Non-target-site mechanism of glyphosate resistance in Italian ryegrass (*Lolium multiflorum*)

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In Shizuoka Prefecture, Japan, glyphosate-resistant *Lolium multiflorum* is a serious problem on the levees of rice paddies and in wheat fields. The mechanism of resistance of this biotype was analyzed. Based on LD₅₀, the resistant population was 2.8–5.0 times more resistant to glyphosate than the susceptible population. The 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) gene sequence of the resistant biotype did not show a non-synonymous substitution at Pro106, and amplification of the gene was not observed in the resistant biotype. The metabolism and translocation of glyphosate were examined 4 days after application through the direct detection of glyphosate and its metabolite aminomethylphosphonic acid (AMPA) using liquid chromatograph-tandem mass spectrometer (LC-MS/MS). AMPA was not detected in either biotype in glyphosate-treated leaves or the other plant parts. The respective absorption rates of the susceptible and resistant biotypes were 37.90 ± 3.63% and 41.09 ± 3.36%, respectively, which were not significantly different. The resistant biotype retained more glyphosate in a glyphosate-treated leaf (91.36 ± 1.56% of absorbed glyphosate) and less in the untreated parts of shoots (5.90 ± 1.17%) and roots (2.76 ± 0.44%) compared with the susceptible biotype, 79.58 ± 3.73%, 15.77 ± 3.06% and 4.65 ± 0.89%, respectively. The results indicate that the resistance mechanism is neither the acquisition of a metabolic system nor limiting the absorption of glyphosate but limited translocation of the herbicide in the resistant biotype of *L. multiflorum* in Shizuoka Prefecture.

**Keywords:** glyphosate, LC–MS/MS, *Lolium multiflorum*, nontarget-site resistance, translocation.

Glyphosate (N-[phosphonomethyl] glycine) is the most widely and intensely used non-selective herbicide in both agricultural fields and non-agricultural land. Since glyphosate came on the market in 1974, no weed biotypes resistant to the herbicide evolved until 1996 because of the unique properties of glyphosate: its transition-state mimicry, lack of metabolism in plants and absence of known active transporters of the herbicide in plants (Bradshaw *et al.* 1997; Sammons & Gaines 2014). Currently, however, resistant biotypes have been reported in 41 weed species, including both dicots and monocots (Heap 2018).

The level of glyphosate resistance differs depending on the resistance mechanisms, which can be classified into two categories: target-site resistance (TSR) and non-target-site resistance (NTSR). TSR against glyphosate results from either amino acid substitutions replacing Pro106 with Ser, Ala, Thr or Leu in 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) or EPSPS gene amplification (Shaner *et al.* 2012; Sammons & Gaines 2014). A single amino acid mutation confers two- to sevenfold glyphosate resistance (Sammons & Gaines 2014). Double amino acid substitutions at Pro106Ser and Thr102Ile in EPSPS result in more than 180-fold resistance compared with the wild type in *Eleusine indica* (L.) Gaertn. (Yu *et al.* 2015). The gene amplification mechanism has been reported in 41 weed species, including both dicots and monocots (Heap 2018). This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
glyphosate-resistant biotypes of several species, including *Amaranthus palmeri* S. Wats., *A. tuberculatus* (Moq.) Sauer, *Kochia scoparia* (L.) Schrad. and *Lolium perenne* L. (Gaines et al. 2010; Salas et al. 2012; Lorentz et al. 2014; Wiersma et al. 2015). The resistance level is correlated with the number of EPSPS gene amplified.

