Stress-induced evolution of herbicide resistance and related pleiotropic effects

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Stress-induced evolution of herbicide resistance and related pleiotropic effects

William Edward Dyer*

Abstract

Herbicide-resistant weeds, especially those with resistance to multiple herbicides, represent a growing worldwide threat to agriculture and food security. Natural selection for resistant genotypes may act on standing genetic variation, or on a genetic and physiological background that is fundamentally altered because of stress responses to sublethal herbicide exposure. Stress-induced changes include DNA mutations, epigenetic alterations, transcriptional remodeling, and protein modifications, all of which can lead to herbicide resistance and a wide range of pleiotropic effects. Resistance selected in this manner is termed systemic acquired herbicide resistance, and the associated pleiotropic effects are manifested as a suite of constitutive transcriptional and post-translational changes related to biotic and abiotic stress adaptation, representing the evolutionary signature of selection. This phenotype is being investigated in two multiple herbicide-resistant populations of the hexaploid, self-pollinating weedy monocot Avena fatua that display such changes as well as constitutive reductions in certain heat shock proteins and their transcripts, which are well known as global regulators of diverse stress adaptation pathways. Herbicide-resistant populations of most weedy plant species exhibit pleiotropic effects, and their association with resistance genes presents a fertile area of investigation. This review proposes that more detailed studies of resistant A. fatua and other species through the lens of plant evolution under stress will inform improved resistant weed prevention and management strategies.

Keywords: herbicide resistance; non-target site resistance; plant stress response; systemic acquired herbicide resistance; weeds
other pleiotropic effects. Plants have evolved under a wide range of biotic and abiotic stresses and in response have evolved adaptive strategies to deal with both. To combat biotic invasions by pathogenic bacteria and fungi, they have evolved highly effective innate and induced immune responses. The components and pathways of pattern-triggered immunity and systemic acquired resistance (SAR) are well characterized, involving recognition, signal transduction, and transcriptional reprogramming in response to biotic challenge. Systemic acquired acclimation describes a constitutive upregulation of proteins that confer enhanced resistance to subsequent abiotic stresses. There are a number of commonalities between the two responses, including cell-surface receptors that recognize stress-related molecules, components of signal transduction cascades, and the central role of reactive oxygen species (ROS). Priming of pattern-triggered immunity and SAR can require multiple, consecutive exposures to modest, perhaps hormetic (see below) stresses, as has been noted for the evolution of NTSR. An intriguing and potentially related initiator of NTSR was recently proposed: microbial plant endophytes that either directly metabolize xenobiotics or prime host plant stress responses that include NTSR.

When exposed to sublethal herbicide doses by improper applications or environmental constraints, susceptible plants respond in much the same as they do to other abiotic stresses (reviewed in Alberto et al. ). Stress resulting from abiotic sources including several herbicides induces rapid ROS generation, which in turn impacts multiple pathways and cellular components. SAR and systemic acquired acclimation are characterized by the constitutive, heritable upregulation of genes/enzymes for redox maintenance, xenobiotic metabolism, and other protective pathways, likely as a result of rapid yet persistent changes in abiotic stress-specific transcription factors and non-coding RNAs. Similarly, stress-induced changes in protein PTMs like phosphorylation and cysteine oxidation modify enzymatic and regulatory activities, allowing plants to rapidly adjust carbon flux. Plant transcriptome, proteome, and PTM changes caused by herbicide stress are quite similar to those resulting from abiotic and biotic stresses, supporting the existence of the SAHR phenotype proposed here. Although not the focus of this review, herbicide safeners also induce similar gene expression changes likely through oxylipin-mediated signaling and the unclear relationship between safeners and NTSR evolution is currently under investigation. Given that systemic acquired acclimation and SAR are better understood than NTSR, this review proposes that the plant stress literature may be fertile ground for generating hypotheses about NTSR evolution and its pleiotropic effects.

The widely accepted theory for the origin of NTSR is that herbicides impose Darwinian selection on standing genetic variation in weed populations, and sublethal doses select for pre-existing individuals able to evolve resistance. Surviving individuals are thought to subsequently accumulate additional alleles for NTSR and/or MHR in succeeding generations, mostly through outcrossing. However, because the majority of the world’s worst annual weeds are self-pollinating, an additional theory is proposed here that does not rely on outcrossing and incorporates a deeper understanding of the plant stress response. As discussed further below, the theory proposes that stress imposed by sublethal herbicide exposure creates a profoundly altered physiological state that provides novel sources of variation upon which selection for MHR and NTSR can act, without the need for additional alleles obtained through outcrossing.

