Koo Dal-Hoe (Orcid ID: 0000-0003-4578-8519)
Roma-Burgos Nilda (Orcid ID: 0000-0002-6977-6873)
Jugulam Mithila (Orcid ID: 0000-0003-2065-9067)

Research Article

Title: Extrachromosomal DNA-mediated glyphosate resistance in Italian ryegrass

Running title: *EPSPS* gene in extrachromosomal DNA

Dal-Hoe Koo[1]*, Yoonha Ju[1], Karthik Putta[2], Rajendran Sathishraj[1], Nilda Burgos[3], Mithila Jugulam[2], Bernd Friebe[1], and Bikram S. Gill[1]

[1]Wheat Genetics Resource Center and Department of Plant Pathology, Kansas State University, Manhattan, KS 66506

[2]Department of Agronomy, Kansas State University, Manhattan, KS, 66506

[3]Department of Crop, Soil and Environmental Sciences, University of Arkansas, Fayetteville, AR, 72701

***Corresponding author:** Dal-Hoe Koo

Email: dkoo@ksu.edu

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ABSTRACT

BACKGROUND: A Italian ryegrass population from Arkansas, USA developed glyphosate resistance due to *EPSPS* gene amplification. The plants in this population with ~70 *EPSPS* copies were used in the present study for the physical mapping of amplified copies of *EPSPS* gene to determine the possible mechanism of *EPSPS* gene amplification confering glyphosate resistance in Italian ryegrass.

RESULT: Fluorescence *in situ* hybridization (FISH) anlaysis of glyphosate resistant (GR) Italian ryegrass plants with ~70 *EPSPS* copies displayed *EPSPS* hybridization signals randomly on most of the metaphase chromosmes. Whereas, glyphosate susceptible (GS) Italian ryegrass plants with one *EPSPS* copy displayed single prominent *EPSPS* hybridization signal which was co-localized with 5S rDNA locus along with few additional signals on outside of chromosomes. Pulsed-field gel electrophoresis (PFGE) followed by DNA blot using *EPSPS* gene as a probe identified a prominent *EPSPS* hybridization around 400 kb region in GR DNA samples but not in GS DNA samples.

CONCLUSION: We report the extrachromomal DNA-mediated glyphosate resistance in Italian ryegrass. Physical mapping of amplified copies of *EPSPS* gene in Italian ryegrass by FISH gives us a clue that the amplified copies of *EPSPS* gene may be present in the extrachromosomal DNA elements. Further analysis by PFGE followed by DNA blotting revealed that the extrachromosomal DNA containing *EPSPS* is of ~400 kb similar in size with that of eccDNA replicon in *Amaranthus palmeri*.

1. INTRODUCTION

Lolium perenne ssp. *multiflorum* (Lam.) Husnot (Italian ryegrass) is primarily grown as a forage grass.[1] However, the characteristics such as fast growth, prolific seed production and adaptation to diverse environmental conditions transformed this species into an important weed in different cropping systems.[2, 3] It is a diploid (2n=14), self-incompatible and outcrossing species.[4, 5] It outcrosses readily with other *Lolium* and *Festuca* species producing fertile hybrids.[6] This ability to outcross with a variety of different species and genera has resulted in the creation of vast genetic diversity in this species, which predisposes it to evolve resistance to herbicides. The first case of glyphosate resistance in Lolium was reported in a rigid ryegrass (*Lolium rigidum*) population from Australia.[7] Since then, glyphosate resistance has been reported in several Lolium species including Italian ryegrass. Glyphosate resistance in Italian ryegrass is due to *5-enolpyruvylshikimate-3-phosphate synthase* (*EPSPS*) gene mutation[8] and/or *EPSPS* gene amplification.[9, 10] Although most of the cases for evolved target site glyphosate resistance in Italian ryegrass has been explained by point mutations or enhanced expression/copy number in the *EPSPS* there are also reports of other functional genes playing significant role in selection of glyphosate resistance.[11] Recent study on evolution of glyphosate resistance in Italian ryegrass populations from Orgeon reported independent origins ruling out gene flow among populations.[12] Cytological characterization of *EPSPS* gene amplification was reported in *Kochia scoparia,*[13] *Amaranthus tuberculatus*[14, 15] and *Amaranthus palmeri.*[16] Cytogenetic analysis of glyphosate-resistant *A. palmeri* identified the presence of extrachromosomal circular DNA (eccDNA) harboring *EPSPS* gene copies.[16, 17] However, chromosomal basis of gene amplification in Italian ryegrass has not been investigated. Based on molecular and cytogenetic analyses, we report the extrachromosomal DNA driven *EPSPS* gene amplification leading to glyphosate resistance in glyphosate-resistant Italian ryegrass.

