced from the common PCR product containing genomic DNA fragments of both *ALS1* and *ALS2*.

5-2. Materials and Methods

Plant materials

The plant materials used in this Chapter are shown in Table 5-1, which shows two accessions of SU-susceptible *S. juncoides* biotypes and 11 accessions of SU-resistant *S. juncoides* with a single-nucleotide substitution in either *ALS1* or *ALS2* (all were homozygous at the site of mutation). The accessions were *Kyo* (susceptible), *Tak* (susceptible), *Min* (P197S1), *Kak* (P197L1), *Aji* (P197H1), *Mit* (P197A1), *Sug* (P197R1), *Mik* (P197S2), *Mih* (P197L2), *Kas* (P197R2), *Tok* (P197T2), *Krs* (D376E2) and *Chi* (W574L2). The SU-resistant accessions used were chosen from the ones reported in Chapter 1, in order to cover all the known amino acid substitutions in the ALS gene of *S. juncoides* (six Pro_{197} substitutions, D376E, and W574L).

PCR-RFLP

Theoretical validation of the restriction enzymes

In *S. juncoides*, Pro_{197} in both ALS1 and ALS2 is coded by the codon, CCT, which, with the foregoing sequence, GGTT, forms a recognition sequence of the restriction enzyme, *Bsp*LI (GGN|NCC) in the genomic DNA. As reported for *R. raphanistrum* (Tan and Medd 2002) and *M. vaginalis* (Inagaki *et al.* 2008), it is theoretically expected that *Bsp*LI can be used to detect amino acid substitutions at Pro_{197} with other amino acids in *S. juncoides* because amino acid substitutions here always are accompanied with the loss of the adjacent double C at the recognition site of *Bsp*LI.

In *S. juncoides*, Asp_{376} in both ALS1 and ALS2 is coded by the codon, GAT, which, with the following nucleotide C, forms a recognition sequence of the restriction enzyme, *Mbo*I (|GATC) in the genomic DNA. As reported for *A. hybridus* (Corbett 2004) and *M. vaginalis* (Imaizumi *et al.* 2008b), it is theoretically expected that *Mbo*I can be used to detect the D376E substitution in *S. juncoides* because the substitution involves the codon, GAA or GAG, which disrupts the recognition sequence of *Mbo*I.

In *S. juncoides*, Trp_{574} in both ALS1 and ALS2 is coded by the codon, TGG. In the W574L substitutions of *S. juncoides*, leucine is coded by TTG, as consistently reported in other weed species (Tranel and Wright 2002). The nucleotide substitution, with the foregoing sequence, CAA,

introduces a recognition sequence of the restriction enzyme, *Mun*I (C|AATTG) in the genomic DNA. As reported in several species (Corbett and Tardif 2006), it is theoretically expected that *Mun*I can be used to detect the W574L substitution in *S. juncoides*.

PCR-RFLP experiments

The genomic DNA was extracted from green, mature stems of *S. juncoides* by using an extraction kit (Isoplant; Nippongene, Tokyo, Japan). The PCR primers used in this study are shown in Table 5-2. In order to obtain strong bands in the electrophoresis images, a nested PCR was carried out. For the amplification of the genomic DNA fragments of *ALS1* and *ALS2* at the same time, a first PCR was carried out with primer set 1 and a second PCR was carried out with primer set 2 by using the first PCR product as a template (the resultant nested PCR product was called "*ALS1*/*ALS2* mixed fragments" in this study). For the selective amplification of the genomic DNA fragments of *ALS1*, a first PCR was carried out with primer set 3 and a second PCR was carried out with primer set 4 by using the first PCR product as a template (the resultant nested PCR product was called "*ALS1* fragments"). For the selective amplification of the genomic DNA fragments encoding *ALS2*, a first PCR was carried out with primer set 5 and a second PCR was carried out with primer set 6 by using the first PCR product as a template (the resultant nested PCR product was called "*ALS2* fragments"). The *ALS1* and *ALS2* fragments were longer than the *ALS1/ALS2*-mixed fragments at the 5'-ends by 57 bp and 29 bp, respectively, while at the 3'-ends, they had identical sequences (Table 5-2, Fig. 5-1). All the PCRs had 40 cycles of 95°C for 30 s, 55°C for 1 min and 72°C for 1 min (TaKaRa Extaq kit; Takara Bio, Otsu, Japan), in accordance with the manufacturer's instructions. In each of the second PCR procedures of the nested PCR, the reaction solution of the first PCR was diluted by 50-fold with deionized water and was loaded as a template.

These nested PCR products were digested with *Bsp*LI (Fermentas, Burlington, Canada), *Mbo*I (Takara Bio) or *Mun*I (Fermentas) at 37°C for 3 h. The digested products were visualized by electrophoresis in 3% or 1% agarose gel and illuminated under ultraviolet light after an ethidium bromide staining.

Isogene-specific direct sequencing of the Pro_{197} site

The genomic DNA fragments were amplified by PCR using a primer set of Fw1 and Rv2 (Table 5-2) in order to amplify the fragments from *ALS1* and *ALS2* simultaneously. For the *ALS1*-specific sequencing, the PCR product was sequenced directly with the primer, Fw4, as a sequencing primer. For the *ALS2*-specific sequencing, the same PCR product was sequenced directly with the primer, Fw6, as a sequencing primer. A BigDye terminator cycle sequencing kit v 3.1 (Applied Biosystems, Foster City, CA, USA) was used for the direct sequencing. As shown in Figure 5-1, the genomic *ALS1* and *ALS2* differed in length and in sequence at the upper regions

(intron regions) of the Pro_{197} site. The primer, Fw4, is designed to selectively anneal to *ALS1* in this region, while the primer, Fw6, is designed to selectively anneal to *ALS2* in the region.

5-3. Results and Discussion

PCR-RFLP of the *ALS1*/*ALS2*-mixed fragments

For the validation of the PCR–RFLP of the *ALS1*/*ALS2*-mixed fragments, all the accessions in Table 5-1 were used. The restriction maps in *ALS1* and *ALS2* are shown in Figure 5-2 in the range of the *ALS1*/*ALS2* mixed fragments. In this range, *ALS1* and *ALS2* do not differ in the location of the recognition sites, except for a 1 bp-long difference in the fragments near the 5'-ends (Fig. 5-1, 5-2).

An electrophoresis image of the *Bsp*LI-digested products of the *ALS1*/*ALS2* mixed fragments for all the accessions tested is shown in Figure 5-3a. The three major bands observed in each lane of the SU-susceptible accessions (lanes 1 and 2) were 685 bp, 275 bp and 215 bp, judging from the restriction map (Fig. 5-2a). In each lane of all the accessions with a substitution at Pro_{197} in either ALS1 or ALS2, an additional band was observed above the 275 bp band (lanes 3–11). This additional band coincides well with a theoretical fragment of 313 bp or 312 bp, which is a combination of 215 bp and 98 bp or 97 bp, generated from a mutated *ALS1* or *ALS2* gene, respectively, with the Pro_{197} site intact (Fig. 5-2a). The lanes of the other SU-resistant accessions (lane 12 for D376E and lane 13 for W574L) showed the same band pattern as the SU-susceptible accessions because there is no mutation at the Pro_{197} site. A 215 bp band seen in the lanes of any accession with a Pro_{197} substitution (lanes 3–11) was derived from a Pro_{197}-unmutated ALS gene of each accession. These results indicate that the *ALS1*/*ALS2* mixed fragments indeed were generated from both *ALS1* and *ALS2* without a significant deviation.

An electrophoresis image of the *Mbo*I-digested products of the *ALS1*/*ALS2* mixed fragments for the tested accessions is shown in Figure 5-3b. The apparent three bands observed in each lane of the SU-susceptible accessions (lanes 1 and 2) were 530 bp, an overlapping of 375 bp and 374 bp and an overlapping of 238 bp and 225 bp, judging from the restriction map (Fig. 5-2b). In the lane of the accession, *Krs* (D376E2), an additional band was observed above the 530 bp band (lane 12). The additional band was 563 bp, as a total of 530 bp and 33 bp, with the Asp_{376} site intact in ALS2 (Fig. 5-2b). The 530 bp band in the lane of *Krs* was derived from unmutated *ALS1*. The lanes of the other SU-resistant accessions (lanes 3–11 and 13) showed the same band pattern as the SU-susceptible accessions.