Sublethal herbicide doses create a continuum of responses that have implications for resistance evolution, as illustrated by a typical dose–response curve for herbicides (or almost any inhibitor of any organism; Fig. 1). The stimulatory effect seen at very low doses of the response curve (region A) is termed hormesis, a poorly understood but likely universal response. A recent plant study seeking a mechanistic explanation showed that hormetic doses of a disease resistance-inducing chemical enhanced Arabidopsis root growth and suppressed the expression of photosynthesis- and respiration-related genes, while higher sublethal doses induced a very different suite of defense-related transcripts. This finding, along with the fact that reduced herbicide applications can select for resistance in Avena fatua and other species supports the idea that hormetic and sublethal doses are part of a continuum that induces overlapping adaptive responses like NTSR. Interestingly, point B in Fig. 1 is often termed the no observable adverse effect level, although the absence of observable damage should not suggest that this dose is physiologically neutral. In fact, this dose of glyphosate induced numerous proteome changes in the fungus Aspergillus nidulans with no other observable effects. Hormetic herbicide doses may play a part in resistance evolution in weeds, because highly resistant and less resistant subpopulations from a heterogeneous acetyl CoA carboxylase inhibitor-resistant Alopecurus myosuroides metapopulation responded differentially to hormetic herbicide doses (Belz R, personal communication). Further, the recent widespread weed exposure to very low (perhaps hormetic) volatilized dicamba doses in the Midwestern United States may have implications for resistance evolution.

Plants exposed to (probably) hormetic doses and (certainly) higher sublethal doses of herbicides experience stress, and stress is well known to induce profound physiological changes. Stress-induced mutagenesis, originally thought to operate only in prokaryotes, is widely documented in plants in response to both biotic and abiotic stressors. Mutations are caused by rapid ROS generation, which causes random breaks in DNA and induces other systemic stress responses. The idea that herbicides and other pesticides can increase mutation frequencies is not new, although it is rarely discussed. In addition to ROS-mediated DNA damage, sublethal herbicide exposure can also lead to genetic and epigenetic changes such as gene amplification and altered DNA methylation patterns. And as
discussed above, changes in ROS-mediated signal transduction, transcriptional remodeling, and PTM patterns create a significantly altered physiological environment during herbicide stress, with attendant widespread pleiotropic effects.

If herbicide stress is like other abiotic stresses, weeds exposed to sublethal doses unleash a vast array of heritable epigenetic, biochemical, and physiological defense/repair mechanisms, and together these changes provide novel sources of variation upon which selection for NTSR can act. As shown for systemic acquired acclimation and SAR, this variation allows selection on genome, transcriptome, and proteome changes that confer a suite of stress-response adaptations, resulting in NTSR and the SAHR phenotype. As previously proposed, the genetic association between stress and adaptation challenges widespread assumptions about the evolution of herbicide resistance. More specifically, estimates for the initial frequency of resistance alleles and constancy of mutation rates used in traditional population genetic models may be very different in a stressed plant from those of standing genetic variation, and revised values incorporated into resistance models may lead to novel and useful predictions about NTSR evolution.

3 PLEIOTROPIC FITNESS COSTS

Of the potential pleiotropic effects associated with resistance, fitness costs as predicted by Fisher in 1930 have been the most studied and have shaped our expectations about herbicide resistance for decades. Under annual applications of herbicides with the same mode of action, weed populations are expected to fix resistance alleles regardless of their cost, because continued resistance is necessary for survival. However, more realistic rotations among modes of action, non-herbicide integrated weed management strategies, and other weed suppression tactics cause fluctuating selection pressure on resistance alleles with pleiotropic fitness costs. Under varying selection pressures, overall population genetic variation and cycling of allele frequencies should be maintained. Thus, the theoretical relationship between NTSR alleles and pleiotropic fitness cost alleles can become disassociated over time, or their effects modified through compensatory adaptation. Understanding this relationship is essential to predict the evolutionary fate of NTSR alleles.