As the NTSR mechanisms, reduced translocation, increased vacuolar sequestration, enhanced metabolism and/or acquisition of metabolic system were known to endow resistance against glyphosate. Treated glyphosate is not translocated to the roots in glyphosate-resistant *Lolium rigidum* Gaud. and *Conyza canadensis* L. (Lorraine-Colwill et al. 2003; Feng et al. 2004; Preston & Wakelin 2008). Resistant plants with reduced translocation are generally more resistant than those with Pro 106 substitution of the EPSPS gene (Preston & Wakelin 2008). Vacuolar sequestration is related to a reduction in the translocation of glyphosate in glyphosate-resistant *C. canadensis*. The M10 and M11 ABC transporter genes are highly upregulated in the resistant biotypes and not in a susceptible biotype (Peng et al. 2010; Nol et al. 2012), but these two ABC transporters are suggested to be a part of the stress response induced by the herbicide rather than a resistance mechanism (Moretti et al. 2017). Enhanced metabolism is also involved in glyphosate resistance in *Digitaria insularis* (L.) Fedde (De Carvalho et al. 2012; Da Costa et al. 2014) and *Parthenium hysterophorus* L. (Bramantore et al. 2016).

The degree of resistance differs from species to species, in addition to depending on the mechanism of resistance; some resistant biotypes have more than two different resistance mechanisms simultaneously. NTSR sometimes shows multiple resistance (Yu & Powles 2014). To establish rational resistant weed control programs, it is very important to clarify the mechanisms of resistance.

In the western part of Shizuoka Prefecture, Japan, naturalized Italian ryegrass (*Lolium multiflorum* Lam.) is a serious problem on the levees of rice paddies and in wheat fields. To control this weed, glyphosate has been used for approximately 20 years, but some populations of the weed are poorly controlled. These populations showed at least a 78% survival rate after foliar application of glyphosate at 2.3 kg ai ha\(^{-1}\) (Niinomi et al. 2013). Both TSR and NTSR mechanisms to glyphosate have been reported in ryegrass species: amino acid substitutions at Pro 106 of EPSPS (Perez-Jones et al. 2007; Jasieniuk et al. 2008), EPSPS gene amplification (Salas et al. 2012) and a change of glyphosate translocation pattern (Ghanizadeh et al. 2016). Thus, the mechanisms of glyphosate resistance might differ from population to population of the same species, and case studies are necessary for the control of resistant weeds.

Here, we examined a mutation that endows glyphosate resistance and EPSPS gene multiplication in the resistant Italian ryegrass biotype. We also examined the translocation of glyphosate to parts other than the treated leaf and the metabolism of glyphosate in the resistant Italian ryegrass first reported in Japan.

**MATERIALS AND METHODS**

**Whole-plant dose–response experiment**

On 10 June, 2011, we collected seeds from two populations of Italian ryegrass in Fukuroi, in the western part of Shizuoka Prefecture, Japan, to assess their resistance to glyphosate. One population (PL) was located on paddy levees (34.706°N, 137.922°E), where glyphosate had been applied twice a year for approximately 20 years. The other population (RS) was growing on the roadside 4.5 km north (34.742°N, 137.906°E) of the PL population, where glyphosate had not been applied. Seeds were collected from 30 individuals at every 1 m during fruiting in each population and were stored in a desiccator at room temperature (approx. 20–28°C) until the experiments. In addition, seeds of Tachimusha\(^\circledast\) (CV; Snow Brand Seed Company, Sapporo, Japan), an Italian ryegrass cultivar for livestock feed, were used as a susceptible control.

The seeds were sown on moistened filter paper in 9-cm plastic Petri dishes and incubated in an incubator (LH-30-8CT; Nippon Medical & Chemical Instruments, Osaka, Japan) under a 12-h alternating 25/15°C cycle with a 12-h photoperiod. After germination, the seedlings were individually transplanted into cell trays (2.4 × 2.4 cm and 5.0 cm deep) filled with culture soil (Takii & Co., Kyoto, Japan) and were placed in a greenhouse at Kyoto University, Kyoto, Japan (35.032°E, 135.783°N). At the two- to four-leaf stage, seedlings were treated with glyphosate potassium salt solution (Touchdown iQ\(^\circledast\) 44.7% w/w; Syngenta Japan, Tokyo, Japan) at rates of 0, 0.01, 0.1, 0.4, 0.8, 1.6, 3.2 and 6.4 kg ai ha\(^{-1}\). Twenty-four plants were treated at each rate and for each population. One month after the glyphosate application, we assessed plant survival. A seedling was judged to be dead when the aerial part of the plant was completely browned. The experiment was repeated three times: the first experiment was started on November 12, 2012; the second one on November 30, 2012; and the third one on November 7, 2013. Glyphosate was applied at four-, two- and three-leaf stages in the first, second and third experiment, respectively. The survival data of each plant were analyzed.