An electrophoresis image of the *Mun*I-digested products of the *ALS1*/*ALS2* mixed fragments for the accessions tested is shown in Figure 5-3c. A single band observed in each lane of the SU-susceptible accessions (lanes 1 and 2) was 1264 bp, judging from the restriction map (Fig. 5-2c). In the lane of the accession, *Chi* (W574L2), an additional band was observed below the 1264 bp band (lane 13). The band was 1088 bp, generated by digestion at the Trp_{574} site in ALS2 (Fig. 5-3c).

A 1264 bp band in the lane of the accession, *Chi*, was derived from unmutated *ALS1*. The lanes of the other SU-resistant accessions (lanes 3–12) showed the same band pattern as the SU-susceptible accessions.

As shown above, the known SU-resistant mutations that exist in *ALS1* or *ALS2* were detected successfully with this new PCR–RFLP method. It also was demonstrated that a single PCR product, covering the Pro_{197}, Asp_{376} and Trp_{574} sites, which was amplified from genomic *ALS1* and *ALS2* simultaneously, can be commonly digested with *Bsp*LI, *Mbo*I and *Mun*I separately. These results indicate that the PCR–RFLP of the *ALS1*/*ALS2* mixed fragments is a rapid and labor-saving genetic analysis tool for SU-resistant *S. juncoides*, although it cannot be determined which ALS gene has the mutation.

PCR-RFLP for the separate analyses of *ALS1* and *ALS2*

The *ALS1* and *ALS2* fragments of the accessions, *Kyo*, *Min* and *Mik*, were digested with *Bsp*LI (Fig. 5-4a). Similarly, those of the accessions, *Kyo* and *Krs*, were digested with *Mbo*I (Fig. 5-4b) and those of the accessions, *Kyo* and *Chi*, were digested with *Mun*I (Fig. 5-4c).

When the *ALS1* or *ALS2* fragments of the SU-susceptible accession, *Kyo*, were digested with *Bsp*LI (lanes 1 and 2 in Fig. 5-4a), three major bands identical to the ones in lanes 1 and 2 in Figure 5-3a were observed. The *Bsp*LI digestion of the *ALS1* fragments of the accession, *Min* (lane 3 in Fig. 5-4a), and of the *ALS2* fragments of the accession, *Mik* (lane 6 in Fig. 5-4a), gave a specific band of 370 bp or 341 bp, respectively, with the disappearance of the 215 bp band. As the *ALS1* and *ALS2* fragments were longer than the *ALS1*/*ALS2* mixed fragments and the extended portions at the 5'-ends had no recognition site for *Bsp*LI (Fig. 5-1), the specific bands of *Min* and *Mik* were longer than the corresponding band of the *ALS1*/*ALS2* mixed fragments (lane 3 and lane 8 in Fig. 5-3a, respectively) by the respective extended lengths (i.e. 313 + 57 bp for *Min* and 312 + 29 bp for *Mik*).

In the digestion with *Mbo*I, the apparent three bands, which were identical to those of the *Mbo*I-digested *ALS1*/*ALS2* mixed fragments (lane 1 in Fig. 5-3b), were observed in the lanes for *ALS1* and *ALS2* of the SU-susceptible accession, *Kyo* (lanes 1 and 2 in Fig. 5-4b). The lane for *ALS2* of the accession, *Krs* (lane 4 in Fig. 5-4b), had a 563 bp band instead of the 530 bp band, compared to the corresponding lane for the accession, *Kyo* (lane 2 in Fig. 5-4b). In the digestion with *Mun*I, a 1264 bp band was observed in the lanes for *ALS1* and *ALS2* of the SU-susceptible accession, *Kyo* (lanes 1 and 2 in Fig. 5-4c), which was identical to the one for the *ALS1*/*ALS2* mixed fragments (lane 1 in Fig. 5-3c). The lane for *ALS2* of the accession, *Chi* (lane 4 in Fig. 5-4c), had a 1088 bp band instead of the 1264 bp band. The specific bands observed in *Krs* and *Chi* indicating the existence of the mutations in the separate analyses of the *ALS1* and *ALS2* fragments are identical to the bands observed in the analysis of the *ALS1*/*ALS2* mixed fragments, because the location at which the forward primers combined did not affect the size of the bands (Fig. 5-2).

In conclusion, as a result of the PCR–RFLP analyses of the SU-resistant accessions, the mutated ALS gene and the mutated site could be decided according to which specific band appeared and the unmutated ALS gene having the same bands as the SU-susceptible accession. These suggest that the PCR works isogene-specifically to amplify the fragments selectively from *ALS1* or *ALS2*.

Isogene-specific direct sequencing

The accessions, *Kyo* and *Kak* (P197L1) and *Mih* (P197L2), were applied in the isogene-specific direct sequencing. The sequencing chromatograms are shown in Figure 5-5. As *ALS1* and *ALS2* differ consistently in the codon that codes Arg_{199}, which is CGC for *ALS1* and CGT for *ALS2* (Fig. 5-1), *ALS1* and *ALS2* can be distinguished according to the third nucleotide of this codon. When the PCR product containing fragments of both *ALS1* and *ALS2* of the accession, *Kyo*, was applied for sequencing with the primer, Fw4, only *ALS1* was sequenced, while with Fw6, *ALS2* was selectively sequenced, judging from the third nucleotide of Arg_{199} (Fig. 5-5a). Then, the Pro_{197} sites of the two SU-resistant accessions, *Kak* and *Mih*, were sequenced with the primer, Fw4, to result in CTT for the accession *Kak* and CCT for the accession *Mih* (Fig. 5-5b,c). Similar sequencing with the primer, Fw6, resulted in CCT for the accession *Kak* and CTT for the accession *Mih*. These results at the Pro_{197} sites were completely identical to the sequences that already had been analyzed by separate PCR of *ALS1* and *ALS2* (Table 5-1, Chapter 1).

Thus, this study has established a method to selectively sequence *ALS1* or *ALS2* with the isogene-selective primers from common templates containing both *ALS1* and *ALS2*. This sequencing method is more rapid and labor-saving than a current sequencing method commonly used because neither a gene cloning nor separate PCR of *ALS1* and *ALS2* is necessary. In most cases, the method was applicable for sequencing the Asp_{376} sites as well, although the signals in the chromatogram were not as clear as for the Pro_{197} sites. This sequencing method utilized a specific positional relationship between the annealing sites of the isogene-specific sequencing primers and the Pro_{197} sites in the ALS genes, where the former are located upstream closely to the latter (Fig. 5-1).

Proposed diagnostic procedures for SU-resistant *S. juncoides*

In summary, the following new methods to analyze the mutation sites of SU-resistant *S. juncoides* were successfully developed: (i) PCR–RFLP with *ALS1*/*ALS2* mixed fragments; (ii) PCR–RFLP for separate analyses of *ALS1* and *ALS2*; and (iii) isogene-specific direct sequencing.

PCR-RFLP with *ALS1*/*ALS2* mixed fragments is the most efficient method and is recommended for the detection of the known mutations at the Pro_{197}, Asp_{376} and Trp_{574} sites in either ALS1 or ALS2, regardless of the two ALS genes being indistinguishable. When it is necessary to investigate which site in which ALS gene has mutated, PCR–RFLP for separate analyses of *ALS1* and *ALS2* is recommended.