The first well-characterized resistant weed species (Senecio vulgaris L. with target site resistance to photosystem II inhibitors) fit Fisher’s prediction of fitness costs exactly and shaped our expectations for the next 20 years. However, more recent examples from multiple disciplines show that fitness costs are not necessarily a consequence of evolved resistance to insecticides or fungicides, and the situation for herbicides remains unclear. In entomology, there are ongoing controversies concerning the fitness of laboratory- versus field-selected insecticide-resistant populations and in weed science, determining how to properly make fitness comparisons has been discussed at length. Thus, the correlation between resistance and fitness costs is not absolute, but the co-occurrence of other pleiotropic effects with resistance is consistent across many species, as shown below. For target site resistance, pleiotropic effects are likely conferred by the resistance gene, while NTSR pleiotropic effects are due to combinations of known and unknown genes.

4 PLEIOTROPIC EFFECTS OF RESISTANCE

Table 1 shows some known pleiotropic effects associated with herbicide resistance. For the purposes of this review, the effects are restricted to those with a non-obvious association with resistance mechanisms, are not necessarily associated with fitness costs, and are constitutively present prior to herbicide exposure. Populations with both target site and non-target site resistance are included, to illustrate that unexpected pleiotropic effects are associated with both. It should be noted here that it is unclear if the pleiotropic effect is directly conferred by resistance gene(s), or if it is associated with the action of other genes unrelated to resistance.

Whole-plant pleiotropic effects include a phenological association between seed dormancy/germination and resistance in several species. MHR A. myosuroides, Lolium rigidum, and Kochia scoparia populations had lower germination than their susceptible counterparts in certain environments. By contrast, acetolactate synthase inhibitor-resistant K. scoparia germinated more rapidly than susceptible counterparts at low temperatures, indicating that this association may be complex. Studies of 32 natural Ipomoea purpurea populations showed that glyphosate-resistant populations displayed lower anther–stigma distances and thus had higher rates of self-pollination than susceptible populations. Although such changes in seed germination and pollination rates would no doubt create fitness effects in all environments, negative cross-resistance of triazine-resistant populations to other herbicides would confer a fitness benefit only under ongoing herbicide selection.

At the biochemical level, triazine-resistant populations of several species display altered photosynthetic inhibition characteristics, chloroplast membrane lipid composition, and elevated heat tolerance, features that do not seem to be directly related to their well-characterized target site mutations. Paraquat resistance is associated with the monogenic upregulation of several ROS detoxification enzyme activities in Conyza bonariensis, indicating the involvement of a pleiotropic transcription factor. At the molecular level, essentially all transcriptome analyses of resistant populations show some kind of constitutive gene expression changes unrelated to biochemical resistance mechanisms. For example, several species display upregulation of SAR-associated transcripts for protein kinases and transcription factors involved in disease resistance, salicylic acid response, and others. In particular, MHR A. fatua contains a number of constitutive transcript, protein, and PTM changes related to the stress response, as discussed below. This phenotype may be more common than currently recognized, because most transcriptome and proteome surveys to date have focused on known mechanisms of resistance, to the exclusion of other possibilities. Pleiotropic effects of genes conferring resistance to fungicides and insecticides are well documented, and so their presence in herbicide-resistant populations should not be surprising.

Resistance conferred by amplification of the gene encoding the herbicide target enzyme deserves extra attention, due to the high potential for pleiotropic effects. Detailed investigations of glyphosate-resistant Amaranthus palmeri populations reveal that resistant plants possess multiple copies of a 297-kb cassette containing one copy of the 5-enol-pyruvylshikimate 3-phosphate synthase gene and 72 additional open reading frames. The amplified cassette shows little homology to sequences from glyphosate-sensitive A. palmeri plants, indicating that it is a recent evolutionary event and was not present before glyphosate use. Among the additional predicted cassette genes are those similar to stress response genes, while others are involved in transposon mobility and DNA replication. (Molin W, and Saski C, personal communication). If shown to be functional, the potential for pleiotropic effects of these additional genes is
Table 1. Constitutive pleiotropic effects associated with target site and non-target site herbicide resistance

