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The Basis for Glyphosate Resistance in Rigid Ryegrass (*Lolium rigidum*) from California

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The occurrence of glyphosate-resistant weeds has been reported after more than 20 yr of extensive use. Rigid ryegrass that evolved resistance to glyphosate was found in Australia and in California. Glyphosate-resistant rigid ryegrass plants were collected from northern California and selected through generations 8 and 5 to segregate the most resistant (R) and sensitive (S) biotypes. The eighth generation of R and the fifth generation of S biotypes survived 6.72 and died from 0.11 kg ae ha^{-1} glyphosate, respectively. The objectives of this study were to evaluate the role of metabolism in the observed resistance, to study the effect of glyphosate on the activity of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19), and to characterize the EPSPS gene in R and S rigid ryegrass. Neither quantitative nor qualitative difference was observed in the metabolism of ^{14}C-glyphosate between the biotypes. Activity of constitutive EPSPS decreased more significantly in the S than R biotype in the presence of 5, 50, 500, and 5,000 μM glyphosate. Inhibition of 50% (I_{50}) of the EPSPS activity by glyphosate was more than 90-fold in S compared to R biotype. Decreased EPSPS sensitivity in the R biotype appeared to be a major contributor to glyphosate resistance in rigid ryegrass from California. Fragments of the EPSPS gene containing 1,320 nucleotides were isolated from mRNA of S and R biotypes. A single nucleotide mutation from cytosine (C) to thymine (T) was identified at nucleotide 301 of the truncated EPSPS gene of the R biotype. This mutation changed the amino acid code from proline (Pro) to serine (Ser), which was similar to that reported for the glyphosate-resistant goosegrass from Malaysia and correlated with glyphosate insensitivity of EPSPS.
Nomenclature: Glyphosate; goosegrass, *Eleusine indica* (L.) Gaertn. ELEIN; rigid ryegrass, *Lolium rigidum* Gaud. LOLRI.
Key words: EPSPS, glyphosate, metabolism, herbicide resistance.

Glyphosate resistance in rigid ryegrass was reported in Australia (Powles et al. 1998; Pratley et al. 1996) and in California (Simarmata et al. 2001). The approach to investigate the glyphosate-resistance mechanism included (1) evaluation of physiological mechanisms including absorption, translocation, sequestration of absorbed glyphosate inside the plant cell, and metabolism; and (2) biochemical and molecular investigation of the target site, which is considered to be the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; Heap and LeBaron 2001).

Glyphosate metabolism in plants is very limited (Malik et al. 1989). Although in some studies, metabolism of glyphosate was demonstrated in plants, transformation products did not significantly reduce its phytotoxicity (Sandberg et al. 1980). Aminomethylphosphonic acid (AMPA), an intermediate metabolite of glyphosate, has limited phytotoxicity to rigid ryegrass (Simarmata et al. 2003). That glyphosate metabolism did not contribute to glyphosate resistance has been reported in rigid ryegrass from Australia (Feng et al. 1999; Lorraine and Colwill 2003), in goosegrass from Malaysia (Tran et al. 1999), and in horseweed [*Conyza canadensis* (L.) Cronq.] across the United States (Feng et al. 2004).

Differential glyphosate absorption by rigid ryegrass was not the major contributor for the observed resistance (Baerson et al. 2002a; Feng et al. 1999; Lorraine-Colwill et al. 1999, 2003; Simarmata et al. 2003); however, translocation may play a partial role. Simarmata et al. (2003) reported that sequestration of absorbed glyphosate into chloroplasts was not significantly different between R and S biotypes. Lorraine-Colwill et al. (2003) reported differences in the pattern of glyphosate translocation between S and R biotypes in the Australian ryegrass. They concluded that glyphosate accumulated in the roots of the S biotype and accumulated in the leaf tips of the R biotypes. They proposed that this difference could contribute to the observed glyphosate resistance in rigid ryegrass from Australia. A similar pattern of glyphosate translocation was also reported in horseweed (Feng et al. 2004).

