Evolution of herbicide resistance mechanisms in grass weeds

Maor Matzrafi, Yaron Gadri, Eyal Frenkel, Baruch Rubin, Zvi Peleg *  
The Robert H. Smith Institute of Plant Sciences and Genetics in Agriculture, The Hebrew University of Jerusalem, PO Box 12, Rehovot 7610001, Israel

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**A B S T R A C T**

Herbicide resistant weeds are becoming increasingly common, threatening global food security. Here, we present BrIFAR: a new model system for the functional study of mechanisms of herbicide resistance in grass weeds. We have developed a large collection of Brachypodium accessions, the Brl collection, representing a wide range of habitats. Wide screening of the responses of the accessions to four major herbicide groups (PSII, ACCase, ALS/AHAS and EPSPS inhibitors) identified 28 herbicide–resistance candidate accessions. Target-site resistance to PSII inhibitors was found in accessions collected from habitats with a known history of herbicide applications. An amino acid substitution in the psbA gene (\textit{Ser} to glycine) conferred resistance and also significantly affected the flowering and shoot dry weight of the resistant accession, as compared to the sensitive accession. Non-target site resistance to ACCase inhibitors was found in accessions collected from habitats with a history of herbicide application and from a nature reserve. In-vitro enzyme activity tests and responses following pre-treatment with malathion (a cytochrome-P450 inhibitor) indicated sensitivity at the enzyme level, and give strong support to diclofop-methyl and pinoxaden enhanced detoxification as NT resistance mechanism. BrIFAR can promote better understanding of the evolution of mechanisms of herbicide resistance and aid the implementation of integrative management approaches for sustainable agriculture.

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1. Introduction

Weeds are the most important biotic factor affecting agricultural production; they are responsible for over 34% of crop yield losses worldwide [1]. Herbicide application is considered the most cost-efficient and effective method of weed control. The biggest challenge in weed control is finding selective herbicides for use in crops. It is particularly difficult to selectively control grass weeds in cereal crops. In recent years, over-use, misapplication and/or inappropriate practices have led to increased selective pressure toward herbicide-resistant weeds species [2]. Moreover, the introduction of genetically modified crops has resulted in increased use of herbicides, especially glyphosate, and, as consequence, enhanced evolution of glyphosate-resistant weeds [3]. To date, about 220 herbicide-resistant weed species have been reported worldwide, when almost one-third (32%) of them are grass weeds (reviewed in Heap [4]).

Herbicide resistance can be endowed by either alterations of the target site (TS) (reviewed by Beckie and Tardif [5]), or non-target site (NTS) mechanisms (reviewed by Délye et al. [6]). TS resistance is endowed by structural changes due to point mutations in herbicide-binding proteins, such as the D1 protein in the photosystem II (PSII) complex [7], acetylactate synthase (ALS, also known as acetohydroxy acid synthase, AHAS) [8], acetyl-CoA carboxylase (ACCase) [9] or 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) [10]. Additionally, TS resistance is sometimes the result of an increased number of copies of the target gene, such as EPSPS [11,12]. NTS mechanisms involve detoxification of the herbicides by glutathione S-transferase (GST) [13] or cytochrome P450 monooxygenase [14,15], reduced absorption [16] or reduced translocation in the plant [17] and sequestration into vacuoles [18]. Unlike TS, NTS resistance mechanisms can sometimes be more general and confer resistance to several herbicide mode-of-action (MOA) groups [19,20]. An understanding of the environmental and/or anthropogenic evolutionary factors that affect the evolution of herbicide resistance in plants is crucial for understanding the mechanisms of resistance [21].

Annual ryegrass (\textit{Lolium rigidum} Gaud.) is one of the most troublesome weeds in the world. In wheat fields, \textit{L. rigidum} can cause yield reductions of more than 40% [22]. Like many other grass weeds, \textit{L. rigidum} is an obligate out crosser with a gametophytically controlled self-incompatible reproduction system [23,24]. While this type of reproductive system can enhance the spread of resistance in the population, it also makes it difficult to obtain
homoyzgous plants for the molecular dissection of herbicide resistance mechanisms.