<table>
<thead>
<tr>
<th>Species</th>
<th>Resistance</th>
<th>Target (T) or non-target (N)</th>
<th>Pleiotropic effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole plant effects</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lolium rigidum</em></td>
<td>MHR</td>
<td>Both</td>
<td>Altered seed germination</td>
<td>63</td>
</tr>
<tr>
<td><em>Alopecurus myosuroides</em></td>
<td>ALS inhibitors</td>
<td>T</td>
<td>Altered seed germination patterns</td>
<td>64</td>
</tr>
<tr>
<td><em>Kochia scoparia</em></td>
<td>Glyphosate</td>
<td>T</td>
<td>Reduced seed longevity and germination</td>
<td>65</td>
</tr>
<tr>
<td><em>Kochia scoparia</em></td>
<td>ALS inhibitors</td>
<td>?</td>
<td>Faster germination at low temperatures</td>
<td>66</td>
</tr>
<tr>
<td><em>Ipomea purpurea</em></td>
<td>Glyphosate</td>
<td>?</td>
<td>Reduced anther–stigma distance</td>
<td>67</td>
</tr>
<tr>
<td><em>E. crus-galli, C. canadensis</em></td>
<td>Triazines</td>
<td>T</td>
<td>Negative herbicide cross-resistance</td>
<td>68</td>
</tr>
<tr>
<td>Biochemical effects</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. vulgaris, C. album</em></td>
<td>Atrazine</td>
<td>T</td>
<td>Altered chloroplast lipids</td>
<td>69</td>
</tr>
<tr>
<td><em>Glycine max (cell culture)</em></td>
<td>Atrazine</td>
<td>T</td>
<td>Altered chloroplast lipids; heat tolerance</td>
<td>70</td>
</tr>
<tr>
<td><em>Brassica napus</em></td>
<td>Atrazine</td>
<td>T</td>
<td>Increased sensitivity to photoinhibition</td>
<td>71</td>
</tr>
<tr>
<td><em>Brassica campestris</em></td>
<td>Triazines</td>
<td>T</td>
<td>Increased chlorophyll a/b complex</td>
<td>72</td>
</tr>
<tr>
<td><em>Cynza bonariensis</em></td>
<td>Paraquat</td>
<td>N</td>
<td>Increased ROS detoxification enzymes</td>
<td>73</td>
</tr>
<tr>
<td><em>Kochia scoparia</em></td>
<td>Dicamba</td>
<td>?</td>
<td>Increased chalcone synthase expression</td>
<td>74</td>
</tr>
<tr>
<td><em>Eleusine indica</em></td>
<td>Glyphosate</td>
<td>N</td>
<td>Phosphofructokinase gene amplification</td>
<td>75</td>
</tr>
<tr>
<td><em>Amaranthus palmeri</em></td>
<td>Glyphosate</td>
<td>T</td>
<td>Numerous co-amplified predicted genes</td>
<td>48</td>
</tr>
<tr>
<td><em>Alopecurus myosuroides</em></td>
<td>ALS inhibitors</td>
<td>N</td>
<td>Disease resistance and peroxidases</td>
<td>76</td>
</tr>
<tr>
<td><em>Alopecurus aequalis</em></td>
<td>Mesosulfuron</td>
<td>N</td>
<td>Altered flavonoid metabolism</td>
<td>77</td>
</tr>
<tr>
<td><em>Avena fatua</em></td>
<td>MHR</td>
<td>N</td>
<td>Salicylic acid-responsive kinases</td>
<td>78</td>
</tr>
<tr>
<td><em>Lolium rigidum</em></td>
<td>MHR</td>
<td>N</td>
<td>Numerous differential genes</td>
<td>79</td>
</tr>
<tr>
<td><em>Ambrosia trifida</em></td>
<td>Glyphosate</td>
<td>?</td>
<td>Altered protein and PTM profiles</td>
<td>26</td>
</tr>
<tr>
<td><em>Eleusine indica</em></td>
<td>Paraquat</td>
<td>?</td>
<td>Overexpressed protein kinases</td>
<td>31</td>
</tr>
<tr>
<td><em>Echinochloa crus-galli</em></td>
<td>Quinclorac</td>
<td>?</td>
<td>Altered pathogen response transcripts</td>
<td>80</td>
</tr>
<tr>
<td><em>Cicer arietinum</em></td>
<td>Imazethapyr</td>
<td>?</td>
<td>Photosynthetic and defense proteins</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Transcription factors; core metabolism</td>
<td>82</td>
</tr>
</tbody>
</table>

substantial. In the related species *Amaranthus tuberculatus*, an extra ring chromosome containing amplified copies of the 5-enol-pyruvylshikimate 3-phosphate synthase gene appears to be the result of stress-induced chromosomal alterations. Further detailed investigations of gene amplification mechanisms and pleiotropic effects in these and other resistant species will no doubt lead to important insights into NTSR evolution.