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replication were fitted to a two-parameter logistic model (Ritz & Streibig 2005) using the drc package in R ver. 3.3.2 (R Development Core Team 2016) to estimate LD50, the herbicide dose required to kill 50% of the plants. The response to glyphosate of the two wild populations (RS and PL) was compared with that of the susceptible cultivar (CV) using a log-likelihood ratio test. The goodness of fit of two statistical models, one of which is a model merging the survival data of CV and RS (or PL), and the other is a model separating those data.

**DNA extraction and EPSPS gene sequencing**

Because Italian ryegrass is an obligate outcrosser, its genetic heterogeneity is high. Therefore, a population can contain both susceptible and resistant plants. Consequently, we first identified herbicide-susceptible and herbicide-resistant plants within the resistant PL population.

On September 21, 2012, 30 seeds of the PL population were sown individually in cell trays (4.0 × 4.0 cm² and 5.0 cm depth) filled with culture soil and were placed in a greenhouse at Kyoto University. At the five- to eight-tiller stage, the plants were excavated from the trays, and one tiller per plant (one clone) was excised. These clones were replanted in different cell trays. One week later, they were sprayed with 2.3 kg a.i. ha⁻¹ glyphosate potassium salt solution (Touchdown 44.7% w/w; Syngenta). One month after this treatment, the dead clones were classified as herbicide-susceptible and the live clones as herbicide-resistant. The remaining tillers were replanted (one plant per 10.5-cm-diameter pot) and allowed to continue growing. After classifying the susceptible and resistant phenotypes, the corresponding tiller plants were identified, and the leaves of three susceptible and four resistant tiller plants were harvested and stored at −80°C. RNA was isolated from leaf tissue using the RNeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA). Complementary DNA was synthesized from the RNA using the SuperScript III First-Strand Synthesis System (Life Technologies Japan, Tokyo, Japan). A set of primers (Perez-Jones et al. 2007) was used to amplify a 564-kb fragment of the EPSPS gene containing codon 106, which is associated with glyphosate resistance in several weed species (Ng et al. 2003; Nandula et al. 2008). A polymerase chain reaction (PCR) was carried out in 20-μL reaction volumes containing 2.0 μL of template cDNA, 0.5 μM each of the forward and reverse primers, 0.2 mM of each dNTP and 0.5 units of Ex Taq DNA polymerase (TaKaRa Bio, Otsu, Japan) in 1× concentration of the supplied buffer. The cycling program was as described in Perez-Jones et al. (2007). The PCR products were sequenced by the FASMAC DNA sequencing service (FASMAC, Atsugi, Japan). The EPSPS gene was sequenced in three susceptible and four resistant plants from the PL population.

**EPSPS gene copy number**

Genomic DNA was extracted from eight plants from the PL population and two CV plants using a modified version of the cetyltrimethylammonium bromide (CTAB) method (Murray & Thompson 1980). Quantitative real-time PCR was used to measure the EPSPS copy number relative to the cinnamoyl-CoA reductase gene (CCR), which is present as a single-copy gene in perennial ryegrass (McInnes et al. 2002). The primer pairs EPSPS F2 and EPSPS R2 (Salas et al. 2012) and LpCCR1 F2 and LpCCR1 R2 (McInnes et al. 2002) were used to amplify the EPSPS and CCR genes, respectively. Quantitative real-time PCR was carried out in a 20-μL reaction volume containing 2.5 ng of genomic DNA, 0.5 μM of each forward and reverse primer and 4 μL of LightCycler FastStart DNA Master PLUS SYBR Green I (Roche Diagnostics, Tokyo, Japan) using a LightCycler 1.5 Instrument (Roche Diagnostics). The cycling program followed that described in Salas et al. (2012). The relative quantity of EPSPS was calculated as ΔCt = (CtCCR – CtEPSPS) according to the method described by Gaines et al. (2010). The increase in EPSPS copy number was expressed as 2ΔΔCt.

