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Evolution of herbicide resistance mechanisms in grass weeds

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ABSTRACT

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 *BrI 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 ($serine_{264}$ 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 NTS 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.

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], acetolactate 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 (*Lolium rigidum* Gaud.) is one of the most troublesome weeds in the world. In wheat fields, *L. rigidum* can cause yield reductions of more than 40% [22]. Like many other grass weeds, *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

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homozygous 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 (BrI-FAR), to better understand the mechanisms that confer resistance to herbicides in grass weeds.

2. Materials and methods

2.1. Plant material

Seeds of 989 *Brachypodium* spp. plants were collected from different habitats across Israel[1]. Accessions were classified according to six types of habitat: cultivated fields, nature reserves, planted forests, roadsides, urban areas and unclassified 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$_{1781}$ to leucine) showed a high level of enzyme resistance to diclofop, but not to pinoxaden [26]. Population NO (cysteine$_{2088}$ 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: sulfonylurea (SU)–iodosulfuron-methyl + mesosulfuron-methyl (Atlantis®, 2 + 10 g L^{-1} OD, Bayer, Germany)—X = 25 + 120 g ha^{-1}; triazines–atrazine (Atranex® 50% SC, ADAMA Agan, Israel)—X = 1000 g ha^{-1}; glycines–glyphosate (Roundup® 360 g L^{-1} SL, Monsanto, USA)—X = 720 g ha^{-1}; and aryloxyphenoxypropionate (Fop)–diclofop-methyl (Iloxan®, 360 g L^{-1} EC, Bayer, Germany)—X = 720 g ha^{-1}, respectively. Herbicides were applied using a chain-driven sprayer delivering 300 L ha^{-1}. 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, triazinone–metribuzin (Sencor® 70% WG, BAYER, Germany)—X = 350 g ha^{-1}; phenylurea–diuron (Diurex® 800 g L^{-1} SC, ADAMA Agan, Israel)—X = 1200 g ha^{-1}; diclofop-methyl as mentioned above; cyclohexandione (CHD)–cycloxydim (Focus®, 100 g L^{-1} EC, ADAMA Agan, Israel)—X = 100 g ha^{-1} and phenylpyrazole (Den)–pinoxaden (Axial®, 45 g L^{-1} EC, Syngenta, Switzerland)—X = 30 g ha^{-1}. 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 (Gentra, 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_2O (control) or a solution of one of three different concentrations (10^{-4}, 10^{-5} and 10^{-6} M) of technical atrazine [Atranex® (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_2O for 30 min in the dark. We added 0.05% of alkylaryl polyether alcohol surfactant (DX® 800 g L^{-1}, 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

[1] All accessions included in the BrI collection are available upon request.