Although not yet documented in resistant populations, herbicide-induced epigenetic effects have been confirmed, first by low-resolution techniques showing evidence for glyphosate-induced and atrazine-induced alterations in DNA methylation patterns. More recently, detailed investigations of Arabidopsis plants treated with sublethal glyphosate doses showed that methylation changes were not uniformly spread across the genome, but many were clustered in differentially methylated regions associated with transposons, and the majority were related more specifically to glyphosate injury than to other abiotic and biotic stresses, suggesting a level of precision in DNA methylation response. Current research will determine if transposons are in fact activated by these changes, and if their activation is associated with pleiotropic effects as seen elsewhere (Westwood J, personal communication).

5 MULTIPLE HERBICIDE RESISTANT AVENA FATUA

We recently described the MHR3 and MHR4 populations of *A. fatua* that are resistant to members of all selective (in small grains) herbicide families available in North America for *A. fatua* control. Specifically they are resistant to the acetyl-CoA carboxylase inhibitors fenoxaprop-P-ethyl, tralkoxydim, and pinoxaden, the acetolactate synthase inhibitors imazamethabenz-methyl and flucarbazone, the growth inhibitor difenoconazol, the photosystem I inhibitor paraquat (MHR3 only), and the very long chain fatty acid biosynthesis inhibitors triallate and prosulfocarb, with resistant/susceptible ratios ranging from 1.4 to 57. MHR plants do not contain known target site mutations for acetyl-CoA carboxylase or acetolactate synthase inhibitors, and the cytochrome P450 monoxygenase inhibitor malathion partially reversed the resistance phenotype for a subset of herbicides, indicating the involvement of P450-mediated NTSR mechanisms. Resistance to flucarbazone, imazamethabenz-methyl, and pinoxaden is controlled by three separate but linked nuclear genes. Although all the specific biochemical mechanisms conferring NTSR to this wide array of herbicides are not yet known, resistance to fenoxaprop-P-ethyl and imazamethabenz-methyl may be due to reduced rates of carboxylesterase activity, an enzymatic step necessary for cellular uptake and conversion of these pre-herbicides into active inhibitors. Enhanced herbicide catabolism does not appear to play a major role in MHR *A. fatua*, because glutathione S-transferase specific activities towards fenoxaprop-P-ethyl and imazamethabenz-methyl were not different between untreated MHR and susceptible plants and only a relatively small number of constitutively elevated xenobiotic catabolism transcripts and proteins were detected in MHR plants. The *A. fatua* NTSR phenotype is inheritable and stable for multiple generations in the absence of herbicide treatment. Specific herbicide use histories from the fields where the MHR *A. fatua* populations were collected are not available, but regional practices indicate that they evolved under 40 years of chronological rotations of selective herbicides.
Additional insights into MHR *A. fatua* evolution may be gleaned from comparison with the previously characterized FG93R22. *A. fatua* population derived from the same location as MHR with resistance to only triallate and difenzoquat. Resistant/susceptible ratios to these two herbicides are similar in MHR and FG93R22, suggesting commonalities in resistance mechanisms. Triallate resistance in FG93R22 is conferred by reduced rates of sulfoxidase activity, a pre-herbicide activation step required for toxicity, and is controlled by two recessive nuclear genes. Difenzoquat resistance is associated with herbicide efflux from the cytoplasm and irreversible binding to cell wall components. Although it is not known if MHR plants have the same mechanisms as FG93R22, it is tempting to speculate that the constitutive elevation of xenobiotic transporter transcripts in MHR plants is related to difenzoquat resistance.

Potential fitness costs associated with the MHR *A. fatua* phenotype are unclear. Under non-competitive conditions in the greenhouse, photosynthetic and relative growth rates were not different between susceptible and MHR plants, but MHR plants produced fewer tillers and seeds than susceptible plants. However, a second greenhouse study showed that there were no differences in competitive abilities between susceptible and MHR plants under gradients of biotic (competition with *Triticum aestivum*) and abiotic (limiting nitrogen) stresses. Fitness comparisons have not been conducted under field conditions.