**Glyphosate metabolism, absorption and translocation**

After the previously described glyphosate treatment at 0.8 kg a.i. ha⁻¹ in the whole-plant dose–response experiment, survivors of the PL population were crossed with each other within a non-woven fabric bag that prevented pollen penetration, and seeds were collected. Plants of the RS population were also crossed with each other as described above, and seeds were collected without glyphosate treatment. These seeds were used in the experiment.

A seed bioassay was carried out to select resistant individuals from the crossed PL population as L. multiflorum is self-incompatible, and the population is heterogeneous. Ten seeds of the crossed PL population were sown on 20 mL of 0.6% agar medium with 40 mg L⁻¹ glyphosate potassium salt solution (Roundup Maxload® AL 0.96% w/w; Nissan Chemical, Tokyo, Japan), which inhibits the shoot growth of susceptible biotypes (Ninomi et al. 2013), in 9-cm-diameter plastic dishes, which were put in an incubator (25/15°C, 12-h photoperiod). Four days
after germination, shoot length was measured. Seedlings with 20–30-mm shoots were selected as resistant plants. Seeds of the crossed RS population were sown on a 0.6% agar medium without glyphosate in 9-cm-diameter plastic dishes, and 4 days after germination, seedlings with shoot lengths similar to those of resistant plants were selected as susceptible controls. These seedlings were then transplanted into cell trays (5.5 × 5.5 cm² and 5.0-cm depth) filled with culture soil and grown in an incubator (20/15°C, 9-h photoperiod).

At the three-leaf stage of susceptible and resistant plants, 5 μL of glyphosate potassium salt solution (Roundup Maxload® 48% w/w diluted 100 times in Milli-Q water) was put on the adaxial side of the second leaf of each plant. Five seedlings per biotype were treated. After the treatment, the plants were grown in an incubator (20/15°C, 9-h photoperiod). Four days after the treatment (4DAT), the amounts of glyphosate and its metabolite aminomethylphosphonic acid (AMPA) in the treated leaf, non-treated parts of shoots and roots were measured to investigate the metabolism, absorption and translocation of glyphosate in the plant. The absorption rate was calculated as a percentage of total amounts of glyphosate in the treated leaf, non-treated parts of shoots and roots to the amounts of glyphosate used for treatment.

The glyphosate and AMPA were quantified on an LCMS-8030 mass spectrometer (Shimadzu Corporation, Kyoto, Japan) equipped with an electrospray ionization (ESI) probe, according to Yoshioka et al. (2011) with some modifications. High-performance liquid chromatography separations were carried out using an Obelisc N column (150 mm × 2.1 mm i.d., 5 μm; SIELC Technologies, Prospect Heights, IL, USA). The mobile phase included 0.1% formic acid-acetonitrile (80:20, v/v) at a flow rate of 0.2 mL min⁻¹, and the injection volume was 25 μL. The column was maintained at 40°C in the column oven. The mass spectrometer was operated in selected reaction monitoring mode under the following conditions: the nebulizing and drying gases were nitrogen at flow rates of 2 and 15 L min⁻¹, respectively; the interface voltage was set to 4.5 kV; and the DL and heat block temperatures were set at 250 and 400°C, respectively. Table 1 summarizes the MS/MS parameters.