2. MATERIALS AND METHODS

2.1 Plant materials

The glyphosate susceptible (GS) and glyphosate resistant (GR) Italian ryegrass seeds were obtained from Dr. Nilda Burgos, University of Arkansas. The characterization of GS and GR Italian rygrass population was done by Putta.[20] The seeds were germinated on moist filter paper in small Petri dishes at room temperature in dark room. After establishing 2-3 cm roots, seedlings were transferred to individual pots (4 cm x 4 cm) containing Miracle-Gro potting mix (Scotts Miracle-Gro Company, Marysville, OH, USA) and the plants were grown in a greenhouse maintained at 25/20°C temperature; 15/9 h light day/night, supplemented with 120 mmol m^{-2} s^{-1} illumination using sodium vapor lamps. A total of 23 GS and 60 GR plants were grown until one tiller stage and tillers were separated to make one clone for each plant respectively. A total of 23 GS and 60 GR Italian ryegrass clones derived from separate GS and GR plants were treated with a discriminating dose (1x) of glyphosate along with appropriate adjuvant [Roundup Weathermax® @ 840 g ae ha^{-1} in 2% (v/v) ammonium sulfate (AMS)] at three-leaf stage. The herbicide was applied using a track sprayer (Research Track Sprayer, Generation III, De Vries Manufacturing., MN) equipped with a flat-fan nozzle tip (80015LP TeeJet tip, Spraying Systems Co., IL) delivering 168 L ha^{-1} at 222 kPa in a single run at 4.8 km h^{-1}. An untreated check of 3-4 plants of both GS and GR Italian ryegrass were included. Sprayed plants were maintained at the same growth conditions as mentioned above. Plants were assessed for percent survival at 28 days after treatment (DAT). There were no survivors of GS plants treated with glyphosate indicating a homogenous glyphosate susceptible population. On the other hand, the response of GR plants showed a variation in the level of resistance to glyphosate indicating that this population was segregating for glyphosate resistance. Out of the 60 GR plants treated with 1x glyphosate dose, only 24 plants survived. The seventeen GR plants that survived the treatment of 1x glyphosate were tested for *EPSPS* gene copy number

along with GS. The GR plant with ~70 *EPSPS* copy number along with GS plant with one *EPSPS* copy were selected and root tips were sampled for cytogenetic analysis.

2.2 Fluorescence *in situ* hybridization (FISH)

Mitotic and meiotic chromosome preparations were done based on the published protocol[19] with minor modifications. Root tips were collected from GR and GS plants and treated in a nitrous oxide gas chamber for 1.5 h. The root tips were fixed overnight in a 3:1 ethanol:glacial acetic acid and then squashed in a drop of 45% acetic acid. All the preparations were stored at -70°C until use. FISH hybridization and detection was carried out as described previously.[15, 16] The maize 5S rDNA[21] was labeled with biotin-16-dUTP (Roche, San Francisco, CA, USA) and *EPSPS* gene[20] was labeled with digoxigenin-11-deoxyuridine triphosphate (Roche) using a standard nick translation reaction. The biotin- and digoxigenin-labeled probes were detected with Alexa Fluor 488 streptavidin antibody (Invitrogen, Carlsbad, CA, USA) and rhodamine-conjugated anti-digoxigenin antibody (Roche), respectively.