### 6 Pleiotropic Features in Resistant Weeds

As shown in Table 1, all resistant populations characterized to date exhibit various pleiotropic effects at the whole plant, biochemical, or molecular level. Of these, MHR *A. fatua* contains more documented molecular changes than other species, as well as several unusual resistance mechanisms, and so it has been used as a model to investigate the primary stress effects associated with the evolution of NTSR. Initial efforts to describe these have focused on constitutive differences between MHR and susceptible populations, because they are most likely to represent changes that would lead to pleiotropic effects. Transcriptome and proteome analyses showed that MHR plants have constitutively higher levels of transcripts and proteins with functions in stress response, disease resistance, redox maintenance, xenobiotic transport and catabolism, transcriptional regulation, and signal transduction.

For example, transcripts for three protein kinases including the lectin S-receptor-like serine/threonine-protein kinase LecRK2, a well-characterized SAR receptor/signal transducer, were constitutively elevated in MHR plants. By contrast, levels of heat shock protein (HSP) transcripts and proteins were constitutively lower than in susceptible plants. In the first phosphoproteome and redox proteome comparisons between resistant and susceptible plants of any species, PTMs of proteins with functions in core cellular processes were shown to be constitutively reduced in MHR *A. fatua* plants, perhaps in association with certain fitness costs. PTMs of proteins involved in xenobiotic and stress response, ROS detoxification/redox maintenance, heat shock response, and intracellular signaling were more abundant in MHR than susceptible plants. Overall, this suite of constitutive changes in MHR *A. fatua* populations could represent pleiotropic effects of NTSR, or it is more likely that they are an evolutionary signature of ongoing stress responses like systemic acquired acclimation or SAR, of which NTSR is only one aspect.

### 7 NTSR: A Role for Heat Shock Proteins?

Of all the changes associated with MHR *A. fatua* discussed above, perhaps the most intriguing is the constitutive reduction of HSP transcripts and proteins and altered HSP PTM patterns. In all eukaryotes, HSPs are typically induced and subjected to PTMs in response to heat shock as well as a wide variety of biotic and abiotic stresses, and they are implicated in hormetically induced epigenetic effects on phenotype. HSPs primarily function as molecular chaperones that repair misfolded client proteins during stress, although a subset regulate transcription factors for a diverse set of genes. For example, several HSPs are involved in adaptation of the yeast *Saccharomyces cerevisiae* to the auxinic herbicide 2,4-D. In the same species and related fungi, the Ssa1p HSP70 binds to and represses the Pdr3p transcription factor controlling expression of an ATP-binding cassette transporter that confers multiple drug resistance. Thus, a reduced level of Ssa1p derepresses transporter expression and leads to enhanced resistance in fungi. In MHR *A. fatua*, constitutively reduced HSP transcripts and proteins, and elevated xenobiotic transporter transcripts may indicate the presence of an orthogonal regulatory system in higher plants that contributes to herbicide resistance.

Constitutively reduced HSP profiles have also been reported in stress-tolerant plant biotypes. For example, a comparison of natural *Chenopodium album* biotypes showed that those adapted to stressful environments contained lower levels of certain HSPs than those from moderate habitats, both constitutively and in response to thermal stress. Comparable ecological studies of *Drosophila* and *Arabidopsis* reported similar findings, suggesting that this is an evolutionarily conserved strategy for stress acclimation. In a series of papers starting in 1998, it was proposed that HSP90 functions as a global regulator for stress adaptation by acting as a capacitor for phenotypic variation. Under stress, HSP90 levels are typically induced but under some conditions cannot keep pace with demands for client protein re-folding and repair of regulatory pathway components. Limiting HSP90 levels thus allows normally buffered polymorphisms to persist, providing novel sources of variation upon which selection can act, and the polymorphisms that are adaptive in the new stressed environment become fixed. Experimental evidence shows that pharmacologic reductions of HSP90 levels in *Drosophila* and *Arabidopsis* produced a wide variety of novel phenotypes, and the same treatment in *S. cerevisiae* and *Candida albicans* led to the evolution of resistance to two classes of fungicides. The role of HSP90 as a global regulator of diverse stress-induced signaling pathways is now well accepted.