Here, we suggest using *Brachypodium* spp. as a model species for the study of herbicide resistance mechanisms in grass weeds such as *L. rigidum*. *Brachypodium* is a temperate wild grass that possesses many characteristics required for a tractable model system including self-fertility, simple growth requirements and a small and fully sequenced genome. In addition, efficient transformation methods are available for use with this plant [25]. We have developed a large *Brachypodium* collection from Israel (BrI collection) comprised of ~1000 accessions representing a wide range of habitats, including cultivated fields, roadsides, planted forests and nature reserves. In the current study, the BrI collection was used to (i) examine the effect of herbicide application history on the evolution of weeds exhibiting resistance to herbicides with different modes of action (PSII, ACCase, ALS/AHAS and EPSPS inhibitors) and (ii) investigate the physiological, biochemical and molecular mechanisms of resistance. Our results demonstrate the potential of the BrI collection as a powerful tool for Functional Analysis of herbicide Resistance (BrFAR), to better understand the mechanisms that confer resistance to herbicides in grass weeds.

2. Materials and methods

2.1. Plant material

Seeds of 889 *Brachypodium* spp. plants were collected from different habitats across Israel. Accessions were classified according to six types of habitat: cultivated fields, nature reserves, planted forests, roadsides, urban areas and uncultivated habitats. Seeds were air-dried and stored at 4 °C until they were used. Seeds from every accession were germinated in trays filled with commercial growth mixture (Pele-Shacham, Israel), with three replicates of each accession in the same tray. The trays were placed in a cold room (16 °C) to break the seeds’ dormancy and then transferred to a greenhouse (18 °C night/25 °C day) and watered as needed. Since we could not identify any TS resistance to ACCase inhibitors among the BrI collection, for the in-vitro enzyme activity analyses, we used two *L. rigidum* populations that were previously shown to exhibit TS resistance to different ACCase inhibitors. Population MH (substitution of isoleucine<sup>1781</sup> to leucine) showed a high level of enzyme resistance to diclofop, but not to pinoxaden [26]. Population NO (cysteine<sup>2088</sup> to arginine substitution) was highly resistant to pinoxaden (Matzrafi, unpublished results), but not to diclofop.

2.2. Species identification

Species identification and ploidy level characterization were conducted for all of the accessions that were used in different experiments. Two complementary identification methods were used. Nuclei were isolated from young leaves (40 mg) by chopping with a razor blade in ice-cold LB01 buffer [27–29]. Nuclei were then separated using cotton squares that were placed on the top of 10-ml glass columns and total DNA was stained with propidium iodide [30]. Flow cytometry (FACS Calibur, BD Biosciences) was used to determine DNA content and confer ploidy levels [31], in comparison with two previously known accessions Bd-21 (*Brachypodium distachyon*) and Hawalid (*Brachypodium stacei*) [32].

DNA was extracted from fresh leaves of the same accessions and PCR was carried out under known conditions with microsatellite marker *XALB165* [33]. Amplification products from each accession were analyzed in a 3% agarose gel. Species classification was determined by comparing the isolated sequences with the following reference sequences: Bd-21 (*B. distachyon*), BRA102 (*B. stacei*) and BRA143 (*Brachypodium hybridum*) (see [32]).

2.3. Screening the BrI collection for responses to herbicides

Plants in each tray were treated at the early stage (3 to 4 leaves) with half of the recommended dose of commercial herbicide formulations of ALS/AHAS, PSII, EPSPS and ACCase inhibitors: sulfometuron-methyl + mesosulfuron-methyl (Atlantis®, 2 + 10 g L<sup>-1</sup> OD, Bayer, Germany)—X=25 + 120 g ha<sup>-1</sup>; triazines—atrazine (Atranex® 50% SC, ADAMA Agan, Israel)—X= 1000 g ha<sup>-1</sup>; glycines—glyphosate (Roundup<sup>®</sup> 360 g L<sup>-1</sup> SL, Monsanto, USA)—X= 720 g ha<sup>-1</sup>; and aryloxyphenoxypropionate (Fop)—diclofop-methyl (lloxan® 360 g L<sup>-1</sup> EC, Bayer, Germany)—X= 720 g ha<sup>-1</sup>, respectively. Herbicides were applied using a chain-driven sprayer delivering 300 L ha<sup>-1</sup>. Plant shoot fresh weight (FW) was recorded 21 days after treatment (DAT).