The level of expression of EPSPS was reported not to be significantly different between S and R biotypes of rigid ryegrass from Australia, and the enzyme activities were equally sensitive to glyphosate inhibition (Lorrraine-Colwill et al. 2003). Increase of enzyme activity levels of basal EPSPS mRNA was observed in the R biotype of rigid ryegrass from Australia, but differences in the glyphosate sensitivity of EPSPS between S and R biotypes were not apparent (Baerson et al. 2002a). Both studies concluded that expression levels of EPSPS were indistinguishable between S and R lines and suggested that the mechanism of glyphosate resistance in rigid ryegrass from Australia could be nontarget-site based. The only similarity among the studies was the appearance of shikimic acid accumulation in the sensitive biotypes after glyphosate treatment (Baerson et al. 2002a; Lorraine-Colwill et al. 1999, 2003; Simarmata et al. 2003). This evidence supports the hypothesis that the basis of the glyphosate-resistant mechanism involves the shikimic acid pathway.

A report on a truncate of the EPSPS gene isolated from the mRNA of rigid ryegrass from Australia has been published on the website of the National Center of Biotechnology Information (NCBI, 2007; accession AF349754). That report found no mutation in plastid mRNA of EPSPS. Recently, Wakelin and Preston (2006) identified a single mutation in the EPSPS from the Australian resistant rigid ryegrass that changed the amino acid 106 from proline (Pro) to threonine (Thr). In another report, Perez-Jones et al. (2005) identified no differences in the EPSPS cDNA between R and S biotypes of Italian ryegrass (*Lolium multiflorum* Lam.). This was different from the results on glyphosate-resistant goosegrass reported from Malaysia that found an altered EPSPS gene in the R biotype (Baerson et al. 2002b). The mutation in the R

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contributor to the observed resistance in rigid ryegrass from California (Figure 2). Fragments of EPSPS gene were isolated, sequenced, and aligned between S and R biotypes (Table 2). Substitution of nucleotide 301 from C to T changed the amino acid code from Pro to Ser (Table 2). This change contributed to the decreased level of glyphosate sensitivity of EPSPS in the R biotype of rigid ryegrass from California.

Sources of Materials

[1] BACCTO professional planting mix, Michigan Peat Co., P.O. Box 980129, Houston, TX 77098.

[2] Radiolabeled glyphosate, Sigma Co., 11542 Fort Mims Drive, St Louis, MO 63146-3510.

[3] TeeJet 8001E, Spraying Systems Co., P.O. Box 7900, Wheaton, IL 60189.

[4] Class Act next-generation surfactant, Agriliance, LLC, P.O. Box 64089, St. Paul, MN 55164-0089.

[5] UNIPLATE thin-layer chromatography plate, Analtech, Inc., P.O. Box 7558, Blue Hen Drive, Newark, DE 19714.

[6] AMBIS radioanalytical imaging system, Ambis System, Inc., 3939 Ruffin Road, San Diego, CA 92123.

[7] SAS software, SAS Institute, 100 SAS Campus Drive, Cary, NC 27513-2414

[8] MICRO-SPINS Shephadex G-50 column, Life Science Products, Inc., 5989 Iris Parkway, P.O. Box 1150, Frederick, CO 80530.

[9] Oligotex mRNA mini kit, and bacterial DNA purification kit, QIAGEN, Inc., 28159 Stanford Avenue, Valencia, CA 91355.

[10] 5′/3′ RACE PCR Kit, Roche Applied Science, 9115 Hague Road. P.O. Box 50414, Indianapolis, IN 46209-1387.

[11] Oligonucleotides specific primers, Integrated DNA Technologies, Inc., 1710 Commercial Park, Coralville, IA 52241-2760.

[12] dNTP (mix), PCR buffer, Taq polymerase, Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711-5399.

[13] pGEM T-Easy Vector II, Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711-5399.

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