By the PCR–RFLP methods with *Bsp*LI or *Mbo*I as described above, it cannot be determined which amino acid is substituting at the Pro_{197} or Asp_{376} site, although glutamate is the only known substituting amino acid at the Asp_{376} site of the ALS genes of field-selected, SU-resistant weeds, including *S. juncoides*. Therefore, if any mutation were to be found at the Pro_{197} or Asp_{376} site by PCR–RFLP analyses, subsequent isogene-specific direct sequencing would be necessary in order to determine which amino acid is substituting at the site. As a matter of course, it is also possible to implement isogene-specific direct sequencing from the beginning without doing PCR–RFLP for Pro_{197} and Asp_{376}.

In the PCR–RFLP method, each of the three restriction enzymes has a recognition sequence that covers the region coding the target amino acid to be investigated and an extra region. Thus, if any variation exists in such an extra region, a false diagnosis could happen theoretically. However, the author investigated the ALS genes of > 100 *S. juncoides* plants collected from 24 sites across Japan (Chapter 1) and the sequences of such extra regions were conserved according to what was described in "Theoretical validation of the restriction enzymes" in the Materials and Methods. Thus, it would be reasonable to think that the methods described in this report have a relatively lower risk of this kind of fault.

From field-selected, upland weeds resistant to ALS inhibitors, such as imidazolinones, mutations at the Ala_{122}, Ala_{205} or Ser_{653} site in the ALS gene are frequently reported, but they have not been regarded as conferring high (>10-fold) resistance to SU herbicides (Tranel and Wright 2002). Field-selected SU-resistant *Monochoria vaginalis* (a paddy weed) with an Ala_{205}Val mutation was reported in a rice field in Japan (Inagaki *et al*. 2009). In addition, it was reported that new amino acid substitutions at Ala_{122}, such as Ala_{122}Val and Ala_{122}Tyr (Krysiak *et al*. 2011; Han *et al*. 2012, respectively) as well as new amino acid sites such as Arg_{377} (Massa *et al.* 2011), and they conferred high resistance to SU-herbicides in upland weeds. The PCR–RFLP methods reported in this study are not designed to detect such new mutations if they occur in *S. juncoides*. Thus, the proactive development of diagnostic methods for such possible new mutations for *S. juncoides* is desirable.

In this study, PCR–RFLP was carried out separately for each restriction enzyme. Further studies might make the analysis more efficient, by which RFLP using simultaneous digestion by two or more enzymes (double or triple digestion) is carried out. As for the plant materials, the ALS genes of *S. juncoides* tested in this study were homozygous without exception, although the infrequent existence of heterozygous SU-resistant *S. juncoides* has been reported (Chapter 1). The reported methods here are theoretically applicable for such heterozygous *S. juncoides* plants. The author is confident that the methods reported in this study, with future improvements, if any, could play a significant role in the genetic analysis of SU-resistant *S. juncoides* by providing rapid and accurate diagnosis.

5-4. Summary

Rapid diagnostic methods to detect known mutations in ALS genes that confer SU resistance to *S. juncoides* were developed in this Chapter. By using 11 SU-resistant accessions (nine accessions with a Pro_{197} substitution in *ALS1* or *ALS2*, one accession with an D376E substitution in *ALS2* and one accession with a W574L substitution in *ALS2*), polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) analysis for DNA fragments amplified simultaneously from genomic *ALS1* and *ALS2* and PCR–RFLP analysis for DNA fragments amplified from either of the genomic *ALS1* or *ALS2* were carried out. In each of the two PCR–RFLP analyses, a common PCR product was digested separately with the restriction enzymes, *Bsp*LI, *Mbo*I and *Mun*I, in order to detect Pro_{197} substitutions, an D376E substitution and a W574L substitution, respectively. In each of the lanes where the detection of SU-resistant substitutions was aimed, a specific band to suggest the existence of the said substitutions was observed in theoretically assumable ways. Separately, a direct sequencing method also was established, which was able to selectively sequence *ALS1* or *ALS2* from common templates containing both *ALS1* and *ALS2* by the isogene-selective primers designed to anneal either of the ALS genes. It is expected that these methods could be used for the genetic analysis of SU-resistant *S. juncoides* by providing rapid and accurate diagnosis.

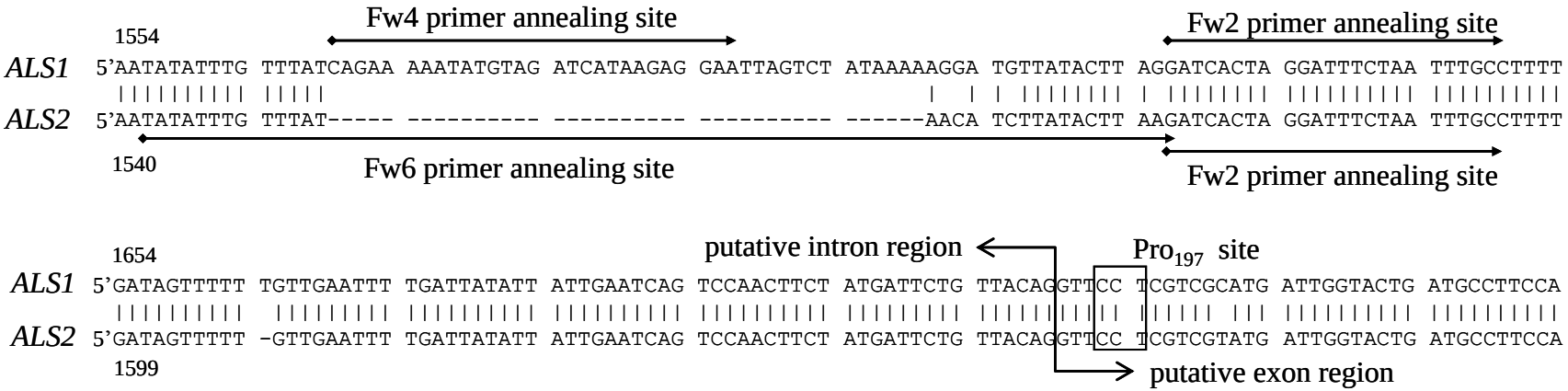

Fig. 5-1. Multiple alignment of the partial genomic DNA sequences around the Pro_{197} sites of *ALS1* and *ALS2* of *S. juncoides*. The vertical lines between the two sequences indicate the nucleotides that are matched between *ALS1* and *ALS2*. The hyphen means the non-existence of corresponding nucleotides in *ALS2* (differing in length). The numbers at the 5'-ends of *ALS1* and *ALS2* are the nucleotide numbers in GenBank accession numbers AB257440 and AB257442, respectively.