Chromosomes were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) in Vectashield antifade solution (Vector Laboratories, Burlingame, CA, USA). The images were captured with a Zeiss Axioplan 2 microscope (Carl Zeiss Microscopy LLC, Thornwood, NY, USA) using a cooled CCD camera CoolSNAP HQ2 (Photometrics, Tucson, AZ, USA) and AxioVision 4.8 software (Carl Zeiss, White Plains, NY, USA). The final contrast of the images were processed using Adobe Photoshop CS5 software (Adobe Inc., San Jose, CA, USA).

2.3 Pulsed-field gel electrophoresis (PFGE) and DNA blotting

Nuclei were isolated by grinding the leaf tissue in liquid nitrogen. The macerate was suspended in nuclei isolation buffer (10 mM Tris-HCl pH 9.5, 10 mM EDTA, 100 mM KCl, 0.5 M sucrose, 4 mM spermidine, 1.0 mM spermine, 0.1% 2-mercaptoethanol) and filtered through nylon mesh.[22] The nuclei were subsequently embedded in agarose plugs and treated with Proteinase K (Sigma, St. Louis, MO, USA) to remove any proteins present in the sample. The high molecular

weight-DNA in agarose plugs was used for PFGE in CHEF-DR II system (Bio-Rad, Hercules, CA, USA) to separate the DNA fragments. The separated DNA fragments were subjected to DNA blot analysis with *EPSPS* gene as a probe by AP chemiluminescent blotting kit (GE Healthcare, Chicago, IL, USA) following the manufacturer's instruction.

3. RESULTS AND DISCUSSION

A glyphosate resistant (GR) Italian ryegrass population was reported by Salas et al.[9, 10] This population was found in a field with a history of several burndown applications of glyphosate.[18] Further characterization of GS and GR Italian ryegrass population identified that the GS population was homogenous whereas, GR population was heterogenous and showed a variation in the level of resistance to glyphosate indicating that GR population was segregating for glyphosate resistance. Resistant plants in this GR Italian ryegrass population had *EPSPS* gene copy numbers ranging between 11 to >80 compared to GS plants[10, 20] and the plant with ~70 *EPSPS* gene copies was used in the present study to determine the possible mechanism of EPSPS gene amplification conferring glyphosate resistance in Italian ryegrass.

3.1 *Amplified EPSPS gene copies in GR Lolium perenne is located in the extrachromosomal DNAs*

FISH analysis of GS Italian ryegrass plants with one *EPSPS* copy displayed single prominent *EPSPS*-FISH hybridization signal, which was co-localized with 5S rDNA locus (Fig. 1A-D). Interestingly, in addition to native locus, additional prominent *EPSPS*-FISH signals were often observed on the chromosomes in different cells derived from a single root tip (arrowheads in Fig. 1C). These signals were not located on any specific chromosomal region but were randomly distributed on different chromosomes. The number of additional prominent *EPSPS*-FISH signals varied in different cells, ranging from 1 to 3 and are different from weak background FISH signals. The weak background signals are diffused and caused due to non-

specific hybridization of *EPSPS* gene probe to the chromosome spreads. These additional *EPSPS*-FISH signals in GS plants is quite natural in species that develop glyphosate resistance mediated by extrachromosomal DNA. High frequency of additional *EPSPS*-FISH signals were also reported in the root tip cells of GS *Amaranthus palmeri* whereas the cells from leaf tissue of GS *A. palmeri* recorded occurance of *EPSPS*-FISH signal at very low frequency.[16] The copy number of *EPSPS* gene in the present study was calculated using the gDNA isolated from leaf tissue. Whereas, the cytology procedures to investigate the presence of ecDNA was carried out using root tip cells. Inspite this deviation, Salas et al.[10] reported that >10 *EPSPS* copies were necessary for Italian ryegrass to survive the recommended field dose of glyphosate. Though additional *EPSPS* copies were observed outside the native locus in GS Italian ryegrass (Fig. 1C), they were less than the threshold level of 10 copies. The additional copies of *EPSPS* in GR Italian ryegrass may have additive effects in conferring glyphosate resistance.[10] GR Italian ryegrass had positive correlation between *EPSPS* copy number and the level of resistance to glyphosate[9] similar to that of glyphosate resistant *A. palmeri.*[23] In GR Italian ryegrass plants with ~70 *EPSPS* copies, the FISH signals were often observed randomly on most of the chromosomes (Fig. 1H). The FISH signals were often observed away from the metaphase chromosomes and were frequently observed as a single signal overlaying a chromosome. Two *EPSPS* hybridization signals are expected on sister chromatids if *EPSPS* gene was integrated into a specific chromosomal region. But hybridization patterns differed between homologous chromosomes, indicating that the amplified *EPSPS* copies in GR Italian ryegrass may not be integrated into the chromosome but exist in extrachromosomal elements. Similar hybridization patterns were also observed in meiotic pachytene cells (Fig. 1I-P). Accounting the pattern of *EPSPS*-FISH hybridization signals in GS and GR Italian ryegrass, it can be hypothesized that the additional signals of *EPSPS* gene observed outside the chromosomes are due to the presence of *EPSPS* gene in the extrachromosomal DNA structures whose number and conformations can vary in soma cells. Such distribution patterns of amplified *EPSPS* copies on