2.4. Herbicide dose response

Selected resistant accessions that survived half of the recommended dose of different herbicides in the preliminary tray experiment were exposed to different rates (0, 0.25X, 0.5X, X, 2X and 4X) of three PSII and three ACCase inhibitors, with four replicates of each treatment. The plants were sprayed with atrazine as mentioned above, triazine–metribuzin (Sencor® 70% WG, BAYER, Germany)—X= 350 g ha<sup>-1</sup>; phenylurea–diuron (Diurex<sup>®</sup> 800 g L<sup>-1</sup> SC, ADAMA Agan, Israel)—X= 1200 g ha<sup>-1</sup>; diclofop-methyl as mentioned above; cyclohexanone (CHD)—cycloxydim (Focus<sup>®</sup>, 100 g L<sup>-1</sup> EC, ADAMA Agan, Israel)—X= 100 g ha<sup>-1</sup> and phenylpyrazole (Den)—pinoxaden (Axial<sup>®</sup>, 45 g L<sup>-1</sup> EC, Syngenta, Switzerland)—X= 30 g ha<sup>-1</sup>. Plants' shoot FW values were recorded at 21 DAT. For each herbicide treatment, the shoot FW ± SD values for the X and 4X rates were compared.

2.5. DNA extraction and molecular studies

DNA was extracted from fresh leaf tissue (~200 mg) of three week-old plants with the Puregene DNA isolation kit (Genta, Minnesota, USA) according to the manufacturer's instructions. Known primers were used to sequence the *psbA* gene [34]. For the ACCase gene, primers were designed using known sequence of *L. rigidum* (DQ184640.1; Supplementary Table S7). Genes were amplified and PCR products were sequenced to locate the common point mutations that can endow TS resistance. Sequence analyses and alignment were performed using Bioedit software [35]. The obtained sequences were compared to the known *psbA* and ACCase sequences of *Arabidopsis thaliana* and *B. distachyon*.

2.6. Photosynthetic efficiency

Resistant (BrI-637) and sensitive (BrI-638) accessions were characterized for photosynthetic efficiency in the presence of the PSII inhibitor atrazine. Fully exposed leaves were collected and placed in tubes containing either 2 mL of ddH<sub>2</sub>O (control) or a solution of one of three different concentrations (10<sup>-4</sup>, 10<sup>-5</sup> and 10<sup>-6</sup> M) of technical atrazine [Atranex<sup>®</sup> (tech) 98.5% WP, ADAMA Agan, Israel]. Tubes were placed in full sunlight for 2 h and then transferred to tubes containing 2 mL of ddH<sub>2</sub>O for 30 min in the dark. We added 0.05% of alkylaryl polyether alcohol surfactant (DF® 800 g L<sup>-1</sup>, ADAMA Agan, Israel) to each tube. Photosynthetic efficiency was measured as quantum yield (Y) using a MINI-PAM Portable Chlorophyll Fluorometer (Walz, Germany). The effective

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<sup>1</sup> All accessions included in the BrI collection are available upon request.
PSII quantum yield of photosynthetic energy conversion was calculated as \( Y \frac{F_{n}}{F_{m}} = \Delta F \frac{F_{m}}{F_{m}} \) [36].

2.7. Characterization of fitness costs

Seeds of resistant (BrI-637) and sensitive (BrI-638) accessions were germinated in a dark cold room (16 °C) to break seeds’ dormancy. Six uniform seedlings of each accession were transplanted into pots (7 × 7 × 6 cm), which were moved to a net house (16 °C night/28 °C day). After 71 days, the aboveground biomass was collected, oven-dried (80 °C) and weighed. Plant shoot dry weights were calculated.