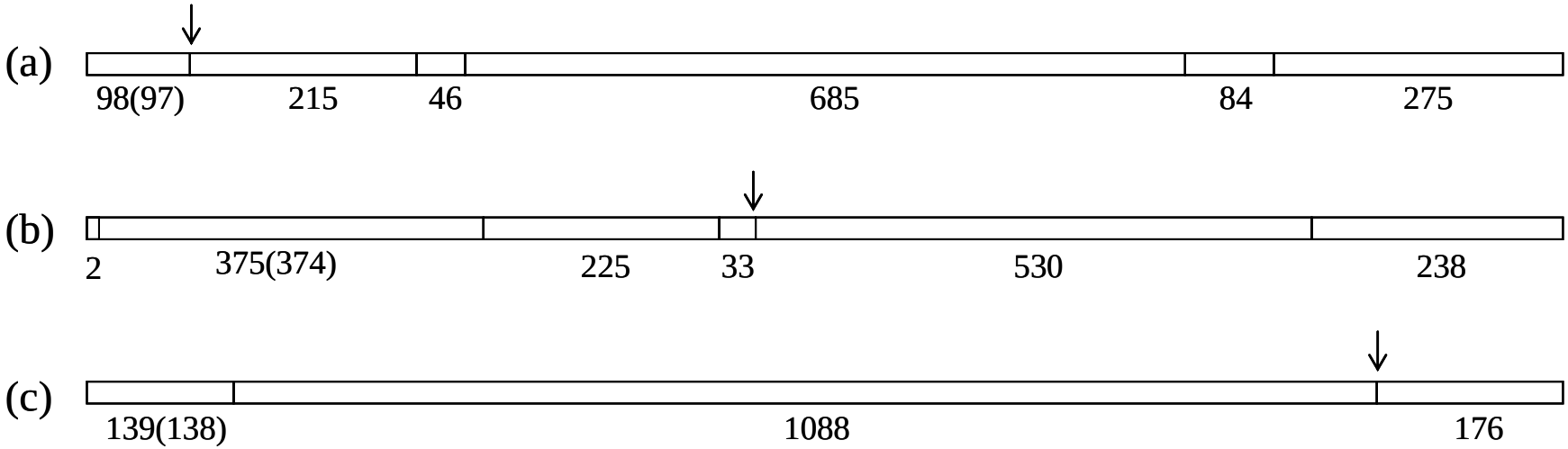

Fig. 5-2. Restriction maps of (a) *Bsp*LI, (b) *Mbo*I and (c) *Mun*I in the *ALS1*/*ALS2* mixed fragments. Both of *ALS1* and *ALS2* are included in each bar as they are substantially equivalent in the location of the recognition sites of the restriction enzymes within the region. The vertical lines in the bars are the recognition sites. The numbers are the length of fragments (bp). *ALS1* and *ALS2* differ in the length of fragments near the 5' -ends by 1 bp. The numbers in parentheses are the fragment sizes of *ALS2*. The arrows are the $Pro_{197,}$ Asp_{376} and Trp_{574} sites in (a), (b) and (c), respectively. The Trp_{574} site in (c) becomes a recognition site of *Mun*I only when it mutates to cause a $Trp_{574}Leu$ substitution by a single-nucleotide substitution.

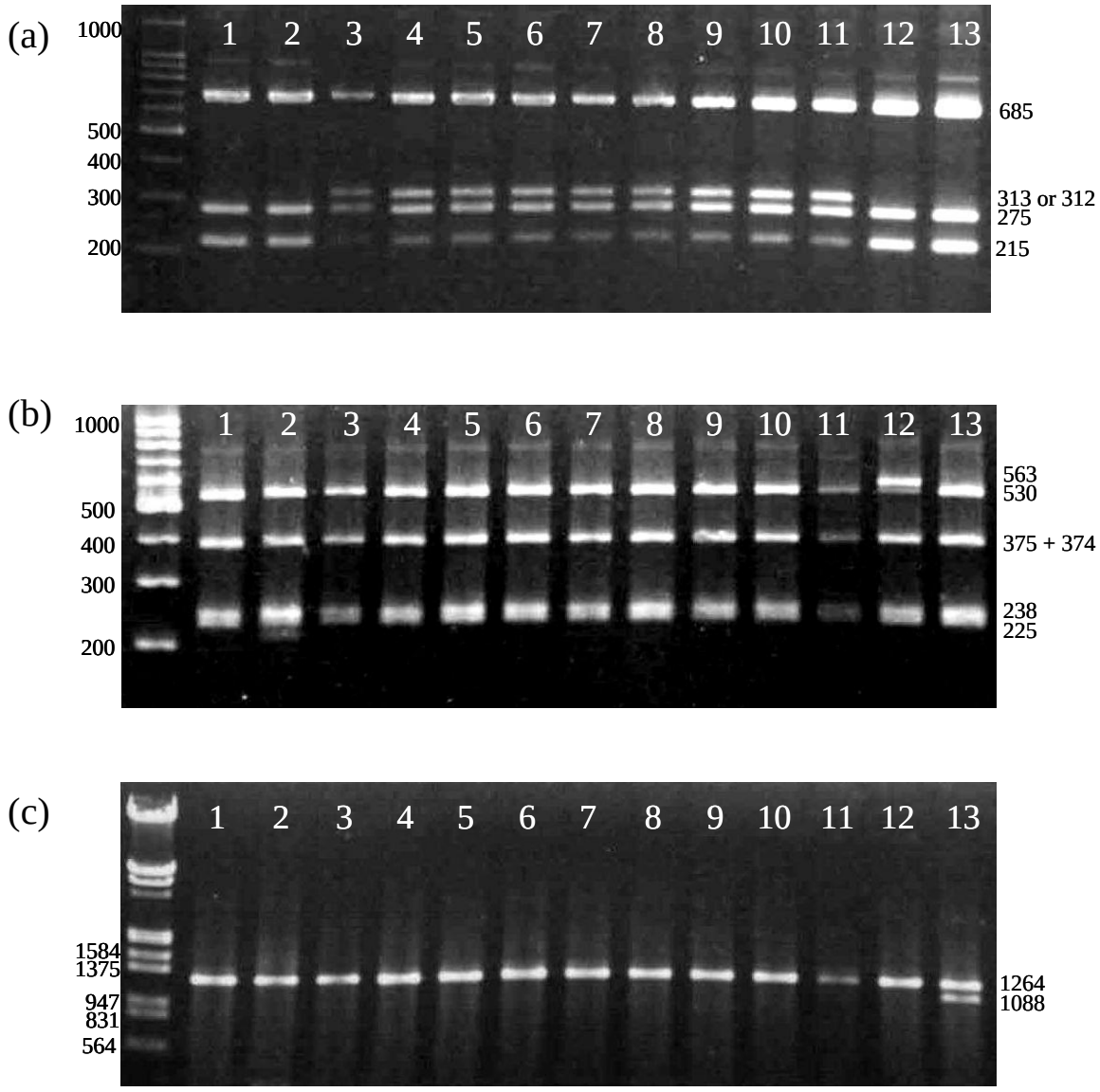

Fig. 5-3. Agarose gel electrophoresis of the digest products of the *ALS1*/*ALS2* mixed fragments. (a) *Bsp*LI digestion (3% agarose); marker: 100 bp ladder. (b) *Mbo*I digestion (3% agarose); marker: 100 bp ladder. (c) *Mun*I digestion (1% agarose); marker: λ-*Eco*RI/*Hin*dIII. Lane 1: *Kyo* (susceptible); Lane 2: *Tak* (susceptible); Lane 3: *Min* (P197S1); Lane 4: *Kak* (P197L1); Lane 5: *Aji* (P197H1); Lane 6: *Mit* (P197A1); Lane 7: *Sug* (P197R1); Lane 8: *Mik* (P197S2); Lane 9: *Mih* (P197L2); Lane 10: *Kas* (P197R2); Lane 11: *Tok* (P197T2); Lane 12: *Krs* (D376E2); Lane 13: *Chi* (W574L2) (see Table 5-1 for explanations about the accessions that were used in the study).