the metaphase chromosome of GR *A. palmeri* were reported previously[16, 24] and unambiguously proved to be eccDNAs conferring glyphosate resistance.[16] The eccDNAs in GR *A. palmeri* exist in various conformations in linear or circular.[16] Cytogenetic analysis of glyphosate resistant Italian ryegrass similar to that of glyphosate resistant *A. palmeri*[16] may reveal conserved structural and functional elements regarding the biology of extrachromosomal DNA structures, possibly an extrachromosomal circular DNA (eccDNA) as a source of copy number variation (CNV).

3.2 *Confirmation of EPSPS gene as a part of ~400 kb size extrachromosomal DNA*

Pulsed-field gel electrophoresis (PFGE) separated the DNA fragments of about 10 kilobases up to 9 megabases in length in a size-dependent manner on the agarose gel.[25] Two plants each from GR (~70 *EPSPS* copies), and GS Italian ryegrass population were used in PFGE (Fig. 2). Hybridization of the *EPSPS* gene probe to DNA gel blots from PFGE displayed prominent *EPSPS* hybridization around 400 kb region in GR DNA samples but not in GS DNA samples (Fig. 2B). Large intact circular molecules such as double minute chromosomes[26] and plant organelle genomes[27, 28] do not migrate freely and stay in the well in PFGE analysis. In this regard, the 400 kb sized hybridization bands observed in this study were not believed to contain circular molecules. It may be linearized DNAs from broken circles or true linear molecules. Fiber-FISH analysis in GR *A. palmeri* showed that ~38% of eccDNA replicons are linear structured molecules.[16] DNA blot analysis also revealed significant hybridization signals in the wells too (Fig. 2B) indicating the possibility of the existence of extrachromosomal DNAs in circular conformation. Intriguingly, the size of *EPSPS* extrachromosomal DNA of GR Italian ryegrass was similar to that eccDNA replicon in *A. palmeri* which was also ~400 kb in size.[16, 29] The eccDNA replicon of *A. palmeri* contained 59 genes and many other sequence domains that may play a role in eccDNA replication and transmission.[29] Future experiments on comparative analysis of Italian ryegrass *EPSPS* extrachromosomal DNA with *A. palmeri* eccDNA replicon will

provide important information about the conserved structural and functional domains of eccDNAs.

4. Conclusion

FISH and PFGE analyses of glyphosate susceptible and resistant Italian ryegrass population identified that the amplified copies of *EPSPS* may be present in the extrachromosomal DNAs. This was evident from the *EPSPS*-FISH hybridization signals outside the metaphase chromosomes. The possibility of presence of amplified copies of *EPSPS* in extrachromosomal DNAs with ~400 kb was further confirmed by PFGE followed by DNA blot analysis. Further molecular and cytogenetic analysis of amplified *EPSPS* copies as an extrachromosomal structure in Italian ryegrass will shed light on the evolution of extrachromosomal DNA-driven glyphosate resistance and has vast implications for the evolution and management of glyphosate resistance.