2.8. Pinoxaden and malathion treatments

Metabolic processes of CYT P450 involved in the resistance mechanism were tested with the help of the organophosphate insecticide malathion, a known CYT P450 inhibitor [37]. Pinoxaden was applied at a rate of 30 g ha\(^{-1}\) with and without pre-treatment with 1000 g ha\(^{-1}\) of the organophosphate insecticide malathion 1 h before treatment. Pesticides were applied in a spraying cabinet as described above.

2.9. In vitro enzyme activity tests

Fresh shoot tissue (5 g) of 3 to 4 seedlings was used for a test of in-vitro enzyme activity. Tissue was ground and total proteins were extracted. Crude ACCase was extracted and purified as described [38]. Enzyme activity was assayed following the incorporation of \(^{14}\)C from NaH\(^{14}\)CO\(_3\) into acetyl-CoA to produce heat- and acid-stable malonyl-CoA. Four different concentrations (0.1, 1.0, 10 and 100 \(\mu\)M) of diclofop (98.5% acid, Fluka Analytical, Germany) and pinoxaden (99.7%, Fluka Analytical, Germany) were used to inhibit ACCase activity, with three replicates for each concentration. The concentrations of diclofop and pinoxaden (in \(\mu\)M) that inhibit ACCase by 50% \((I_{50})\) in different accessions/populations were calculated from concentration-response curves.

2.10. Statistical analysis

The JMP (ver. 10) statistical package (SAS Institute Inc., Cary, NC, USA) was used for statistical analyses. Dose–response curves were constructed by plotting the shoot FW data 21 DAT, from the different accessions as a percentage of untreated control (UTC). The data were analyzed using SigmaPlot (ver. 10) software (Systat Software Inc., San Jose, CA, USA). A nonlinear curve model (sigmoidal logistic, three parameters [39]) was adjusted to analyze the effects of the tested herbicides in the different experiments.

\[
Y = \frac{a}{1 + \left(\frac{x}{X_0}\right)^b}
\]

In the model, if \(b > 0\), then \(a\) describes the upper limit of \(Y\). \(X_0 = ED_{50}\) and \(b\) describes the slope of the curve in \(ED_{50}\). The \(R/S\) ratio of the \(ED_{50}\) (RI) was calculated to determine the level of resistance of the resistant plants to that of the sensitive plants.

3. Results

3.1. Survey of herbicide resistance in the BrI collection

A wide survey of 989 Brachypodium accessions (124 habitats) was conducted during spring of 2012–2013 (Fig. 1). The BrI collection includes accessions collected from undisturbed habitats (i.e., nature reserves; Fig. 1A–C) and habitats with a known history of herbicide applications (e.g., cultivated fields, roadsides, urban areas and planted forests; Fig. 1E–G). Most of the accessions were collected from roadsides and nature reserves (36% and 35%, respectively; Fig. 1D and H). It is worth noting that only a few of the accessions were collected from cultivated fields (7%) and most of those were collected from the margins of the fields. From the BrI collection, we selected a core collection of 364 accessions representing all types of habitats and characterized their responses to four different herbicide MOAs. The core collection was screened using half of the recommended dose of each herbicide. Accessions that survived half of the recommended herbicide rate (X) were defined as herbicide-resistance candidates and were further tested in herbicide dose–response assays. This screen resulted in the identification of 28 accessions as herbicide-resistance candidates (i.e., high survival rate; Fig. 1I and J; Supplementary Table S1).

In order to further characterize the resistance of these candidate lines, we tested them under increasing herbicide rates (i.e., dose–response methodology). We selected resistance to the recommended dose as the validation criterion, which resulted in the selection of 11 resistant accessions (Supplementary Table S1). Accessions BrI-637, 670, 671 and 672 showed resistance to PSII inhibitors and accessions BrI-242, 646, 647, 737, 738, 739 and 782 showed resistance to ACCase inhibitors. None of the accessions that showed resistance to ALS/AHAS (10 accessions) or EPSPS (3 accessions) inhibitors survived the recommended dose.