Each plant part was washed with 0.1 M HCl (50% (v/v) MeOH), cut into approximately 3-mm-long pieces, and put in 50% MeOH (v/v) in a 14-mL plastic tube. The plastic tubes were set on a shaking machine at 1800 rpm for 30 min and were centrifuged using TOMY LC-100 with a TS-7 rotor at 2500 rpm for 5 min. Then, 3 mL of the supernatant in the tube was transferred to a new tube and placed in a centrifugal evaporator for approximately 2 h to remove the excess MeOH. The concentrated extract was diluted 10-fold with 10 mM of ammonium formate (pH = 3.8) and placed in a 0.5-mL polypropylene vial (Osaka Chemical, Osaka, Japan). A vial containing 5 μL glyphosate was prepared as a control. Standard vials of glyphosate (0.01, 0.03, 0.1, 0.3, 1.0, 3.0 and 10.0 μg mL⁻¹) and AMPA (0.1, 0.3, 1.0, 3.0 and 10.0 μg mL⁻¹) were also prepared to make calibration curves. The experiments were replicated three times.

The data were analyzed using LabSolutions LC/GC software (Shimadzu Corporation). The area under the peak is correlated with the amount of a specific compound, so the amounts of glyphosate and AMPA were estimated from the calibration curves. The amount of glyphosate in each plant part was compared between the PL and RS populations using Aspin–Welch’s t-test (α = 0.05).

## RESULTS

### Whole-plant dose–response experiment

All the plants survived when no herbicide was applied. The leaf stage at glyphosate application had a significant effect on the survival rate; the efficacy of glyphosate was profound when applied at the two-leaf stage in the second experiment, and the average LD₅₀ was 0.124. It was 0.547 when glyphosate was applied at the four-leaf stage in the first experiment. The log-likelihood ratio test showed that there was no significant difference between the survival rates of CV and RS populations (P = 0.36, 0.83 and 0.10 for the first, second and third experiments, respectively); 80–100% of the plants were killed at 0.4, 0.1 and 0.4 kg a.i. ha⁻¹ in the first, second and third experiments, respectively. While the responses of the PL population were significantly different from those of CV (P < 0.0001 for all experiments), more than 85% of the plants from the PL population survived at the doses described above (Fig. 1). Based on LD₅₀, the PL population was 2.8–5.0 times more resistant to glyphosate than the CV and RS populations (Table 2).

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| Glyphosate | 168.1 | 150.1 | 17 | 14 | 17 |
| AMPA | 110.2 | 81 | 11 | 14 | 16 |

Table 1. Optimized parameters of glyphosate and aminomethylphosphonic acid (AMPA)
Target-site mutation in the EPSPS gene

The aligned sequences were approximately 530 bp long. The EPSPS sequence of the resistant plants did not show a non-synonymous substitution at Pro106 (data not shown). A non-synonymous Ile85Val substitution was detected in one resistant plant. However, this mutation is not common to resistant plants and has not been reported in association with glyphosate resistance.

EPSPS gene copy number

Agarose gel electrophoresis of the real-time PCR products showed single bands for both the CCR and EPSPS PCR products, indicating that the primers used for the target sequences were specific. The estimated copy number of the EPSPS gene relative to CCR gene was 0.55 for plants in the PL population and 0.75 for susceptible CV plants. Thus, amplification of the EPSPS gene was not found in this resistant population.

Glyphosate metabolism, absorption and translocation

The chromatographic peaks of glyphosate and AMPA were resolved with retention times of 5.7 and 2.7 min, respectively. Figure 2 shows the chromatogram of these compounds in a treated leaf. AMPA was not detected in either biotype in the treated leaf or the other plant parts, which means that little glyphosate was metabolized to AMPA. The respective absorption rates of the RS and PL populations were 37.90 ± 3.63% and 41.09 ± 3.36% (mean ± SE), respectively, which were not significantly different (Table 3). These results indicated that the resistance mechanism of the PL population is neither the acquisition of metabolic system nor the limited absorption of glyphosate.