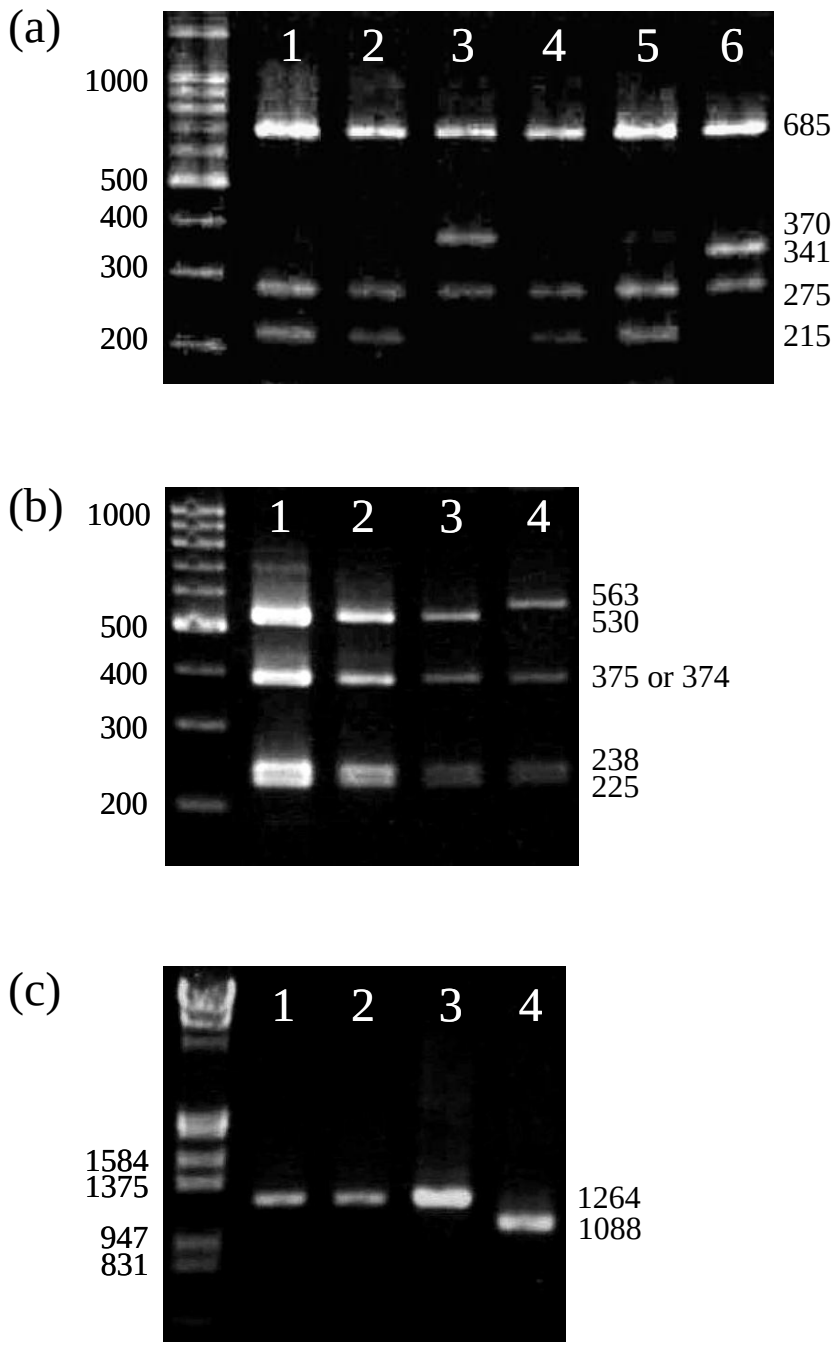

Fig. 5-4. Agarose gel electrophoresis of the digest products of the *ALS1* and *ALS2* fragments. The odd-numbered lanes and the even-numbered lanes have the digest products of the *ALS1* and *ALS2* fragments, respectively. (a) *Bsp*LI digestion (3% agarose); marker: 100 bp ladder; lanes 1 and 2:*Kyo*, lanes 3 and 4: *Min*, Lanes 5 and 6: *Mik*. (b) *Mbo*I digestion (3% agarose); marker: 100 bp ladder; lanes 1 and 2: *Kyo*, lanes 3 and 4: *Krs*. (c) *Mun*I digestion (1% agarose); marker: λ-*Eco*RI/*Hin*dIII; lanes 1 and 2: *Kyo*, lanes 3 and 4: *Chi*.

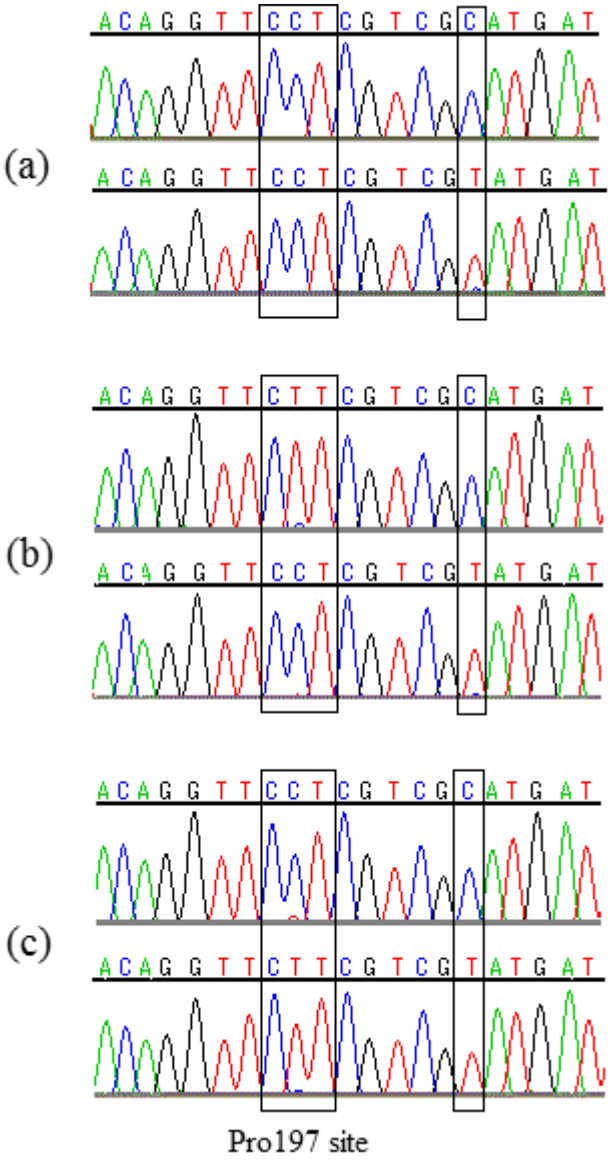

Fig. 5-5. Sequence chromatogram of isogene-specific direct sequencing. (a) *Kyo*, (b) *Kak* and (c) *Mih*. In each accession, the upper chromatogram was obtained by the sequencing primer, Fw4, and the lower chromatogram was obtained by the sequencing primer, Fw6. The smaller box shows the third nucleotide of Arg_{199}, which differs between *ALS1* and *ALS2*.

Table 5-1. Background of *Schoenoplectus juncoides* accessions used in this study

Accession	Site of collection		biotype	*ALS1*			*ALS2*		
	City/Town	Prefecture		Pro_{197}	Asp_{376}	Trp_{574}	Pro_{197}	Asp_{376}	Trp_{574}
Kyo	Kyoto	Kyoto	S	CCT (Pro)	GAT (Asp)	TGG (Trp)	CCT (Pro)	GAT (Asp)	TGG (Trp)
Tak	Takarazuka	Hyogo	S	CCT (Pro)	GAT (Asp)	TGG (Trp)	CCT (Pro)	GAT (Asp)	TGG (Trp)
Mit	Mitsuke	Niigata	R	GCT (Ala)	—	—	—	—	—
Sug	Sugagawa	Fukushima	R	CGT (Arg)	—	—	—	—	—
Chi	Chiyokawa	Ibaraki	R	—	—	—	—	—	TTG (Leu)
Min	Minoo	Osaka	R	TCT (Ser)	—	—	—	—	—
Kas	Kasai	Hyogo	R	—	—	—	CGT (Arg)	—	—
Mik	Miki	Hyogo	R	—	—	—	TCT (Ser)	—	—
Kak	Kakogawa	Hyogo	R	CTT (Leu)	—	—	—	—	—
Krs	Kurashiki	Okayama	R	—	—	—	—	GAA (Glu)	—
Mih	Mihara	Hiroshima	R	—	—	—	CTT (Leu)	—	—
Aji	Yamaguchi	Yamaguchi	R	CAT (His)	—	—	—	—	—
Tok	Tokushima	Tokushima	R	—	—	—	ACT (Thr)	—	—

A hyphen means identical to SU-susceptible accessions. S, SU-susceptible; R, SU-resistant.