The ploidy levels of all of the accessions that exhibited herbicide resistance were determined and the accessions were identified by species, to enable comparisons between them. This was done using flow cytometry (FACS; [31]) and a specific microsatellite marker (XALB165; [33]; Supplementary Fig. S1). All of the PSII-resistant accessions were individuals of the species B. hybridum (BrI-637, 670, 671, 672). Accessions resistant to ACCase were found among three species: B. distachyon (BrI-242), B. stacei (BrI-647, 646) and B. hybridum (BrI-782, 739). The sensitive accession BrI-638 that was used as a reference in all of our experiments was identified as B. hybridum (Supplementary Table S2).

3.2. Mechanism of target-site resistance

3.2.1. Dose response to PSII inhibitors

We selected one atrazine-resistant accession (BrI-637) to serve as a representative of this group in further investigations. As a reference line, we used a sensitive accession (BrI-638) that was collected from the same location (Mevo Hama, planted forest; Fig. 1G, Supplementary Table S1). It has been previously shown that plants can react differently to herbicides from different chemical groups that act via the same MOA [40]. In order to characterize the magnitude of BrI-637 resistance, additional inhibitors from different chemical groups (metribuzin, a triazinone, and diuron, a phenylurea) were used. Accession BrI-637 was found to be significantly resistant to both atrazine and metribuzin, as compared with the sensitive line BrI-638 (Table 1; Fig. 2A–F). The resistant plants showed a high rate of survival at up to four times the recommended dose (4.0 and 1.4 kg ha\(^{-1}\) for atrazine and metribuzin, respectively), as reflected by the very high resistance index observed (RI > 4000 and 40.6 for atrazine and metribuzin, respectively; Fig. 2A–F). It should be noted that no real \(ED_{50}\) and no statistical model could be fitted for the response of the resistant line to atrazine treatment, since it was unaffected even at the highest dose. On the other hand, the reaction to the third PSII inhibitor, diuron, was similar in the two lines, with RI = 1.74 (Table 1, Fig. 2G–I).

3.2.2. PSII molecular studies

DNA sequencing of the six point mutations in the psb4 gene that are known to endow resistance to PSII inhibitors [7] was conducted to shed light on the mechanism of this resistance. Analysis of part
(933 base pairs) of the psbA sequence coding for the D1 protein of the resistant accession (Brl-637) and the sensitive Brl-638 revealed the presence of a nucleotide substitution (point 790), from A to G, in the resistant line. This substitution results in a serine to glycine amino acid replacement (Fig. 3A). Previously, this replacement was reported to endow high-level resistance to triazines and triazines, but not to substituted urea herbicides [41]. Sequencing of additional atrazine- and metribuzin-resistant accessions (Brl-670,
Table 1
Effect of post-emergence treatment with different PSII inhibitors at the recommended rate (X) and four times the recommended rate (4X) on two Brachypodium accessions. Shoot fresh weight (FW) as a percentage of that of the untreated control (UTC) and ED_{50} values (herbicide rate reducing plant growth by 50%) are presented. Values are means ± SD (n = 4).

<table>
<thead>
<tr>
<th>Rate</th>
<th>Shoot FW (% of UTC)</th>
<th>ED_{50} (g ha^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brl-638</td>
<td>Brl-637</td>
</tr>
<tr>
<td>Atrazine</td>
<td>0.2 ± 0.1b</td>
<td>113.9 ± 21.8a</td>
</tr>
<tr>
<td>Metribuzin</td>
<td>0.1 ± 0.1b</td>
<td>65 ± 14.7a</td>
</tr>
<tr>
<td>Diuron</td>
<td>0.5 ± 0.2b</td>
<td>1.2 ± 0.8b</td>
</tr>
</tbody>
</table>

Different letters indicate significant differences according to Tukey’s LSD test (P ≤ 0.05).

