Weed resistance diagnostic technologies to detect herbicide resistance in cereal-growing areas. A review

Diagnosetechnologien zur Detektion von Herbizidresistenz im Getreideanbau . Ein Überblick

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Summary

Cereals are major crops used for food and feed. By 2050, the world population is expected to be close to 10 billion requiring a doubling of the food production from a fixed area of arable land. The control of weeds in cropping systems is one key step to optimize yield. In the last several decades, herbicides have become the most effective management tool for adequate weed control. However, their repetitive use, as well as the limited number of modes of action (MoA) available, has led to the development of resistance in weeds. It has become imperative to change practices that lead to the development of weed resistance in order to protect those MoAs which are still effective in cereals. Several mechanisms of resistance have been developed by weeds to survive herbicide applications. Among them, gene mutations reducing or inhibiting herbicide binding by conferring amino-acid changes in a target enzyme (Target Site Resistance, TSR) and detoxification of the herbicide (Enhanced Metabolic Resistance, EMR) are the main mechanisms in key grass weeds found in cereal crops. These two mechanisms have been extensively studied during the last years and, thus, enabled the development of analytical tools for resistance diagnosis. Sustainable strategies for weed management using herbicides rely on accurate resistance diagnostics that permit optimization of treatment solutions that will lead to herbicide longevity. Greenhouse and laboratory tests used for resistance diagnosis will be reviewed with an emphasis on biochemical and molecular biology technologies. Cases of resistance to ACCase inhibitor herbicides will be presented as examples. Finally, the future development of these technologies will be discussed in the perspective of more practical uses.

Keywords: ACCase, ALS, bioassays, HPLC analyses, non-target-site resistance, pyrosequencing, target-site resistance

Zusammenfassung

Getreide ist zurzeit die wichtigste Grundlage für unsere Nahrungs- und Futtermittelproduktion. Die Getreideproduktion muss jedoch, im Hinblick auf den bis 2050 erwarteten weltweiten Bevölkerungsanstieg auf nahezu 10 Mrd. Menschen und die gleichbleibende landwirtschaftliche Nutzfläche, mindestens verdoppelt werden, um ausreichend Nahrungsmittel bereit zu stellen. Herbizide haben sich in den vergangenen Jahrzehnten zu einem der wichtigsten Instrumente der Unkrautbekämpfung entwickelt, da sie eine maßgebliche Möglichkeit darstellen den Ertrag zu steigern. In einigen Fällen allerdings, führt sowohl die wiederholte Anwendung von Herbiziden als auch die begrenzte Auswahl an verfügbaren herbiziden Wirkmechanismen (MoA) zu einer ernstzunehmenden Resistenzentwicklung in Unkräutern. Diese Entwicklung macht es erforderlich die Anwendung von Herbiziden so zu verändern, dass eine weitere Resistenzentwicklung vermieden wird und vorhandene Wirkstoffe auch in Zukunft der Unkrautbekämpfung im Getreide zur Verfügung stehen.

Verschiedenste Resistenzmechanismen gegen Herbizide haben sich in Unkräutern herausselektiert und sind in den vergangenen Jahren intensiv untersucht worden. Dazu zählen in Getreideunkräutern vor allem die wirkortspezifische Resistenz (Target Site Resistenz, TSR) und die Metabolisierung von Wirkstoffen (Enhanced Metabolic Resistance, EMR oder Non-Target-Site Resistenz, NTSR). Die erarbeiteten Erkenntnisse lassen sich mittlerweile zuverlässig zur Resistenzdiagnose in Unkräutern verwenden. Zuverlässige Diagnoseverfahren für Herbizidresistenz bei Unkräutern werden insbesondere für die Entwicklung nachhaltiger Strategien im Unkrautmanagement und zur Verlängerung der Produktlebensdauer benötigt. Eine zuverlässige Diagnose hilft zudem in Problemfällen Herbizidapplikationen anpassen oder optimieren zu können.

In dieser Studie werden schwerpunktmäßig biochemische und molekularbiologische Diagnosemethoden für Herbizidresistenz in Unkräutern aus Gewächshaus und Labor vorgestellt und am Beispiel ACCase resistenter Unkräuter verdeutlicht. Schließlich wird die zukünftige Entwicklung dieser Technologien im Zusammenhang mit der praktischen Anwendung diskutiert.