Table 5-2. PCR primers used in the amplifications of the ALS genes of *S. juncoides*

Primer	DNA sequence	Annealing sites in genomic DNA	
		ALS1 (AB257440)†	*ALS2* (AB257442)†
Primer set 1			
Fw1	TTATGTCATCTTAATCGAAGGT	1519-1540	1505-1526
Rv1	GCACACAGTACATCAGCATTACA	3293-3315	3232-3254
Primer set 2			
Fw2	GATCACTAGGATTTCTAATTTGCC	1626-1649	1571-1594
Rv2	GGTGTCTCCAACATTTTCCT	3009-3028	2953-2972
Primer set 3			
Fw3	TCTTTGCTCCCGTTCGACT	17-35	—
Rv3	CAAACGACAAATTCGCATTA	(2276-2295)‡	—
Primer set 4			
Fw4	CAGAAAAATATGTAGATCATAAGAGGAA	1569-1596	—
Rv2	GGTGTCTCCAACATTTTCCT	3009-3028	2953-2972
Primer set 5			
Fw5	TCTTTCGATCGCTGTCC	—	30-46
Rv5	TTGTCAAGTGATCCCTTCCC	—	(2211-2230)‡
Primer set 6			
Fw6	TATATTTGTTTATAACATCTTATACTTAAG	—	1542-1571
Rv2	GGTGTCTCCAACATTTTCCT	3009-3028	2953-2972

† GenBank accession numbers of *S. juncoides* genomic ALS genes; ‡ these annealing sites in *ALS1* and *ALS2* are described by the nucleotide numbers of the cDNA information, AB257441 and AB257443, respectively, as the genomic DNA information (AB257440 and AB257442) does not cover these regions; A hyphen means that an annealing site is not assumed to exist.

GENERAL DICUSSION

Occurrences, resistance profiles (cross-resistance and resistance levels), and characterization of diverse genotypes of SU-resistant *S. juncoides* in Japan were studied. As a result, the author have filled the lacking information that was pointed out in the General Introduction, as follows.

For the frequencies of each mutation, P197S is the most frequently found (ca. 40% of all samples) and P197L (ca. 30% of those) is the second most. Other mutations did not show prominent frequencies. Each of the other mutation was found at the frequencies around 10% of the total accessions, or less.

For the geographical distribution of each mutation, there were no significant biases in the nationwide scale. For example, P197S and P197L were found all through the nation. One of the lower-frequent mutations, W574L that shows broad-scope resistance was also found from the northern, eastern and western parts of Japan. Within the local-field scale, SU-resistant plants from several neighboring paddy fields showed a lower diversity in mutations than in the nationwide scale. So such local infestation in several neighboring fields appeared to have been made by colonization from a few ancestors.

For the diversity of mutations, the author firstly found P197T, P197R, and D376E from *S. juncoides*, so that *S. juncoides* covers all the major known SU-resistant mutations, along with P197S, P197L, P197A, P197H and W574L.

For the resistance profiles (cross-resistance and resistance levels), each mutation shows a unique resistance profile, even among Pro_{197} mutations, so that a mutation can be almost identified from the resistance profile. Since the resistance profile is unique, a universal ranking of Pro_{197} mutations on conferring resistance levels could not be determined, because it depends on SUs. For example, P197H that conferred the highest resistance on metsulfuron-methyl among Pro_{197} mutations did not confer the highest resistance on bensulfuron-methyl or imazosulfuron.

Among SUs, metsulfuron-methyl showed least resistance levels among SUs for all the Por_{197} mutations as well as D376E mutation. So from its specific cross-resistance, the possibility to control such ‘SU-resistant’ *S. juncoides* even with a SU was suggested.

For the difference of ALS1 and ALS2, mutations appeared to have occurred at the similar frequencies in ALS1 and ALS2. In addition, ALS1 and ALS2 contributed to whole-plant resistance levels similarly. On the other hand, a significant difference was found in the sensitivities of extracted ALS depending on whether ALS1 or ALS2 is mutated, probably because ALS1 is more abundantly expressed than ALS2. However, the reason why the enzymatic difference does not reflect on whole plants is yet to be defined.

For the rapid diagnostic methods, the author developed the PCR-RFLP methods that can be applied for all the known mutations. An isogene-specific direct sequencing method was also developed.

Having given the solutions to the lacking information, however, further issues must be solved and discussions were made on the following three points.

1. Quantitative correlation between *in vitro* and whole plant.

It is doubtless that a target-site mutation is a molecular basis of whole-plant resistance for *S. juncoides*. Especially, non-target-site resistance is not known for SU-resistant *S. juncoides*. Then, how quantitatively is the *in vitro* resistance lead to the whole-plant resistance?

In order to visualize a relationship, whole-plant R/S ratios and *in vitro* R/S ratios are plotted in a two-dimensional graph on a log-log basis (Fig. 6-1). Data points were cited from the Table 4-1 for the response of *Krs* (D376E2) to various ALS inhibiting herbicides, as well as from the accessions commonly investigated in Chapter 2 (Table 2-2) and Chapter 3 (Table 3-1). For the whole-plant responses of Chapter 2 (using ED_{90}-based R/S ratios), the ED_{50}-based R/S ratios were newly calculated from the regression equations for this purpose. From the Table 3-1, R/S ratios based on $g(x)$ were used since it is considered a non-contaminated response of mutated ALS1 or ALS2.

The two R/S ratios showed a strong correlation as discussed in the Chapter 4 for the D376E mutation. It is surprising that the correlation stands even when D376E mutation and various Pro_{197} mutations were joined together. Collectively, the correlation prevails through i) which mutations are concerned, ii) which herbicides are concerned, iii) which ALS gene is mutated and iv) whether *in vitro* R/S ratios are obtained from heterologously-expressed ALS or from $g(x)$ constructed on extracted ALS. With this observation, along with the fact that resistance profiles (cross resistance and resistance levels) were almost identified by mutations (Chapter 2), a target-site mutation is considered almost a sole basis of SU-resistance of *S. juncoides*, with little or no effect of other factors such as metabolism.

Therefore a robust and simple mechanism determining whole-plant resistance by a target-site mutation must exist. The data points distribute around the line of *in vitro* R/S ratio = (whole-plant R/S ratio)^2. In the higher R/S ratio region (such as >100 of whole-plant R/S ratio), data slightly deviated from this relationship, to the direction of relatively higher whole-plant R/S ratios than the prediction by *in vitro* R/S ratios. This may be contributed by the following mechanism. In order to obtain 50% growth reductions, imazosulfuron dose rates are very high (around 5,000 g AI ha^{-1}). In such dose rates, herbicide availability for plants would not proportionally increase in parallel with the dose rates, so that R/S ratios could be more than a theoretical prediction.

Anyway, it is interesting that the whole-plant R/S ratios are the squared root of *in vitro* R/S ratios. Squaring may be related to the fact that *S. juncoides* has two ALS genes contributing to

whole-plant resistance almost equally. *S. juncoides* plant exposed to an ALS inhibitor may overdrives ALS1 and ALS2 expression as much as possible as a surviving response, so that the abundance of ALS1 and ALS2 are balanced. In such a situation, the double-sigmoid curve of extracted ALS would have a plateau at 50% inhibition. Then, the I_{50}-based R/S ratio of such extracted ALS would be the squared root of $g(x)$-based R/S ratio. Thus, the squared relationship of whole plants might be a simple realization of the possible ALS1/ALS2-balanced enzyme in living plants exposed to an ALS inhibitor.

This hypothesis, at the same time, could explain the reason why the uneven expression of ALS1 and ALS2 (of the plant that are not exposed to ALS inhibitors) are not correlated to whole plant resistance levels where ALS1-mutations and ALS2-mutations act equally. Further research is desired especially on gene expression at the exposure of ALS-inhibiting herbicides because the author's study (Chapter 3) investigated ALS extracted from herbicide-free plants. In addition, if the double-mutated accessions in ALS1 and ALS2 are available, it is worth investigating the whole-plant dose-response as well as the *in vitro* dose-response of extracted ALS. This would be even possible by 'breeding' such an accession.

2. Occurrence frequencies of ALS mutations: now and future

To strengthen the understandings of occurrence frequencies, the author's results and Uchino *et al.* (2006) results were combined into the Table. 6-1. The collections in Chapter 1 are all original without any obtained accessions from Dr. A. Uchino or any other persons, so double-count should be negligible. The author's results were basically consistent to those of Uchino *et al*. (2006), so overall conclusions keep unchanged for the facts that P197S and P197L are most frequently found, as well as that the frequencies between ALS1 and ALS2 are equivalent. However, by doing this summary, some new indications especially for the lower-frequent mutations were obtained based on the larger population.