3.2.3. PSII physiological characterization

Comparative whole-leaf fluorescence measurements were taken in sensitive and resistant accessions. Photosynthetic efficiency was compared with or without pre-treatment with atrazine. When treated with a low dose of atrazine (10^{-6} M), the two accessions showed similar responses (0.74 and 0.49 Fv/Fm for Brl-638 and Brl-637, respectively; Supplementary Table S3). However, when treated with higher doses (10^{-3} M and 10^{-4} M), the sensitive line showed significant (P = 0.0028 and P = 0.0004, respectively) reductions in photosynthetic efficiency (Fig. 3B, Supplementary Table S3). The atrazine-resistant accession did not show any reduction in photosynthetic efficiency, as compared with the control treatment, even when treated with a high dose of atrazine (0.64 and 0.63 Fv/Fm for the control and 10^{-4} M atrazine treatments, respectively). There was no significant difference between the responses of Brl-637 to the different treatments (Supplementary Table S3). Characterization of fitness costs revealed delayed flowering and significant reductions in growth and biomass production of the resistant accession (Brl-637; Fig. 3C). These growth differences resulted in a significant reduction in the shoot dry weight of the resistant (Brl-637) accession as compared with the sensitive (Brl-638) accession (0.59 vs. 0.89 g plant^{-1}, respectively; Fig. 3D).

Fig. 2. Effect of increasing application rates of three PSII inhibitors on the survival and shoot fresh weights of sensitive (Brl-638, closed circles) and resistant (Brl-637, open circles) accessions. ((A)–(C)) atrazine, ((D)–(F)) metribuzin and ((G)–(I)) diuron.
3.3. NTS resistance mechanisms

3.3.1. Dose response to ACCase inhibitors

We selected one diclofop-methyl resistant accession (Brl-782) that was collected from a cultivated field for further examination. As a reference line, we used the sensitive accession Brl-638. Two additional ACCase inhibitors (cyloxydim and pinoxaden), representing the two other chemical groups in this MOA, were also used. In response to different diclofop-methyl treatments, both accessions showed high survival rates (Fig. 4A and B). However, when treated at the recommended rate, the fresh weight (biomass production) of the resistant accession was significantly greater than that of the sensitive one (35.8 vs. 90.8% for Brl-638 and Brl-782, respectively; Supplementary Table S4). The advantage of the resistant accession was indicated by the fact that even at the highest dose of 2880 g ha$^{-1}$ it did not exhibit a 50% decrease in shoot fresh weight (Fig. 4A–C). It should be noted, however, that we could not calculate the ED$_{50}$ for both accessions under diclofop-methyl treatments. The resistant accession survived the application of a 4X rate of pinoxaden (120 g ha$^{-1}$), as indicated by its RI of 8.9 (Fig. 4D–F). Both accessions showed a significant reduction in fresh weight (99.2% and 90.8% for Brl-638 and Brl-782, respectively (Supplementary Table S4) following treatment with the recommended dose of cyloxydim (100 g ha$^{-1}$). (Fig. 4G–I).

3.3.2. Understanding the type of resistance

Seven substitutions in the ACCase gene that confer TS resistance to ACCase inhibitors have been reported in grass weeds (reviewed by Jang et al. [42]). DNA sequencing of PCR products from four of our resistant accessions [Brl-782 (cultivated field), Brl-242 (unclassified habitat), Brl-647 (planted forest) and Brl-739 (nature reserve)]; 3–5 individual plants from each accession] did not reveal the presence of any substitutions known to endow TS resistance (Matzrafi, unpublished results). This may suggest the involvement of an NTS mechanism. To test this hypothesis, we applied a pre-treatment of the organophosphate insecticide malathion (a known CYT P450 inhibitor), in order to prevent the detoxification of the ACCase inhibitor pinoxaden. All four resistant accessions showed high rates of survival under pinoxaden treatment. However, after pre-treatment with malathion, all of these accessions were highly sensitive to pinoxaden, behaving similarly to the sensitive accession (Brl-638; Table 2).