There was a difference in the translocation pattern of glyphosate between the two biotypes. The resistant plants in the PL population retained more glyphosate in the treated leaf (91.36 ± 1.56% of absorbed glyphosate) and less in the non-treated parts of shoot (5.90 ± 1.17%) and roots (2.76 ± 0.44%) compared with the RS plants, which accumulated less glyphosate in the treated leaf (79.58 ± 3.73%) but more in the non-treated parts of shoot (15.77 ± 3.06%) and roots (4.65 ± 0.89%) (Table 3). Thus, the resistant plants exhibited limited translocation as a glyphosate resistance mechanism.

DISCUSSION

We replicated whole-plant dose–response experiment three times at different periods, and the experiment showed that the PL population was 2.8–5.0-fold more resistant to glyphosate than the CV and RS populations. The differences in resistance level among three replications would be a result of the leaf stages of treated plants and the temperatures during the experiment as the efficacy of glyphosate can be affected by temperature (Jordan 1977; Adkins et al. 1998). Weed management using glyphosate for 20 years has resulted in the evolution of glyphosate resistance in this Italian ryegrass population.

In this study, we assessed the mechanisms of glyphosate resistance, including target-site mutations, amplification of the EPSPS gene, enhanced metabolism and
Table 2. The herbicide concentration required to kill by 50% (LD₅₀) and standard errors (SE) by fitting the survival data of the whole-plant dose–response experiment to a two-parameter logistic model (Ritz & Streibig 2005) for a susceptible cultivar (CV) and two wild populations of *Lolium multiflorum* (RS and PL)

| Replication | Population | LD₅₀   | SE    | t-value | P-value |
|-------------|------------|--------|-------|---------|---------|
| 1st         | CV         | 0.362  | 0.215 | 1.679   | 0.093   |
|             | RS         | 0.236  | 0.038 | 6.196   | <0.001  |
|             | PL         | 1.043  | 0.129 | 8.054   | <0.001  |
|             | PL/CV      | 2.883  | –     | –       | –       |
| 2nd         | CV         | 0.064  | 0.170 | 0.379   | 0.705   |
|             | RS         | 0.073  | 0.155 | 0.471   | 0.638   |
|             | PL         | 0.235  | 0.039 | 6.047   | <0.001  |
|             | PL/CV      | 3.657  | –     | –       | –       |
| 3rd         | CV         | 0.183  | 0.027 | 6.849   | <0.001  |
|             | RS         | 0.330  | 0.364 | 0.907   | 0.364   |
|             | PL         | 0.914  | 0.097 | 9.427   | <0.001  |
|             | PL/CV      | 4.998  | –     | –       | –       |

Q₁(m/z): precursor ion; Q₃(m/z): product ion; Q₁(V): accelerating voltage in Q₁; CE: collision energy in collision cell; Q₃(V): accelerating voltage in Q₃.

Fig. 2. Determination of glyphosate and aminomethylphosphonic acid (AMPA) in the treated leaf (TL) of susceptible (RS) and resistance (PL) individuals. Arrows indicate the position of the peak of AMPA.

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Reduced absorption or limited translocation of glyphosate, and found that glyphosate resistance was conferred by limited translocation in this Italian ryegrass population. The level of glyphosate resistance in this study is almost within the reported range of the resistance conferred by limited translocation for Italian ryegrass populations, ranging from three- to ninefold (Preston et al. 2009). Ichihara et al. (2016) investigated the distribution of glyphosate-resistant Italian ryegrass populations in Shizuoka Prefecture and reported that the percentage of resistant populations was the highest, 58.8%, in Chuen district, and 22.1% of populations from the district included more than 50% of resistant plants in the population. Handayani et al. (2017) reported a significant correlation between the endophyte infection frequency in the seeds and glyphosate resistance of the seedlings in the western part of Shizuoka Prefecture. Clarification of the relationship between the evolution of glyphosate resistance and endophyte infection is required in the future.