P197T is the least frequent in *S. juncoides* among Pro_{197} mutations while it is a rather common mutation for other weed species (Heap 2014). It is interesting that P197T confers the smallest resistance to bensulfuron-methyl (Chapter 2). Bensulfuron-methyl has been the market leader of SU herbicides since its introduction to rice paddies in Japan. So this may be related to the less dominance of P197T by possibly suppressing (even if not controlling) the growth of the accessions with this mutation.

Ala_{122} mutations or Ser_{653} mutations have not been documented for *S. juncoides*. It would be natural because IMI herbicides have not been used in rice paddies in Japan. This is in contrast to the fact that many weeds having such mutations have been documented in the upland weeds especially in USA where IMI herbicides are frequently used for soybeans. Thus, along with the indication from

P197T, it is obvious that occurrences of mutations depend on the usage history of ALS-inhibiting herbicides.

Having mentioned that, the author predict some possible changes in the frequencies near future as follows. Pyrimisulfan is not effective on W574L-mutated *S. juncoides* (Takayama *et al.* 2013). This is predicted to be true with propyrisulfuron based on its chemical structure since a pyrimidine ring of SU and PC that is also possessed by propyrisulfuron and pyrimisulfan makes a stacking with Trp_{574} (Pang *et al.* 2003). Introductions of these new ALS-inhibiting herbicides by replacing conventional SUs, will lead to dominance of W574L mutation.

In addition, from the assumption that a mixture of unmutated and mutated ALS makes whole plants less sensitive than *in vitro*, double-mutated accessions in both ALS genes, which have been very rarely reported, could be dominant in future, even with 'less resistant' mutations (such as Pro_{197} mutations) to these new ALS inhibiting herbicides.

Therefore, continuous monitoring is desired to track changes of the future occurrences of mutations. For such monitoring, the author believe that the methods in Chapter 5 should play an important role because the methods enable us to detect both W574L-mutated and double-mutated accessions.

3. Indication for SU-resistant *S. juncoides* management

SU is still very useful to control perennial sedges such as *Cyperus serotinus* and *Eleocharis kuroguwai*. They are not SU-resistant yet probably because of their seed-free perennial propagation. Therefore, it is not the best way to stop using SU (or ALS inhibitor) for rice paddies in Japan. Then, as a 'side effect' of such useful control on these perennials, *S. juncoides* will be kept exposed to a selection pressure of ALS inhibitors even if these ALS inhibitors are mixed with herbicides with a different mode of actions.

In a local-field scale, the author indicated that colonization from a few ancestors is a factor of multiple-field infestations. So, once a field has resistant plants, preventing them from going out of the field (enclosure) is an important tactics to deter local infestation. It is not clarified whether seeds or pollen are moving in such colonization. However, removing the survived plants by sequential application of herbicides or hand weeding (before flowering if pollen moves) will be effective not only for the field itself but also surrounding fields. Further research for colonization in a local-field scale is needed to draw such an enclosure strategy.

On the other hand, in a wider scale, it is doubtless that SU-resistance *S. juncoides* have multiple sources since there are various mutations. So, occurrence itself cannot be prevented by such enclosure. Needless to say, as a general rule, herbicide rotation and mixture with herbicides with a different mode of action is important to prevent resistance. But in Japanese rice cultivation system, 'specifically cultivated rice' is preferred, in which the number of used pesticide active ingredients

has to be less than the half of the standard (Asakura *et al.* 2013). So to simply increase the number of active ingredients is not what farmers and consumers want.

This study, along with other reports (Ikeda *et al.* 2011; Asakura *et al.* 2012) showed some ALS-inhibiting herbicides are still effective on Pro_{197}-mutated *S. juncoides* plants. So, these ALS-inhibiting herbicides such as propyrisulfuron can be recommended for the control of Pro_{197}-mutated *S. juncoides* for the time being if a preparatory diagnosis is carefully made. Unlike some other paddy weed species such as *Monochoria* in which multiple ALS genes' contribution to resistance is complex (Imaizumi *et al.* 2008b; Wang and Tominaga 2012) and *Sagittaria trifolia* in which non-target-site SU-resistance is found (Iwakami *et al.* 2014), *S. juncoides* SU-resistance has been revealed simple and predictable by genotypes. Thus, a genetic diagnosis is expected to provide a robust conclusion. But finally, as previously said, a dominance of W574L-mutated *S. juncoides* is thought inevitable even with a rotation and mixture with other herbicides with a different mode of action.

Therefore, the new herbicidal active ingredient is really desired. Then, such an ideal new herbicide meeting the demands of recent farmers and consumers will be the one like SUs just after introduction in late 1980s, in terms of non-existence of resistance and a broad spectrum control.

In the past, a Pro_{197}-mutation was referred to as a SU-resistance conferrer (Tranel and Wright 2002) but now it has been revealed not always. So the author think that seeking for an ALS-inhibitor that is effective on *S. juncoides* having W574L should not be abandoned. Ji *et al.* (2008) showed chemical structure designs of ALS inhibiting herbicide molecules that could overcome W574L mutation. ALS inhibition is the mode of action that has a very good profile to be a pesticide such as lower use rates as well as reduced risks to mammals. The author believe that the results and the collections shown in this study will help to seek for new herbicides including ALS inhibiting herbicides that can control SU-resistant *S. juncoides*.

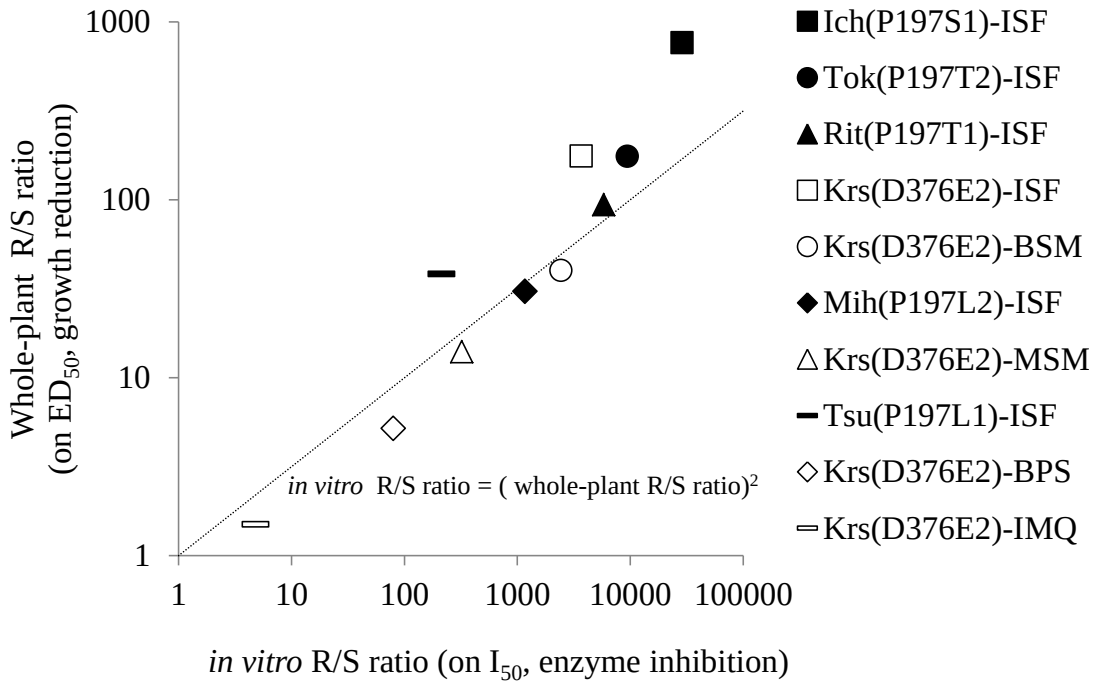

Fig. 6-1 Relationship between *in vitro* R/S ratio and whole-plant R/S ratio. ISF: imazosulfuron, BSM: bensulfuron-methyl, MSM: metsulfuron-methyl, BPS: bispyribac-sodium, IMQ: imazaquin.

