REGIONAL SCALE DISTRIBUTION OF IMIDAZOLINONE HERBICIDE RESISTANT ALLELES IN RED RICE (*Oryza sativa* L.) DETERMINED THROUGH SNP MARKERS

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INTRODUCTION

Red rice is one of the most important weeds in rice paddy fields in Brazil and in most of the irrigated rice areas worldwide. The introduction of imidazolinone (IMI) herbicide-resistant rice cultivars allowed selective control of red rice. Currently, IMI rice cultivars are used in approximately 600,000 ha in Southern Brazil. The benefits of red rice control have resulted in continuous utilization of these cultivars in the same field for several growing seasons. As a consequence, red rice resistant to imidazolinone herbicides has been identified in several rice fields in this region (MENEZES *et al*., 2009). The characterization of the processes involved in the resistance of red rice may be achieved with molecular markers that identified specific resistant alleles present in ALS gene of the IMI rice. The main IMI rice cultivars used in Southern Brazil were IRGA 422 CL, SATOR CL and PUITÁ INTA CL and the ALS mutations identified in this cultivars are respectively $Gly_{654}Glu$, $Ser_{653}Asn$ e $Ala_{122}Thr$ (ROSO *et al*., 2010). The objectives of this study were to determine through single nucleotide polymorphism (SNP) markers the frequency of alleles that confer herbicide resistance in red rice plants collected as escapees of imidazolinone herbicide control in Southern Brazil.

MATERIAL AND METHODS

The plant material of this study consisted of seed samples of red rice plants that survived the application of the herbicides imazethapyr and imazapic in rice paddy fields located in Southern Brazil. Each population corresponded to seeds collected in a single field where IMI rice cultivars had been used for at least two consecutive seasons. Sixteen and 21 populations were collected in the 2006/07 and 2007/08 seasons, respectively. These populations were selected based on a screening for imidazolinone herbicide resistance carried out in 228 populations (MENEZES *et al*., 2009). Populations were selected in order to represent the most important rice growing regions of Southern Brazil. A total of 481 individuals were sampled, 208 plants from the 2006/07 season and 273 plants from the 2007/08 season. Determination of the ALS gene sequences of IMI rice cultivars and the development of SNP molecular markers to identification of ALS alleles of IMI rice cultivars was described by Roso *et al*. (2011).

The SNP markers used were SNP422, SNPSat and SNPPta which identified the mutations: $Gly_{654}Glu$, $Ser_{653}Asn$ e $Ala_{122}Thr$, respectively. Previously, the SNP reactions were validated using, as controls, the DNA sample of the cultivars and a hybrid sample consisting of the artificial mixture of DNA from the susceptible cultivar IRGA 417 and the respective resistant cultivar to which the SNP was designed (Figure 1). PCR assay was performed according the following protocol: 50 ng of template DNA, 0.166 µM of each primer (forward and reverse) (Integrated DNA Technologies, Inc.), 0.166 mM deoxynucleotide triphosphates (dNTPs), 0.2 U Taq DNA polymerase (Invitrogen Corp.), 1x buffer (Invitrogen Corp.), 1.3 µL DMSO 99.9% and 1.5 mM magnesium chloride in a total of 30 µL reaction. PCR cycling condition were: 3 minutes denaturing at 94°C; 30 cycles of 1 minute at 94°C, 1 minute at 55°C and 1.5 minutes at 72°C; and a final 10 minutes at 72°C.

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Each sample was evaluated for each SNP marker in a separate PCR assay. PCR and gel electrophoresis were repeated once. Samples with the absence of amplification for all three SNPs markers were reevaluated in a troubleshooting PCR analysis with the primers Ar1F/Ar1R (ROSO *et al*., 2010) in order to verify the presence and quality of DNA. Samples with absence of amplification of the SNPs but with a positive amplification in the troubleshooting PCR analysis were considered as plants that did not carry any of the ALS resistant alleles evaluated in this study. Frequency (%) of each ALS allele was calculated per each population and across all plants and populations.

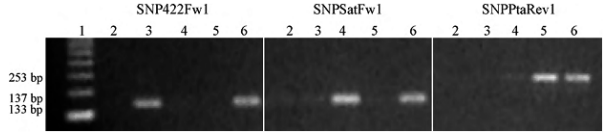

Figure 1. Validation of the SNP markers using the primers SNP422Fw1, SNPSatFw1 and SNPPtaRev1 designed for the identification of the G654E, S653D and A122T mutations, respectively. Lane 1: molecular-size marker (100 bp); lane 2: IRGA 417; lane 3: IRGA 422 CL; lane 4: SATOR CL; lane 5, PUITÁ INTA CL; and lane 6: artificial DNA hybrid of IRGA 417 and the resistant cultivar for which the marker was designed.