3.3.3. In vitro enzyme activity test

To further confirm the resistance mechanism, we tested the in-vitro activity of the ACCase enzyme in response to increasing concentrations of diclofop and pinoxaden [43]. Since we could not identify any Brachypodium accession with TS resistance to ACCase inhibitors, we used L. rigidum populations previously
characterized as possessing TS resistance to ACCase inhibitors (MH for diclofop and NO for pinoxaden tests). The enzyme activity of both resistant (BrI-782) and sensitive (BrI-638) accessions showed the expected reductions in response to increasing concentrations of diclofop ($I_{50} = 1.8 \mu M$ and $1.3 \mu M$, respectively) and pinoxaden ($I_{50} = 0.06 \mu M$ and $0.15 \mu M$, respectively). In contrast, the TS-resistant MH and NO L. rigidum populations were able to maintain high levels of enzyme activity following the diclofop and pinoxaden treatments ($I_{50} = 16.8 \mu M$ and $3.27 \mu M$, respectively; Fig. 5, Supplementary Tables S5 and S6). These results suggest the involvement of an NTS resistance mechanism, as demonstrated previously for L. rigidum [44].

4. Discussion

The ever-increasing human population poses serious challenges for world agriculture. A significant increase (estimated at 50%) in the yield of major crop plants will be necessary to meet the food supply requirements for the projected population of 2050 [45]. In recent years, yield losses due to grass weeds have become a major problem worldwide. Improved techniques for reducing the damage caused by weeds, especially herbicide-resistant weeds, should lead to significant yield increases. Thus, the development of a better understanding of the molecular and physiological mechanisms of herbicide resistance may aid the development

<table>
<thead>
<tr>
<th>Accession (species)</th>
<th>Treatment</th>
<th>Malathion</th>
<th>Pinoxaden</th>
<th>Malathion + Pinoxaden</th>
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<tr>
<td></td>
<td>Control</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>BrI-782 (B. hybridum)</td>
<td>5/5</td>
<td>5/5</td>
<td>3/5</td>
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<tr>
<td>BrI-739 (B. hybridum)</td>
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<td>5/5</td>
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<td>BrI-647 (B. stacei)</td>
<td>5/5</td>
<td>5/5</td>
<td>5/5</td>
<td>0/5</td>
</tr>
<tr>
<td>BrI-242 (B. distachyon)</td>
<td>5/5</td>
<td>5/5</td>
<td>3/5</td>
<td>0/5</td>
</tr>
<tr>
<td>BrI-638 (B. hybridum)</td>
<td>5/5</td>
<td>5/5</td>
<td>0/5</td>
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</tr>
</tbody>
</table>
of new methods for preventing the evolution of new resistant weeds.

Over the last decade, there have been increasing reports of herbicide-resistant grasses; 9 out of 15 of the most herbicide-resistant species (in terms of the number of sites of action) are grass weeds (reviewed in Heap [4]). The study of some of the world’s worst grass weeds such as L. rigidum and Alopecurus myosuroides has been limited by their self-incompatible reproductive systems [24,46]. Brachypodium has been repeatedly shown to be a useful model species for the study of various traits such as responses to abiotic stress [47,48], biofuel potential [49] and biomass production [50]. Here, we propose for the first time, the use of this self-pollinating grass species as a model species for genetic, biochemical and molecular studies of mechanisms of herbicide resistance mechanisms in grass weeds.

Characterization of the accessions from Brl collection in response to four different herbicide modes of action (PSII, ACCase, ALS/AHAS and EPSPS inhibitors) resulted in the identification of a significant and unexpectedly large number of resistant accessions (Fig. 1, Supplementary Table S1). Evolution of TS resistance has been suggested, in most cases, to be a result of strong herbicide selection pressure, which reveals the presence of resistant individuals and gives them an advantage over sensitive ones [6]. Moreover, it has also been shown that different alleles conferring TS resistance can be found in the same field and even in the same plant [51]. TS resistance to ACCase inhibitors in L. rigidum and Phalaris paradoxa [26] and multiple TS resistance to ALS/AHAS and PSII inhibitors in Amaranthus blitoides [52] have been reported in plants collected from habitats with long histories of herbicide applications. In the current study, all of the Brachypodium accessions showing TS resistance to PSII inhibitors (substitution of serine264 to glycine; Fig. 3A) were collected from habitats with known histories of herbicide applications. All accessions collected from the roadside habitat Nafach (Brl-670, 671, 672) exhibited strong TS resistance. Previously, Gressel et al. [53] reported on resistance to PSII inhibitors in Brachypodium accession collected from roadsides, however, in that case, the mechanism of resistance was not confirmed.

