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Regional scale distribution of imidazolinone herbicide-resistant alleles in red rice (*Oryza sativa* L.) determined through SNP markers

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ABSTRACT

Red rice is the main weed in rice paddy fields. Imidazolinone herbicides in resistant rice cultivars currently provide a unique opportunity to control red rice in large-scale rice fields. However, the continuous use of this technology has resulted in imidazolinone-resistant red rice biotypes. This study aimed to identify the mechanism of herbicide resistance and the frequency and spatial distribution of the known imidazolinone herbicide-resistant alleles in red rice. The nucleotide sequence of the ALS gene indicated that the $G_{654}E$, $S_{653}D$ and $A_{122}T$ mutations are present in the imidazolinone herbicide-resistant rice cultivars IRGA 422 CL, SATOR CL and PUITÁ INTA CL, respectively. This information and the nucleotide sequence surrounding these mutations were used for the development of single nucleotide polymorphism (SNP) molecular markers to identify the possible mutations that confer herbicide resistance in red rice. This analysis was carried out in a total of 481 plants from 38 populations collected as individuals that escaped control with the herbicides imazethapyr and imazapic in rice paddy fields in Southern Brazil. The $G_{654}E$ mutation was the most frequent, being found in 100% and 90.9% of the populations in the 2006/2007 and 20007/2008 seasons, respectively. In addition, the $S_{653}D$ and $A_{122}T$ mutations were also present either alone or as double or triple mutations in some plants. Target site insensitivity is the predominant mechanism of resistance in red rice resistant to imidazolinone herbicides in Southern Brazil. The high frequency of the $S_{653}D$ mutation, the same mutation responsible for the resistance in the rice cultivar largely used in Southern Brazil, indicates that gene flow is occurring from the rice cultivar to red rice. Management practices related to increasing crop sanitation and decreasing of herbicide selection pressure through crop rotation should be enforced to prevent the evolution of herbicide resistance in red rice.

1. Introduction

Red rice is one of the most important weeds in rice paddy fields in Brazil (Noldin et al., 2004), the southern United States (Gealy et al., 2002; Norsworthy et al., 2007) and in most of the irrigated rice areas worldwide. The main weedy characteristics of red rice in comparison with cultivated rice are higher seed shattering, longer seed dormancy, taller plants and larger number of tillers. The damage to rice production due to competition with red rice in the state of Arkansas in 2006 was approximately USD 300.00 ha^{-1} (Burgos et al., 2008). Selective control of red rice using herbicides was not possible in the past because red rice belongs to the same species as the cultivated rice. The introduction of imidazolinone herbicide-resistant rice cultivars (IMI rice) allowed selective control of red rice with imidazolinone herbicide (Webster and Masson, 2001). These cultivars were obtained through natural or induced mutation of the ALS gene (Tan et al., 2005). Imidazolinone herbicides inhibit the ALS (ALS; EC 4.1.3.18; also known as AHAS, acetohydroxyacid synthase) enzyme that catalyzes the first step in the biosynthesis of the branched chain amino acids valine, leucine and isoleucine (Mazur et al., 1987).

Several studies have suggested that risk assessment evaluation should be considered before the utilization of the herbicide-resistant rice cultivars to indicate the reliability of the system and to specify the correct management strategies that would ensure the sustainability regarding gene flow to related species and the environmental effects (Lu and Snow, 2005; Kumar et al., 2008; Olofsdotter et al., 2000). Currently, IMI rice cultivars are used in approximately 600,000 ha in Southern Brazil, which represents 55% of the total paddy rice growing area in this region (IRGA, 2008). The benefits of IMI rice cultivars related to red rice control and their interaction with better fertilizer practices and sowing time have increased the average rice grain yield by 2000 kg ha^{-1} in Southern Brazil, resulting in one of the most important technologies since the introduction of dwarf rice varieties. The current recommendations for using IMI rice cultivars are based on crop rotation, utilization of

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certified seeds and control of red rice escapees. The larger benefits of red rice control have resulted in continuous utilization of these cultivars in the same field for several growing seasons in Southern Brazil. As a consequence, red rice resistant to imidazolinone herbicides has been identified in several rice fields in this region. Menezes et al. (2009) screened 228 red rice populations and found that 56% were resistant to imidazolinone herbicides. The processes related to the origin and mechanisms of resistance, population dynamics and occurrence of gene flow from the IMI rice cultivars to red rice in these populations are unknown.

The origin of herbicide-resistant individuals may be related to gene flow or due to the independent process of resistance evolution (Sales et al., 2008). The mechanisms of herbicide resistance are mainly linked to decreased sensitivity of the target enzyme caused by overexpression of the enzyme or by point mutations, changes in absorption and translocation of the herbicide, or by increased metabolism of the herbicide before it reaches the site of action (Gaines et al., 2010; Yu et al., 2009). The effective design of prevention and control practices related to herbicide resistance requires knowledge about the prevalent events related to the origin and the mechanism of resistance occurring at individual and population level of the region under study (Merotto et al., 2010).