RESULTS AND DISCUSSION

SNP markers have been successfully used to discriminate mutants related with ALS inhibitors (KADARU *et al*., 2008) and ACCase inhibitors (DÉLYE *et al*., 2002). A positive result in PCR analysis using these markers indicates that the mechanism of herbicide resistance is related to the insensitivity of the site of action of the ALS enzyme. The $Gly_{654}Glu$ mutation was the most frequent, being found in 100% and 90.9% of the populations in 2006/07 and 20007/08 seasons, respectively (Table 1).

Table 1. Distribution of the ALS gene mutations identified through SNP markers in red rice resistant to imidazolinone herbicides collected in the 2006/2007 and 2007/2008 seasons. Data are presented as percentage of populations carrying at least one of the evaluated ALS mutations and percentage of individual plants across all populations carrying the ALS mutation.

ALS gene mutation	2006/07		2007/08	
	Populations	Plants	Populations	Plants
$G_{654}E$	100.0	56.1	90.9	81.6
$S_{653}D$	12.5	1.5	40.9	6.6
$A_{122}T$	12.5	2.0	18.2	6.6
$G_{654}E$ and $A_{122}T$	18.8	1.5	9.1	0.7
$G_{654}E$ and $S_{653}D$	18.8	1.5	22.7	1.8
$G_{653}E$ and $A_{122}T$	6.3	0.5	4.5	0.4
$G_{654}E$. $S_{653}D$ and $A_{122}T$	0.0	0.0	4.5	0.4
None	100.0	36.9	18.2	1.8

The presence of this mutation was not found in all plants from the evaluated populations, but most populations had at least one individual with this mutation (Figure 2 and 3). This mutation was also the most frequent in all plants collected across all populations (Table 1). The $Gly_{654}Glu$ mutation is the same mutation of the cultivar IRGA 422 CL, which was the main IMI rice cultivar planted in Southern Brazil during the seasons when the red rice plants were collected. Therefore, these data suggest that the high incidence of this mutation in red rice is due to gene flow from the IRGA 422 CL. Several studies have identified red rice resistant to imidazolinone herbicides that originated from gene flow (BURGOS *et al*., 2008; SALES *et al*., 2008). The determination of the origin of the ALS resistant alleles should consider a specific analysis that includes a population dynamics study with neutral molecular markers. However, this was not the aim of the present study. In

addition, gene flow from red rice to the IMI cultivated rice plants can also occur. However the cultivated rice plants that receive the ALS gene are already resistant to ALS-inhibitors and do not represent a new problem for the field that already contains the red rice resistant plants.

The $Ser_{653}Asn$ mutation was present in 12.5% and 40.9% of the populations collected in the 2006/07 and 2007/08 seasons, respectively (Table 1). The $Ala_{122}Thr$ mutation was found in 12.5% and 18.2% of the populations, respectively, in the 2006/07 and 2007/08 seasons (Table 1). Several populations presented more than one mutation (Figure 2 and 3), indicating that several ALS resistant alleles coexist in a single rice paddy field. In addition, several populations presented plants with two mutations in a single plant (Table 1, Figure 2 and 3). Furthermore, population 30, collected during the 2007/08 season, contained one plant carrying all three mutations: $Gly_{654}Glu$, $Ser_{653}Asn$ and $Ala_{122}Thr$ and other plant with the mutations $Ser_{653}Asn$ and $Ala_{122}Thr$ (Figure 3). A large diversity of mutations was found in population 16 collected during the 2006/07 season. This population presented plants with all three mutations individually, one plant with the $Gly_{654}Glu$ and $Ala_{122}Thr$ mutations and another plant with the $Ser_{653}Asn$ and $Ala_{122}Thr$ mutations. The SATOR CL and PUITÁ INTA CL cultivars are resistant to IMI herbicides due to the $Ser_{653}Asn$ and $Ala_{122}Thr$ mutations, respectively. Therefore, these cultivars could be the source of the resistant alleles for the red rice populations described above. However, the SATOR CL and PUITÁ INTA CL cultivars were cultivated in just a small area of Southern Brazil during the evaluated seasons. In addition, none of the evaluated populations was obtained from fields cultivated with these cultivars. Therefore, these analyses indicated that independent evolution of IMI herbicide resistance in red rice is probably occurring in Southern Brazil.

Of the 481 plants evaluated in this study, 83% had one or more of the ALS mutations evaluated, indicating that the main mechanism of imidazolinone herbicide resistance in these populations was related to insensitivity of the ALS enzyme. However, the failure to identify a mutation in some plants previously identified as resistant to imidazolinone herbicides (Table 1; Figure 2 and 3) indicated that another mechanism of herbicide resistance is also occurring.