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Conflict of Interest: Authors report no conflict of interests

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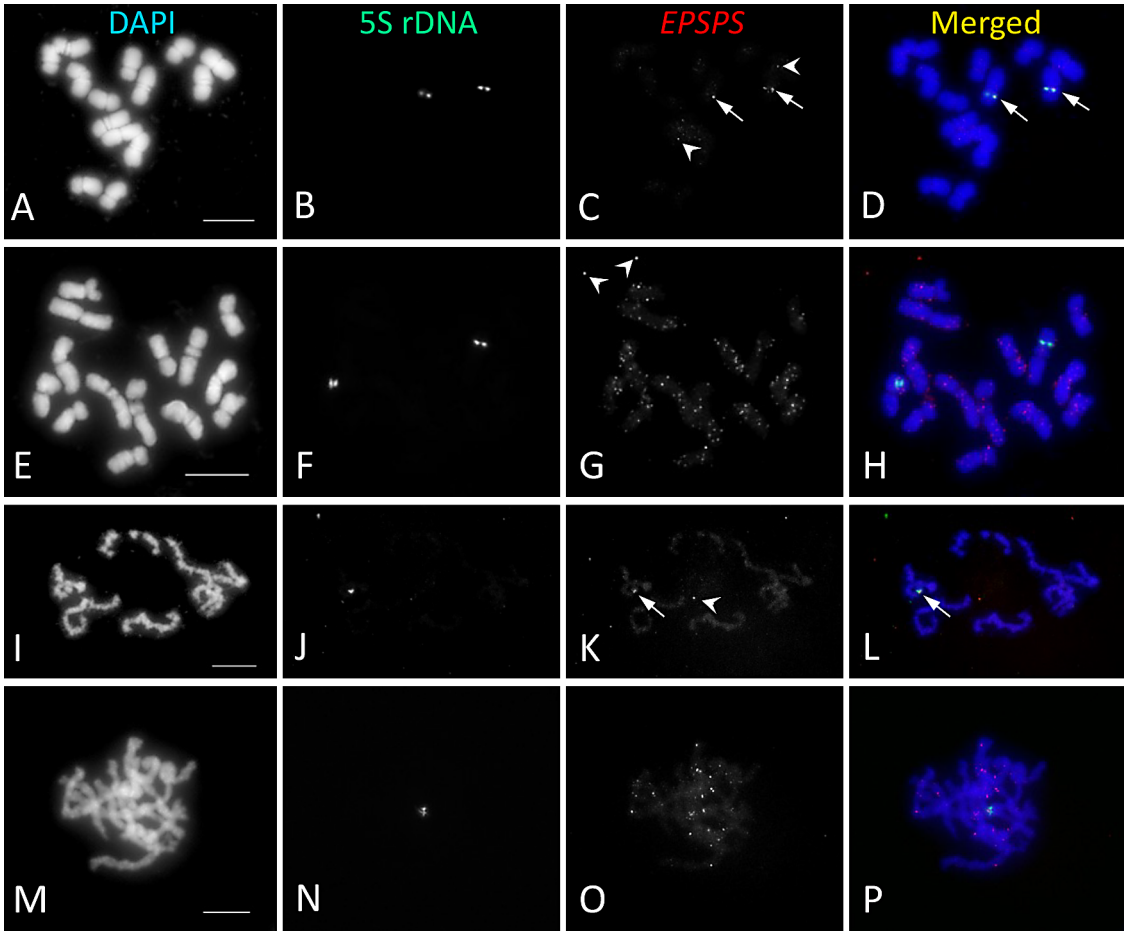
Figure legends

Figure 1. Physical mapping of *EPSPS* gene in mitotic and meiotic pachytene chromosomes of GS- and GR Italian ryegrass. FISH patterns on mitotic metaphase (**A-D**) and meitotic pachytene (**I-L**) cells in GS plant with one *EPSPS* copy. *EPSPS*-FISH signals (arrows in **C** and **K**) were co-

localized with 5S rDNA locus (Arrows in **D** and **L**). Note that additional signals were often detected on different chromosomes or outside of chromosomes (arrowheads in **C** and **K**). FISH patterns on mitotic metaphase (**E-H**) and meiotic pachytene (**M-P**) cells in GR plant with 70 *EPSPS* copies. FISH signals were distributed on most of the chromosomes in GR plant with 70 *EPSPS* copies (red signals in **H**). In addition, several hybridization signals outside of mitotic chromosome (arrowheads in **E**) but not in meiotic pachytene cells (red signals in **P**) were detected. In meiotic pachytene cell, most of *EPSPS*-FISH signals (**O**) were closely associated with pachytene chromosomes (**P**). Bars, 10 μm.