Limited translocation is a glyphosate resistance mechanism found in some weed species, including *C. canadensis* (Feng et al. 2004) and *Lolium* spp. (Lorraine-Colwill et al. 2003; Wakelin et al. 2004; Michitte et al. 2007; Perez-Jones et al. 2007; Nandula et al. 2008; Ge et al. 2012; Ghanizadeh et al. 2016). Glyphosate targets the meristematic zone (Duke & Powles 2008). On reaching this zone, glyphosate inhibits the shikimate pathway in the tissue and kills the plant. Limited translocation can keep glyphosate out of the meristematic zone in resistant plants, enabling them to survive glyphosate treatment.

Only a few studies have clarified how the translocation of glyphosate is limited. Ge et al. (2012) showed that Italian ryegrass populations with a limited translocation mechanism also exhibited vacuolar sequestration, which means that glyphosate accumulates in vacuoles rather than the cytoplasm, so the plant target site can escape from glyphosate. This is also the mechanism that holds glyphosate in the cell so that it does not translocate to other parts of the plant. Vacuolar sequestration might occur in the resistant plants and causes limited translocation found in this experiment, but this requires further research.

Many recent studies have suggested that the modification of membrane transporters is a likely mechanism for some forms of non-target herbicide resistance, including vacuolar sequestration, limited absorption, and limited translocation. These types of resistance are driven by the change in the expression of membrane transporters involved in drug resistance, so-called multidrug resistance (MDR) transporters (Conte & Lloyd 2011; Shoji 2014). For example, it has been suggested that vacuolar sequestration is driven by the ABC transporter, one of the most common MDR transporters (Yuan et al. 2007; Sammons & Gaines 2014). Studies that investigated the difference in the expression pattern of MDR transporters between susceptible and resistant biotypes of *C. canadensis* showed that some MDR transporter genes were upregulated in resistant populations (Yuan et al. 2010; Tani et al. 2015). In *C. bonariensis*, the transporter gene *MFS1* seemed to be a candidate as a part of the glyphosate resistance mechanism (Hereward et al. 2018).

Compared with TSR, which is relatively simple and monogenic, NTSR, including limited translocation and vacuolar sequestration, is a complex, polygenic phenomenon (Délye 2013). The genome information on most weed species, which promotes the study of polygenic resistance mechanisms, is poorer than for model organisms like *Arabidopsis thaliana* (L.) Heynh. and some crop species like *Oryza sativa* L. Because of these difficulties, only a few studies have investigated genes related to NTSR in resistant weeds.

The $^{14}$C method has been used for the analysis on translocation and metabolism of glyphosate in the research on the mechanisms of glyphosate resistance (e.g. Lorraine-Colwill et al. 2003; Feng et al. 2004; Perez-Jones et al. 2007). In this study, we used a new method to measure glyphosate translocation. The direct

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**Table 3.** Percentages of glyphosate absorption and translocation 4 days after treatment from a treated leaf (TL) to untreated parts of shoot (N) and roots (R) in RS and PL populations of *Lolium multiflorum*

| Population | Absorption (%) | Translocation of absorbed glyphosate (%) |
|------------|----------------|----------------------------------------|
|            | TL             | N           | R            |
| RS         | Average 37.90 a | 79.58 b     | 15.77 a      | 4.65 a |
|            | SE 3.63        | 3.73        | 3.06         | 0.89 |
| PL         | Average 41.09 a | 91.36 a     | 5.90 b       | 2.76 b |
|            | SE 3.36        | 1.56        | 1.17         | 0.44 |

Different alphabets show significant differences between RS and PL populations by Aspin–Welch $t$ test ($\alpha = 0.05$).
detection of glyphosate and AMPA with LC-MS/MS is safer and less costly than the conventional 14C method. Therefore, our method has the potential to become a powerful tool for studying herbicide translocation and metabolism.

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DISCLOSURE STATEMENT

The authors declare no conflicts of interest.

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