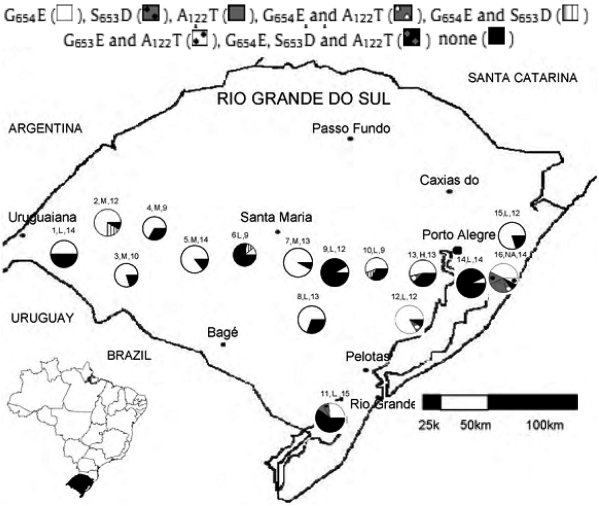

Figure 2. Distribution of ALS alleles and geographical origin of the red rice populations collected in 2006/2007. The location of each population is indicated by the pie positioned as close as possible to the geographical location. Legend of each pie indicates the population number, the level of herbicide resistance evaluated in the phenotypic analysis, and the number of evaluated plants analyzed. Pie size is proportional to number of evaluated plants in each population. Pie charts indicate the percentage of respective resistant ALS alleles.

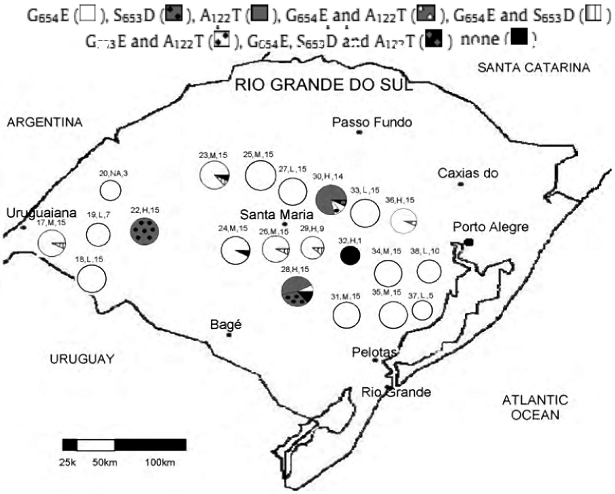

Figure 3. Distribution of ALS alleles and geographical origin of the red rice populations collected in 2007/2008. The location of each population is indicated by the pie positioned as close as possible to the geographical location. Legend of each pie indicates the population number, the level of herbicide resistance evaluated in the phenotypic analysis, and the number of evaluated plants analyzed. Pie size is proportional to number of evaluated plants in each population. Pie size is proportional to number of evaluated plants in each population. Pie charts indicate the percentage of respective resistant ALS alleles.

CONCLUSIONS

The predominant mechanism of herbicide resistance in this species is target site insensitivity due to the $Gly_{654}Glu$ mutation in the ALS gene. High frequency red rice resistant plants carrying the $Gly_{654}Glu$ mutation, which is the same mutation responsible for the resistance in the rice cultivar largely used in Southern Brazil, suggests that gene flow is occurring from the rice cultivar to red rice.

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REFERENCES

BURGOS, N. R. et al. Red rice status after five years of ClearfieldTM rice. **Weed Technology**, Lawrence, v.22, n.1, p.200-208, 2008.

DÉLYE, C.; MATÉJICEK, A.; GASQUEZ, J. PCR-based detection of resistance to acetyl-CoA carboxylase-inhibiting herbicides in black-grass (*Alopecurus myosuroides* Huds) and ryegrass (*Lolium rigidum* Gaud). **Pest Management Science**, Sussex, v.58, n.5, p. 474-478, 2002.

KADARU, S.; ZHANG, W.; YADAV, A.; OARD, J. H. Development and application of allele-specific PCR assays for imazethapyr resistance in rice (*Oryza sativa*). **Euphytica**, Wageningen,v.160, n.3, p.431-438, 2008.

MENEZES, V.G. et al. Arroz vermelho (*Oryza sativa* L.) resistente aos herbicidas imidazolinonas. **Planta Daninha**, Viçosa, v.27, n.sp, p.1047-1052, 2009.

ROSO, A. C., et al. Regional scale distribution of imidazolinone herbicide-resistant alleles in red rice (Oryza sativa L.) determined through SNP markers. **Field Crops Research**, v. 119, n. 1, p. 175-182, 2010.

SALES, M. A.; SHIVRAIN, V. K.; BURGOS, N. R.; KUK, Y. I. Amino acid substitutions in the acetolactate synthase gene of red rice (Oryza sativa) confer resistance to imazethapyr. **Weed Science**, Champaign, v.56, p.485-489, 2008.