The results from MHR *A. fatua* plants indicate that a very different stress, i.e. sublethal herbicide damage, may have caused them to evolve a similar strategy during herbicide selection, leading to a heritable downregulation of certain HSPs and the consequent evolution of NTSR.

It is important to point out that the phenotype just described is much more complex than the acute induction of HSPs in response to a single stress event, which is well documented in eukaryotes. However, neither acute HSP induction nor the constitutive HSP reductions seen in MHR *A. fatua* have been investigated in resistant populations of other species. It is hoped that other transcriptome and proteome datasets will be re-examined for these and other constitutive changes that may inform a better understanding of NTSR evolution. Similarly, it will be of interest to determine whether herbicide-resistant populations are also more tolerant to...
other biotic and abiotic stresses, as is well documented for systemic acquired acclimation and SAR. For example, a coral (Acropora formosa) population adapted to a polluted environment was more tolerant to glyphosate and elevated temperature than one from a pristine environment. For MHR A. fatua, research is underway to determine levels of resistance to other abiotic and biotic stresses.

8 IS MHR AVENA FATUA AN OUTLIER OR HARBINGER?

As noted above, the number and variety of constitutive transcript and protein alterations in MHR A. fatua appears to be larger (or better documented) than in other resistant populations. Also, A. fatua and the MHR phenotype have several characteristics that are not typical of other species. First, unlike L. rigidum, A. myosuroides, and A. tuberculatus, A. fatua is a predominantly self-pollinating species with limited seed dispersal and substantial levels of seed dormancy, features that tend to delay the appearance and spread of novel traits. Second, A. fatua is an allohexaploid, in contrast to the diploid species just mentioned. Polyploidy provides additional standing genetic variation upon which selection can act, but at the same time may buffer novel resistance mutations through expression of homoeologous sensitive alleles, slowing HR evolution. Third, more than 40 years of annual herbicide applications were required before the MHR populations became noticeable, a considerably longer time frame than is typical. Thus, the MHR A. fatua phenotype may be an outlier when compared with other species.

However, the environment, herbicide spectrum, and chronology of herbicide use that selected for MHR A. fatua populations are not unusual, and so other species in similar circumstances may be following a parallel evolutionary path. Certainly, MHR is not unusual, since its incidence is rapidly increasing worldwide especially in monocots. MHR populations of self-pollinating polyploid species in weedy genera like Eleusine and Echinochloa have been reported, and if these traits contribute to a delay in NTSR evolution as argued here for A. fatua, additional resistant populations may continue to appear. Further, transcriptome and proteome surveys of many HR and MHR populations of other species have also detected SAR-like constitutive alterations, as is shown here for MHR A. fatua. By far the largest category of elevated transcripts before and after herbicide treatment of NTSR Apera spica-venti populations was for nucleotide binding site-leucine-rich repeat proteins, a large family of receptors involved in SAR to plant diseases. Although it is possible that such constitutive changes are unrelated to NTSR, it seems more likely that both they and NTSR are part of a general stress response. The commonality of such changes among resistant populations of many species indicates that the MHR A. fatua phenotype may indeed be a harbinger of additional, similar cases in the future.

Figure 2 shows a proposed model for some of the key cellular players in recognition, uptake, signal transduction, and subsequent induced responses to sublethal herbicide doses. Actively absorbed herbicides are transported by cell-surface receptors, which may be similar to receptor-like kinases involved in systemic acquired acclimation and systemic acquired resistance (SAR) that activate a heat shock protein 90 (HSP90)-mediated signal transduction cascade, leading to transcriptional remodeling, post-translational modifications (PTMs), and possibly epigenetic events. These changes in turn cause translation or modification of stress-related proteins with functions in defense, repair, reactive oxygen species (ROS) management, xenobiotic inactivation, and likely non-target site resistance (NTSR). RLKs, receptor-like kinases; CH3, DNA methylation; nc RNAs, non-coding RNAs; TFs, transcription factors.