The diagnosis of the mechanism of resistance related to the sensitivity of the target enzyme can be achieved by molecular techniques when the mutation that caused the resistance is known (Corbett and Tardif, 2006; Délye et al., 2002). Single nucleotide polymorphism (SNP) molecular markers, also known as PCR amplification of specific alleles (PASA), amplification refractory mutation system (ARMS) and allele-specific PCR (AS-PCR), are one of the most appropriated molecular methods to assess herbicide resistance caused by DNA point mutations (Wangkumhang et al., 2007). SNP markers have already been developed to identify red rice resistance to imidazolinone herbicides caused by the amino acid substitutions S_{653}D and G_{654}E (Kadaru et al., 2008). However, another resistance allele, A_{122}T (Livore, 2005), is being used in IMI rice cultivars in Brazil, Argentina, Uruguay and other countries in South and Central America. The characterization of the processes involved in the resistance of red rice in the Southern region of Brazil requires a diagnosis based on all alleles of the ALS gene in the existing rice cultivars used in this region.

The objectives of this study were to identify the mutations and the surrounding nucleotide sequences in the ALS gene of the main IMI rice cultivars used in Southern Brazil and to determine the predominant mechanism of resistance and the frequency of alleles that confer herbicide resistance in red rice plants collected as escapees of imidazolinone herbicide control in this region. These results can contribute to the evaluation of the current utilization of IMI rice cultivars and to the risk assessment of future transgenic or non-transgenic herbicide-resistant rice cultivars that are being evaluated in Southern Brazil and in other rice regions of the world.

2. Materials and methods

2.1. ALS gene sequencing of rice cultivars resistant to imidazolinone herbicides

The plant material was the IMI rice cultivars IRGA 422 CL, PUITÁ INTA CL and SATOR CL and the susceptible cultivar IRGA 417. DNA was extracted using the CTAB (carionic hexadecyl trimethyl ammonium bromide) protocol from approximately 150 mg of young leaves of five plants for each cultivar. The DNA samples were quantified on agarose gel.

The primers used for ALS gene isolation were designed based on the following sequences available in GenBank: AB049822, AK242817, AY885674, DQ516981, NM001053466 and EF576591. The primers were designed by using Primo Pro 3.4 PCR Primer Design (http://www.changbioscience.com). Seven pairs of primers (Table 1) were utilized for the amplification of the domains A to F of ALS gene described in Merotto et al. (2009). PCR assay was performed according with 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.), 1× buffer (Invitrogen Corp.), 1.3 μL DMSO 99.9% and 1.5 mM magnesium chloride in a total of 30 μL reaction. The PCR program was as follows: 3 min at 94 °C; 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 1.5 min at 72 °C; and a final 10-min incubation at 72 °C. The PCR products were separated by electrophoresis on agarose gel (2%) for 120 min at 110 V in TBE 0.5× buffer (40 mM Tris, 1 mM EDTA, pH 8.0) and stained with ethidium bromide (0.02 mL g^{-1}). The gel was photographed with a Kodak Digital Science 1d camera (Eastman Kodak Company). The amplicons corresponding to single bands were purified directly from the solution of the PCR assay using the GFX DNA purification kit (Amersham Biosciences do Brasil LTDA).

DNA sequencing was performed using the ABI 3100 Genetic Analyzer (Applied Biosystems do Brasil). The obtained sequences were edited with the program BIOEDIT 7.0.9 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and aligned using CLUSTAL W (http://www.ebi.ac.uk/clustalw/). The alignment was performed with the standard ALS gene sequence of *Arabidopsis thaliana* (X51514) and with the ALS gene sequence of *Oryza sativa* L. (AB049822). Finally, the rice cultivar sequences were deposited in GenBank Nos. as accessions HM625775, HM625776, HM625777 and HM625778, for the cultivars IRGA 422 CL, SATOR CL, PUITA INTA CL and IRGA 417, respectively.

2.2. Development of SNP markers

The molecular marker used in this study was a variation of the conventional SNP marker called SNAP (single nucleotide-amplified polymorphism). SNAP markers consist of an insertion of an additional mismatched nucleotide in the second or third base from the 3′ end of the SNP primer such that only one allele will amplify in PCR (Drenkard et al., 2000). This procedure increases the reliability of the amplification and discrimination of point mutations between individuals in comparison with the conventional SNP markers. Based on knowledge of the point mutations and the exact nucleotide composition of the ALS gene obtained in previous studies, the SNAP molecular markers were designed for each rice cultivar with the program Websnaper (Wangkumhang et al., 2007) according to the schematic representation in Fig. 1. Two markers were designed for each ALS allele found in the IMI rice cultivars (Table 2). The SNP markers 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. PCR analysis and electrophoresis were performed as described above. PCR cycling condition were 3 min denaturing at 94 °C; 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 1.5 min at 72 °C; and a final 10 min at 72 °C.

2.3. Evaluation of imidazolinone herbicide resistance in red rice populations obtained in rice paddy fields

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 corresponds 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 2006/2007 and 2007/2008 seasons, respectively. These populations