Table 6-1. Number of SU-resistant *S. juncoides* accessions counted by substitution type, as a total of Chapter 1 and Uchino *et al*. (2006)

Amino acid substitution	ALS1	ALS2	Total
P197S	16	15	31
P197L	7	6	13
P197H	5	2	7
P197A	3	2	5
P197R	2	1	3
P197T	1	1	2
D376E	0	1	1
W574L	0	4	4
Total	34	32	66

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ABSTRACT

1. SU-resistant *Schoenoplectus juncoides* plants were collected nation-widely in Japan. DNA sequencing on *ALS1* and *ALS2* found an amino acid substitution occurred at Pro_{197}, Asp_{376} or Trp_{574} in either ALS1 or ALS2 in every SU-resistant plant. Among the mutations, P197S was most frequently found followed by P197L. Their frequencies in ALS1 and ALS2 were similar. W574L that confers a broad-scope resistance was found at a lower frequency (<10% of the samples) but from a wide range of Japan. P197T, P197R, and D376E were reported for the first time in this research for *S. juncoides*. In the local-field scale monitoring in two sites of Hyogo prefecture, less diverse mutations were found than the nation-wide survey. Based on such relatively uniform mutations, it is suggested that colonization from a few ancestors is a basis of local infestation among neighboring fields. On the other hand, several mutations were found from each site in the range of approximately 500m-radius, so multiple origins are involved even in such a local scale.

2. Whole-plant resistance profiles (cross-resistance and resistance levels) were investigated in pot tests in greenhouse, using several Pro_{197} mutations against several SUs as well as other ALS inhibiting herbicides. All the accessions showed high resistance to imazosulfuron and bensulfuron-methyl, relatively lower resistance to metsulfuron-methyl, and no or little resistance to imazaquin. Whole-plant resistance profiles vary on amino acid substitutions at Pro_{197}, but did not vary on which ALS (ALS1 or ALS2) has a mutation. Four pairs of accessions with a common mutation in ALS1 that originated from two distantly-located sites showed very similar resistance profiles in twelve comparisons (four pairs of accessions times three SU herbicides) without exceptions.

3. Enzymatic resistance levels to imazosulfuron were investigated *in vitro* with extracted ALS. Both of the ALS1-mutated enzyme extractions and the ALS2-muatated ones showed resistance to imazosulfuron *in vitro*, re-confirming that a target-site mutation is a basis of resistance. The ALS1-mutated enzyme extractions and the ALS2-mutated ones showed a double-sigmoid dose-response to imazosulfuron inhibition, with a plateau at around 30% and around 70%, respectively. So it is assumed that the abundance of ALS1 is consistently more than that of ALS2 at the timing of extraction.

4. A SU-resistant accession having D376E in ALS2 was characterized. Whole plants showed high resistance to imazosulfuron, bensulfuron-methyl, and metsulfuron-methyl, moderate resistance

to bispyribac-sodium, and little resistance to imazaquin. Heterologously-expressed ALS2 having the mutation showed an *in vitro* resistance profile similar to that of whole-plants. But the R/S ratios (SU-resistant / SU-susceptible ratios of effective doses or effective concentrations) are higher *in vitro* than in whole-plants. Collectively the D376E-mutated accession and the other Pro_{197}-mutated accessions investigated in whole-plant and extracted enzyme assays, whole-plant R/S ratios and *in vitro* R/S ratios (for the extracted enzymes, wild-type-free R/S ratios calculated from the regressions) are in a single strong correlation, where the whole-plant R/S ratios are roughly the squared root of the respective *in vitro* R/S ratios. With this correlation, along with the resistance profiles that could be almost identified by mutations, SU-resistance of *S. juncoides* is considered to be determined by almost all target-site mutations, with little or no effect of other factors such as metabolism

5. The reason is yet to be clarified why whole-plant resistance levels are unbiased between ALS1-mutated and ALS2-mutated accessions having common mutations, despite that enzyme resistance levels are biased. However, a hypothesis is that ALS1 and ALS2 abundance would be similar when plants are exposed to an ALS-inhibiting herbicide. The said squared correlation between whole-plants and *in vitro* would support this hypothesis since such an ALS1/ALS2-balanced enzyme would show an R/S ratio that is the squared root of that of wild-type-free mutated ALS.

6. Rapid diagnostic methods to genetically detect all the known SU-resistant mutations in *S. juncoides* were developed. A PCR-RFLP method is designed to detect Pro_{197} mutations, D376E, and W574L by separately digesting a common PCR product with *Bsp*LI, *Mbo*I and *Mun*I, respectively. The method was applicable for the PCR products having both of *ALS1* and *ALS2*, and the PCR products having either of *ALS1* or *ALS2*. In addition, a direct sequencing method was developed to selectively sequence *ALS1* or *ALS2* from a common template having both of *ALS1* and *ALS2*. This method utilizes a specific positional relationship between the Pro_{197} site and the intron region in genomic DNA. The intron locate in the very close upstream of the Pro_{197} site of both *ALS1* and *ALS2* that have fairly different sequences in the intron.

7. Introduction of new ALS-inhibiting herbicides that can control Pro_{197}-muated plants and cannot control W574L-mutated plants will lead to the dominance of W574L-mutated plants, as well as double-mutated plants in ALS1 and ALS2. The developed diagnostic methods are expected to play an important role to track changes in the monitoring. SU-resistant *S. juncoides* management in near future should be mainly focused on these possibly emerging mutations.

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Finally, though a personal matter, my very first genetic analysis of SU-resistant *S. juncoides* was made in 2005 on the plants that had been collected from my own parents' paddy fields in Miki, Hyogo, where I was born and raised. I appreciate this chance of encounter to my research materials, as the origin of my interest in SU-resistant *S. juncoides*. On the other hand, in the fields, my late father was struggling with SU-resistant *S. juncoides* in early 2000s just before he passed away by an accident in 2003. Though too late for him, I would be delighted if my study could contribute to SU-resistant *S. juncoides* management for those who are struggling with SU-resistant *S. juncoides* like him. This study is dedicated to my late father Kiyoji Sada.

LIST OF PUBLICATION

1. **Sada Y.**, Kizawa S. and Ikeda H. 2012. Varied occurrence of diverse sulfonylurea-resistant biotypes of *Schoenoplectus juncoides* [Roxb.] Palla in Japan, as classified by an acetolactate synthase gene mutation.*Weed Biol. Manag.* **12**, 168–176.

2. **Sada Y.**, Ikeda H. and Kizawa S. 2013. Resistance levels of sulfonylurea-resistant *Schoenoplectus juncoides* [Roxb.] Palla having various Pro_{197} mutations in acetolactate Synthase to imazosulfuron, bensulfuron-methyl, metsulfuronmethyl and imazaquin-ammonium. *Weed Biol. Manag.* **13**, 53-61.

3. Yamato S., **Sada Y.** and Ikeda H. 2013. Characterization of acetolactate synthase from sulfonylurea herbicide-resistant *Schoenoplectus juncoides. Weed Biol. Manag.* **13**, 104-113.

4. **Sada, Y.**, Ikeda H., Yamato S. and Kizawa S. 2013. Characterization of sulfonylurea-resistant *Schoenoplectus juncoides* having a target-site $Asp_{376}Glu$ mutation in the acetolactate synthase. *Pestic. Biochem. Physiol.* **107**,106-111.

5. **Sada Y.**, Ikeda H. and Kizawa S. 2013. Rapid diagnosis of sulfonylurea-resistant *Schoenoplectus juncoides* [Roxb.] Palla using polymerase chain reaction-restriction fragment length polymorphism and isogene-specific direct sequencing. *Weed Biol. Manag.* **13**, 1-9.