Stichwörter: ACCase, ALS, Bioassay, HPLC-Metaboliten-Analytik, Non-Target-Site Resistenz, Pyrosequencing, Target-Site Resistenz

1. Introduction

Modern herbicides make major contributions to global food production by easily removing weeds and substituting for destructive soil cultivation. Weed resistance to herbicides is an increasing problem, not only regarding the number of weeds becoming resistant to a particular mode of action (MOA), but also regarding the appearance of resistance to multiple MOAs (HEAP, 2011). Several factors influence the development of herbicide resistance which follows evolution processes, such as the genetic and biology of a weed species, the herbicide chemistry and its MOA, as well as key agro-ecosystem characteristics and herbicide handling by the users (DARMENCY, 1994; CHRISTOFFERS, 1999; POWLES and YU, 2010). Resistance to herbicides can result from several mechanisms occurring alone or in combination. The first group of mechanisms includes all modifications of the targeted proteins including gene coding sequence mutations, gene over-expression, and gene duplication, collectively known as Target Site Resistance (TSR). The second group comprises processes not directly involving the targeted proteins such as the modification of herbicide penetration into the plant, decreased rate of herbicide translocation, and increased rate of herbicide sequestration or metabolism, collectively known as Non Target Site Resistance (NTSR). TSR is characterized by a relatively narrow resistance to a single MOA or even subgroups of chemical classes showing this MOA whereas NTSR, especially enhanced metabolization, can confer resistance to a broad range of herbicides. In the main herbicide resistant monocotyledonous weeds present in cereal fields *(Alopecurus myosuroides, Apera spica-venti, Bromus* spp.*, Lolium* spp.*)*, mutations of the coding sequences of the acetolactate synthase (ALS) and the acetyl-coenzyme A carboxylase (ACCase) genes have been extensively characterized as well as increased metabolization of herbicides (reviewed by POWLES and YU, 2010). Especially threatening are herbicide-degrading cytochrome P450 enzymes (CytP450) endowing enhanced metabolic resistance towards existing, new, and supposedly herbicides yet to be discovered. Despite extensive studies and reviews of herbicide resistance (POWLES and SHANER, 2001; GRESSEL, 2002), genetic issues associated with resistance evolution need to be investigated further to increase our basic knowledge of this aspect and to generate the necessary tools for accurate resistance diagnostics. The latter will contribute making the best possible decisions for an efficient Integrated Weed Management strategy. Greenhouse and laboratory tests used for weed herbicide resistance diagnosis will be reviewed with an emphasis on biochemical and molecular biology technologies. Cases of resistance to ACCase inhibitor herbicides will be presented as examples. Finally, the future development of these technologies will be discussed in the perspective of more practical uses.

2. Greenhouse bioassays

Weed resistance diagnostics using greenhouse bioassays provide information on whole plant responses to herbicides. Nevertheless, the results often show significant variations between different research groups assessing the same weed biotypes but evaluating different criteria, *e.g*. visual evaluation, mortality, or growth based on fresh weight and using different experimental conditions (e.g. temperature, photoperiod, light intensity) (MOSS et al., 1998). Therefore, screening protocols have to be well defined and standardized methods should be used (BECKIE et al., 2000). A more accurate evaluation of herbicide resistance occurs when the responses of putative resistant (R) biotypes are compared to well characterized sensitive (S) biotypes using a range of six to eight herbicide doses in pot experiments (HEAP, 1994). This allows using appropriate statistical methods to quantify the level of resistance by e.g. the dose required to reduce shoot weight or plant number by 50 % relative to untreated plants (GR_{50} or LD_{50}, respectively) (STREIBIG, 1988; BRAIN and COUSENS, 1989; SEEFELDT et al., 1995). These effective doses are then compared between the R and S biotypes and the relative level of resistance is expressed as the ratio of R/S (resistance factor). To assess a large number of samples in a routine screening approach, this method can be simplified by using a discriminating dose (very often the recommended field dose), i.e. the minimum herbicide dose that provides the appropriate vertical difference between the dose-response curve of R and S biotypes and that results in at least 80 % control of the S biotypes (NORTHWORTHY et al., 1998; BECKIE et al., 2000). Nevertheless,

greenhouse bioassay diagnostics in pots remains time consuming, space consuming, and rather expensive. To overcome these drawbacks, soilless or dish bioassays have been set up (using filter paper or agar), but these are often less accurate (BECKIE et al., 2000) and cannot discriminate between resistance mechanisms (TSR and NTSR).