Figure 2. PFGE analysis of extrachromosomal DNAs from *Lolium perenne*. **A**, Ethidium bromide-stained pulsed-field gel with a λ marker in lanes M1 and M2. **S** and **R** indicate lanes containing embedded nuclei (undigested nuclei) from GS and GR biotypes of *Lolium perenne*, respectively. **B**, Arrow indicates the most prominent band after DNA blotting with *EPSPS* gene as a probe. PFGE condition was 90 sec of switch time at 3 V/cm, with a total run time of 46 hr.



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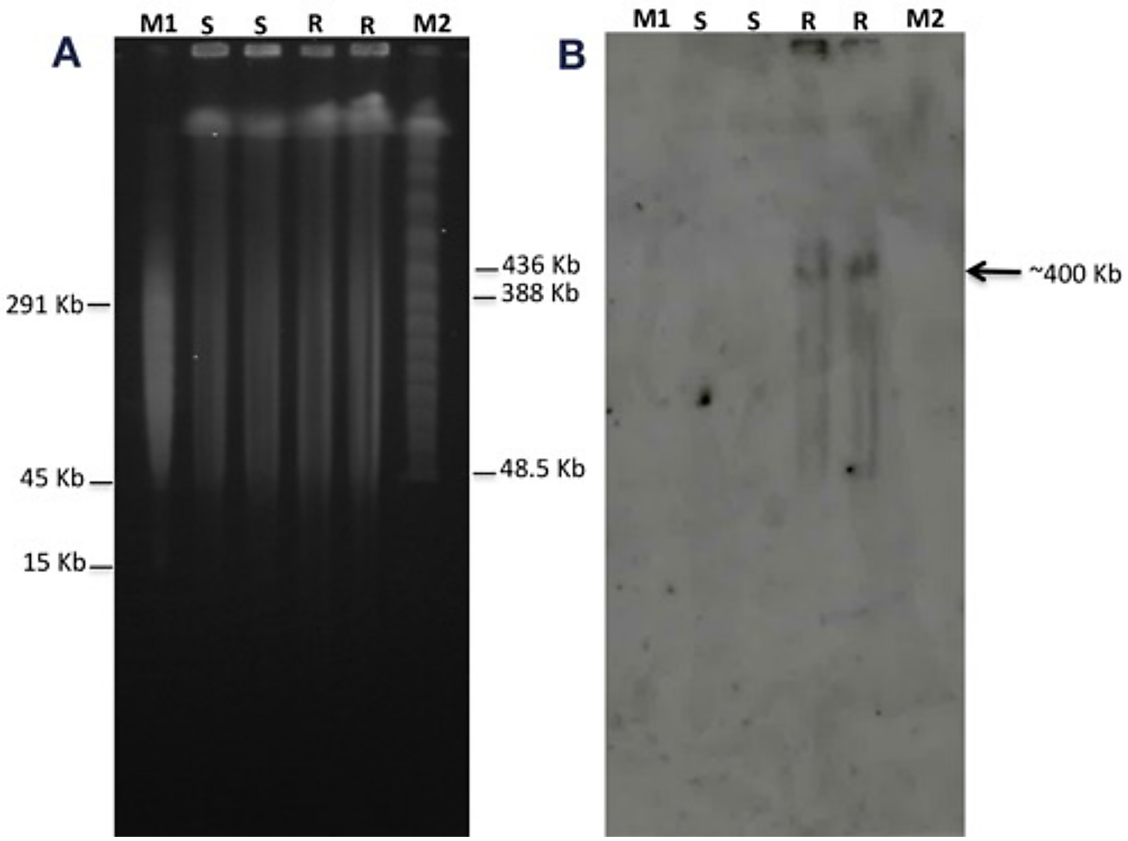

Figure 2.tif