9 FINDING AND STUDYING NASCENT NTSR POPULATIONS

Valuable insights into NTSR and its evolution can be obtained by changing the way that we sample for resistance. Because most if not all field-selected NTSR populations originated from producer complaints of herbicide non-performance, it is likely that the university or company researchers visiting these fields collected only the most robust and therefore most resistant plants/seeds for further study, and the barely surviving weeds under the crop canopy (survivors) were ignored. However, these survivors may have been in the early stages of NTSR evolution, and a conscious effort to sample and study them could provide valuable information. For example, survivor and robust NTSR populations of the same species could be compared to distinguish between two possible chronologies of NTSR evolution. In the traditionally accepted evolutionary chronology, herbicides initially select for direct NTSR evolution.113 Much has been learned about SAR and other plant stress responses by characterizing their receptors, the ensuing signal transduction, and induced effects.
mechanisms such as enhanced metabolism or transport.\textsuperscript{36} However, high fitness costs correlated with these alleles limit population sizes of survivors, so their appearance is usually overlooked. Over time, additional stress responses evolve that compensate for the initial fitness deficits, allowing populations to recover, prosper, and be noticed by producers. The genetic basis of this chronology for fungicide resistance in \textit{Aspergillus nidulans} was recently described.\textsuperscript{119} If this is also true for herbicide selection, then theory predicts that survivor populations would display high fitness costs,\textsuperscript{53} while costs would have been purged or compensated for in robust populations. At the molecular level, constitutive changes in survivor populations would include the downregulation of transcripts/proteins with housekeeping functions related to fitness.

A second possible chronology of NTSR evolution is more aligned with the scenario proposed in this review: general stress responses are first induced by sublethal herbicide doses, and these can simultaneously include both NTSR and any number of pleiotropic effects. If pleiotropic fitness costs are high, limited population growth may eventually be ameliorated through outcrossing and/or compensatory adaptation. However, if fitness costs are moderate, NTSR populations will rapidly predominate under continuing herbicide use. In this scenario, both survivor and robust populations would be expected to display SAR-related constitutive changes in transcripts and proteins, as is seen for the species listed in Table 1 and the MHR \textit{A. fatua} populations described here.

Obtaining evidence for these two possible chronologies of NTSR evolution may be difficult, but both will add to our basic knowledge about plant responses to stress and will also inform improved resistant weed management strategies. If pleiotropic effects are related to primary stress effects as argued above, then a better characterization of pleiotropy in well-characterized NTSR populations of \textit{L. rigidum}, \textit{A. myosuroides}, and species with strong genetic/genomic resources will be informative. Further, because it is established that low-dose impacts cannot be predicted from high dose experiments,\textsuperscript{120} further research on the role of initial sublethal herbicide effects on NTSR evolution is clearly warranted.

10 CONCLUSION
This review proposes that, in addition to the evolution of herbicide resistance through selection on standing genetic variation, it also evolves in response to stress induced by sublethal herbicide exposure. In the latter case, NTSR appears to be only one of many possible stress-induced responses, and together, these responses can lead to the SAHR phenotype and a number of unpredictable pleiotropic effects. This view is supported by phenological changes associated with resistance, and by transcriptome and proteome surveys showing that current resistant populations exhibit a number of constitutive alterations apparently unrelated to NTSR mechanisms. If designed correctly, such surveys generate vast amounts of unbiased data, and then the subsequent challenge often centers on deciding which differences between resistant and susceptible populations to verify and pursue. In the NTSR surveys published to date, this choice was based on previous knowledge about established herbicide resistance mechanisms like enhanced metabolism, and so the results reinforce what is already known. A more informative approach is proposed here that includes a focus on candidates for the primary regulators and physiological markers of stress, with the idea that such features can provide deep insights into the fundamental mechanisms of NTSR evolution and its pleiotropic effects. Resistance due to gene amplification can also present unique opportunities, by providing a window into the potential pleiotropic effects of genomic rearrangements and additional genes that are co-amplified along with herbicide target site genes. The co-occurrence of plants with both target site resistance and NTSR in the same population indicates that current definitions of these terms may be unnecessarily restrictive from a research standpoint\textsuperscript{121} and this author believes that a shifted focus on the primary stress effects of sublethal herbicide exposure will inform both types of resistance. And finally, as both the appearance of NTSR populations and the worldwide occurrence of environmental stress are expected to increase, expanded research on NTSR evolution and its potential for pleiotropic effects should be a high priority.

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