In the planted forest habitat Mevo Hama, we found both resistant (Brl-637) and sensitive accessions. This young planted forest (Pinus pinea) is at the crucial period of establishment and, as is common practice, is being treated with herbicide to minimize the competition of weeds with young trees. PSII inhibitors (such as atrazine) are part of the weed management program in young forests and provide rapid and strong selection pressure toward the development of TS resistant weed populations. Comparisons between the resistant and sensitive accessions revealed significant differences in growth and productivity. The resistant accession showed reduced growth, delayed flowering (Fig. 3C) and lower levels of biomass production (Fig. 3D). Our results, as well as those of others (e.g., [54]), point to a strong correlation between TS resistance to PSII inhibitors and fitness cost(s). A study is currently being conducted in our lab to test the mechanistic basis of these fitness costs and the trade-offs involved in TS resistance to PSII inhibitors.

Surprisingly, in contrast with TS resistance, accessions showing NTS resistance (ACCCase inhibitors) were found in all types of habitats (e.g., roadsides, cultivated fields, planted forests and nature reserves), regardless of the local herbicide application history. It has been suggested previously that NTS resistance is associated with the use of lower than the recommended application rates [55] and misapplication of herbicides [2]. While several cases of NTS resistance to pinoxaden were found among accessions collected from cultivated and disturbed fields (i.e., Brl-647, 782, 242) with histories of frequent herbicide use, we also found an accession with very strong NTS resistance in a nature reserve (Brl-739) with no history of herbicide application (Table 2). Interestingly, we have identified an accession with NTS resistance mechanisms among the different ploidy levels of Brachypodium species (Supplementary Table S2), which may indicate that the resistance mechanism does not involve a different gene copy number. These results suggest that an NTS resistance mechanism is evolving in weed populations regardless of herbicide application history. Natural variation in sensitivity to different herbicides among weed populations has been discussed previously [56]. Moreover, as a polygenic trait, various alleles and molecular mechanisms can accumulate over evolutionary time, especially in self-incompatible species [55]. In a self-fertile species like Brachypodium, these processes are expected to proceed much more slowly. Many studies suggest that the evolution of NTS resistance is based on a gradual selective process (reviewed by Busi and Powels [56] and Délye [57]). However, our results point to a potential risk of the development of NTS resistance weeds in cultivated fields, regardless of herbicide-application practices.

The ACCase inhibitors include three chemically distinct classes: aryloxyphenoxypropionates (Fops), cyclohexanediones (Dims) and the recently developed phenylpyrazoline (Den) group, which includes a single herbicide, pinoxaden [58]. The new herbicide pinoxaden has selectivity characteristics similar to those of fenoxaprop-P-ethyl (Pop) and tralkoxydim (Dim) herbicides [59]. This similarity in crop selectivity makes pinoxaden an excellent tool for managing resistance to other ACCase inhibitors. The strong
Acknowledgments

The rapid development and distribution of herbicide-resistant species is expected worldwide, especially in Europe where regulation is particularly strict. Thus, urgent efforts to develop new tools that will help reveal the complex biochemical, genetic, and molecular means by which plants evolve herbicide resistance are needed. Here, we demonstrate the potential of BrfFAR as a powerful tool for achieving a better understanding of the mechanisms that confer herbicide resistance in grass weeds. This tool can help us to create new and improved weed management practices and find solutions to help farmers maintain sustainable agricultural systems.

5. Conclusions

In an age in which new herbicide registrations are limited [65] and regulation processes aim to minimize herbicide applications [4], the development and distribution of herbicide-resistant species is expected worldwide, especially in Europe where regulation is particularly strict. Thus, urgent efforts to develop new tools that will help reveal the complex biochemical, genetic, and molecular means by which plants evolve herbicide resistance are needed. Here, we demonstrate the potential of BrfFAR as a powerful tool for achieving a better understanding of the mechanisms that confer herbicide resistance in grass weeds. This tool can help us to create new and improved weed management practices and find solutions to help farmers maintain sustainable agricultural systems.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2014.08.013.

References