3. Biochemical assays

Enzyme assays, e.g. acetolactate synthase (EC 4.1.3.18) (ALS) or acetyl-CoA carboxylase (EC 6.4.1.2) (ACCase) assays, require the harvest of living plants in often high quantities, especially when meristematic tissues have to be used, to be able to purify enough protein to perform the assay. This is time consuming and skill-dependent. Although there is a fairly good correlation between the relative resistance of biotypes at the enzyme and whole plant level (DEVINE and EBERLEIN, 1997), enzyme assays provide information only about herbicide resistance conferred by target site changes while NTSR as well as multiple resistance to herbicides with different modes of action remains undetected. Nevertheless, enzyme assays permit detailed studies on the effect of a given mutation in the gene coding sequence on sensitivity to different chemical classes of inhibitors (POWLES and YU, 2010).

4. Molecular assays

Recent developments of gene sequence analyses allow the detection of novel mutations (KAUNDUN et al., 2011), and the analysis of known mutations using CAPS (KAUNDUN and WINDASS, 2006; MASSA et al., 2011; DELYE et al., 2011), real-time quantitative PCR (KAUNDUN et al., 2006), or pyrosequencing (PETERSEN et al., 2010; RIGGINS et al., 2010). Homozygous plants for a given mutation can be differentiated from heterozygous plants for that mutation. High throughput analysis of known ACCase and ALS mutations (POWLES and YU, 2010) has been set up for pyrosequencing in order to provide a more quantitative result than PCR alone. Data using this technology applied to a case study analysis of *Alopecurus myosuroides* in Germany were reported by HESS et al. (2012). Briefly, approximately 20 mg of fresh leaf material was harvested from each plant to determine target site mutations on the ACCase gene. Approximately 1 cm^2 plant material was disrupted in 400 µl 100 mM Tris(HCl) and 1 M KCl (pH 9.5) with stainless steel beads in a swing mill (Retsch 300MM). To amplify the fragment of interest, after a centrifugation step, 0.1 µl of the supernatant was used as template together with Hotstart Taq (Qiagen) and 0.4 µM of each primer to perform the PCR (25 µl). For example, a 483bp long biotinylated (BT) ACCase fragment containing the W2027C mutation was amplified using the forward and reverse primers, respectively P1 (5'tcctgttggtgttatagctgt3'), and P2 (BT-5'ggatcaagcctacccatgca3') at 54.1 °C annealing temperature (40 sec), 72 °C elongation time (40 sec.) in 45 cycles and a final extension step (10 min) at 72 °C. The pyrosequencing reaction was performed as described by PETERSEN et al. (2010) using the pyrosequencing primer P3 (5'cctctgttcatacttgctaac3') in a Pyromark PSQ 96 instrument. The reactions to sequence the 2041, 2078 and 2096 mutation sites in the ACCase sequence were performed using the same fragment but with the sequencing primers 5'gcaaagagatcttttgaagga3' (2041), 5'gtggaggagcctgggtcgtgatt3' (2078) and 5'gctatgctgagaggactgcaaag3' (2096), respectively. To sequence the mutation site 1781 on the ACCase gene, a 183 bp long fragment was amplified by using the forward primer 5'gcacacaagatgcagctagatagt3' and BT-5'tccgattccaacagttcgt3' as the reverse primer and, 5'atggactaggtgtggagaac3' was used as sequencing primer. Similar methods can be developed to test point mutations in any sequence of interest. Other approaches designed to determine gene copy number (GAINES et al., 2010), or gene expression analysis either by using microarrays (YUAN et al., 2010) or a novel RNAseq approach (PENG et al., 2010) offer the opportunity to develop new knowledge about NTSR and use it to develop novel diagnostics approaches with molecular assays that increase throughput at a reasonable cost.

5. Analytical assays

The use of ^{14}C-radiolabelled compounds allows studying the metabolic degradation in planta and is so far the method of choice to perform such studies. In our laboratory, young plants, e.g. *Alopecurus myosuroides* (ALOMY), at developmental stage of one to three leaves were treated either of the leaves

or of the base of the stem with the herbicide to be analyzed. Typical data obtained for ^{14}C-mesosulfuron after extraction and HPLC analyses are shown in Figure 1. This method can be both quantitative and qualitative and offers the possibility to determine Enhanced Metabolic Resistance (EMR) in most monocotyledonous and dicotyledonous weeds found in cereal fields. Similar approaches can be used to study modifications of herbicide transport, uptake, or sequestration. Nevertheless these methods require specific skills, and are time consuming. Genetic markers related to EMR or other NTSR processes will greatly help to improve the diagnostics for such types of herbicide resistance.

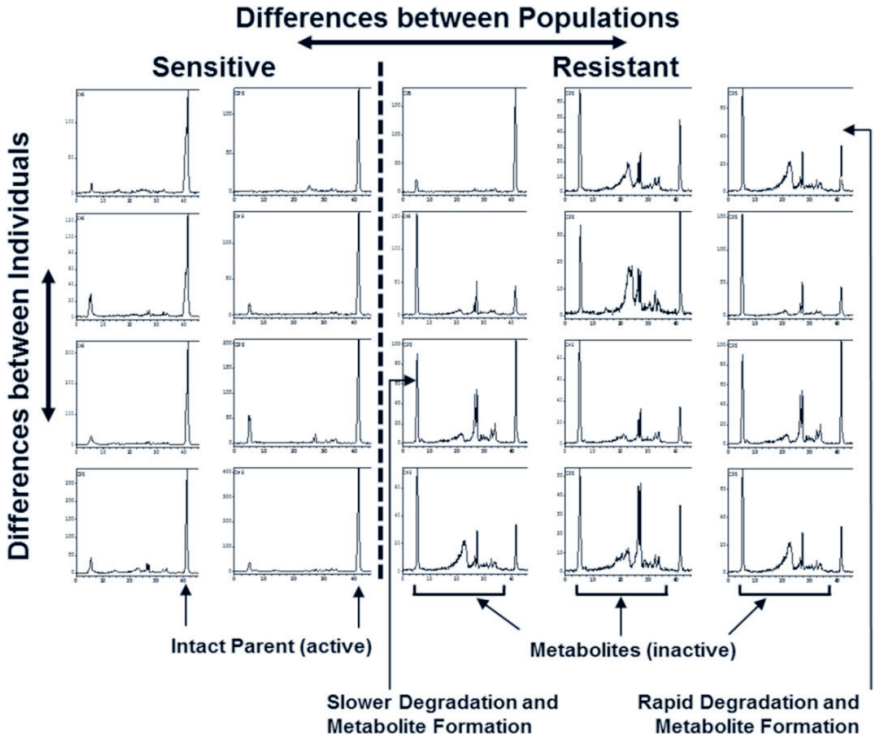

Fig. 1 Example of metabolization of mesosulfuron by five biotypes of *Alopecurus myosuroides* (ALOMY), sensitive (S, left) or resistant (R, right). Almost no metabolites can be detected on the HPLC chromatograms related to the S plants (n = 4 left) compared to those related to the R plants (n = 4 right). Note the high variation between individuals, especially in the case of the R plants.

Abb. 1 *Beispiel einer Mesosulfuron Metabolisierung in fünf verschiedenen sensitiven (S, links) und resistenten (R, rechts) Alopecurus myosuroides (ALOMY)-Biotypen. In dem HPLC-Chromatogramm sind bei den S-Pflanzen (n = 4, links) im Vergleich zu den R-Pflanzen (n = 4, rechts) fast keine Metabolite erkennbar. Auffallend ist die hohe Variation zwischen einzelnen Pflanzen, insbesondere bei den R-Pflanzen.*

6. Conclusion

At this time, greenhouse bioassays remain the basic method to assess resistance to herbicides although they can show variation and are very time consuming. In addition, they cannot be used to deliver a fast answer to growers nor can they definitely discriminate between TSR and NTSR. Novel molecular technologies could offer the tools to overcome these drawbacks and become integrated in novel herbicide resistance diagnostics. This is already the case for TSR. The molecular characterization of NTSR will be the next step and the novel generation sequencing methods offer the opportunity to deliver molecular tools for fast NTSR diagnostics, but require significant investments. Fast diagnostics will help guide the appropriate recommendation in Integrated Weed Management programs.

Nevertheless, one major recommendation, regardless of test results, is the use of active ingredients with different modes of action in rotation, sequences, or in mixtures in combination with soil management tools and the appropriate crop rotations.

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