

# Current Advances in the Action Mechanisms of Safeners

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**Abstract:** Herbicide safeners are a series of agrochemicals that can selectively protect crop plants from herbicide injury without affecting herbicidal efficacy. Understanding mechanisms by which safeners act is significant for discovery of novel types. Safeners primarily alleviate herbicide phytotoxicity to crop plants via several actions: (i) enhancing metabolism of herbicides in crops; (ii) affecting absorption and transportation of herbicides in crops; (iii) competitively binding to herbicide target sites; and (iv) affecting activity of target enzymes. This review describes recent advances in the action mechanisms of safeners, analyzes existing problems, anticipates the future direction of studies of modes of action of safeners, and prospects potential strategies to design safeners related to their reported mechanisms. The aim of this paper is to provide insight into mechanisms of safeners and give tips for development of new safeners.

**Keywords:** herbicide; safener; mechanism; crop plants; metabolize; herbicide injury



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## 1. Introduction

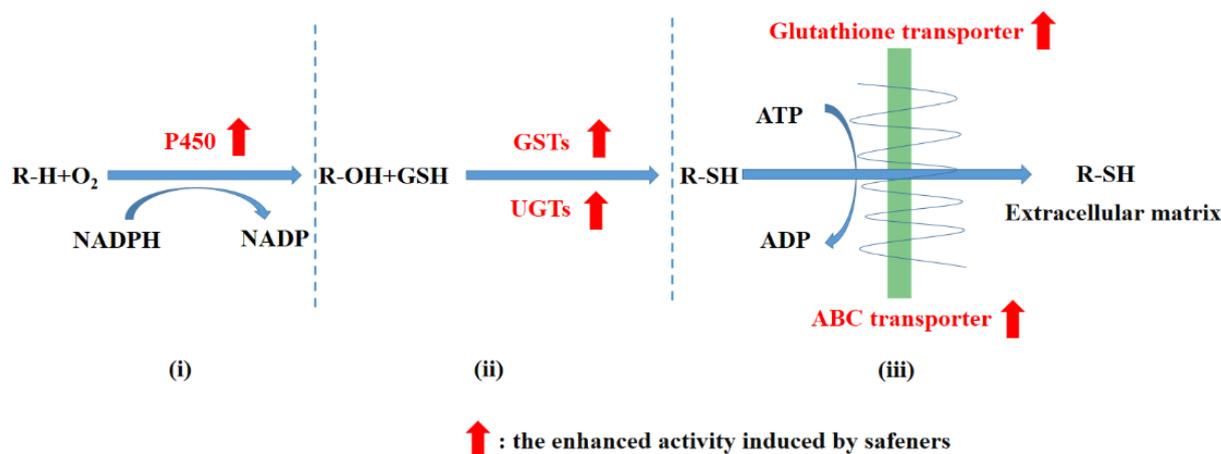
The primary weed species (such as barnyard grass (*Echinochloa crus-galli*)) infesting crop fields are a great challenge due to their competition with crop plants, leading to a significant decrease in yields worldwide [1–3]. Weeds can be managed by herbicide application, which is more efficient than other physical, mechanical, and cultural control methods [4,5]. However, use of herbicides might result in phytotoxicity to crops [6]. The most commonly used strategy for limiting phytotoxicity of herbicides is applying herbicide safeners [7]. Herbicide safeners, known as antidotes and protectants, are commonly used in combination with herbicides or can be added to seeds via pre-sowing seed treatments; they can effectively reduce herbicide-induced toxicity to crops and enhance selectivity of herbicides in crops without affecting efficacy of the herbicide [8]. Safeners only increase herbicide resistance to crop plants and have no effect on weeds. Therefore, use of safeners can broaden the spectrum of herbicide.

To date, over 20 commercial safeners have been developed, and several natural safeners and thousands of safener candidates have been found [9]. However, the exact mechanisms by which safeners act still remain obscure, and multiple pathways have been implicated [10]. Therefore, the current review summarizes recent advances of the main action mechanisms of safeners, which are divided into the following four categories: (i) enhancing metabolism of herbicides in crops; (ii) affecting absorption and transportation of herbicide in crops; (iii) competitively binding to herbicide target sites; and (iv) affecting activity of target enzymes. Moreover, potential strategies to design safeners related to their action mechanisms are also prospected. This will provide some insight into developing novel safener molecules.

## 2. Enhancing the Herbicide Metabolism in Crop Plants

At present, one of the most well-known mechanisms by which herbicide safeners act is enhancing metabolic ability of crops to selectively break down herbicides. Recent studies have also indicated that safeners might have an influence on metabolic rate of herbicides

but not change the metabolic pathway of those herbicides. Metabolism of herbicides in crops is a multi-step process [11]. To better understand safeners' effects on metabolism of herbicides in crops, herbicide metabolism in plants should be introduced and can be further divided into the following stages: (i) hydrolysis or oxidation reactions; (ii) binding; and (iii) conjugates (Figure 1). New functional groups are formed via hydrolysis or oxidation reactions in the chemical structure of herbicides to obtain new metabolites that are able to bind to hydrophilic endogenous substrates in plants. This stage involves metabolic enzymes such as peroxidase (POD) and cytochrome P450s (P450s). As-obtained products from hydrolysis or oxidation reactions bind to glutathione (GSH) or glucose; the main corresponding enzymes involved are glutathione-S-transferases (GSTs) and glycosyl transfer (UGTs). Conjugates produced in stage ii are located in the cell wall or transferred to the vacuole via the ATP binding cassette transporter (ABC transporter), where they pass through the protoplasm membrane and vacuole membrane. The resulting exogenous conjugates might be further processed. Some of them are metabolized by partial degradation or secondary binding reaction. Metabolism of herbicides by crops is a multi-step process that requires the participation of GSH, P450s, GSTs, UGTs, and ABC transporters. As shown in Figure 1, safeners can induce a series of key factors (e.g., GSTs, P450s, UGTs, and ABC transporters) throughout the entire detoxification pathway in the plant, thereby enhancing metabolism, degradation, and isolation of herbicides [11].



**Figure 1.** Herbicide metabolism stages (i–iii) and enhancement of metabolism induced by safeners in crop plants.

### 2.1. Induction of GSTs

The large GST enzyme family, which can catalyze the binding of GSH and various electrophilic foreign compounds such as herbicides, is found both in animals and plants and can obtain derivatives that are less toxic than their original compounds or even obtain non-toxic compounds [12]. GSTs in plants play a role in eliminating herbicide phototoxicity; this has received extensive attention. Induction of enhancement of GSTs activity might be the most accepted action mechanism of safeners because all commercial safeners, some safener candidates, and some natural product safeners are reported to induce improvement of GSTs activities. Table 1 lists some examples of safeners that enhance activity of GSTs. GSTs in some crop plants, such as rice, corn, and wheat [13–53], can be induced in different degrees by safeners; this enhances the herbicide tolerance of these crops. Deng et al. found that after treatment with safener fenclorim, GSTs activity in rice plants was enhanced to 1.3–1.9 times that of the control (only treated with pretilachlor) [29]. Zhang et al. synthesized a series of diazabicyclo derivatives as safener candidates. One of them, compound S15 (3-(2',6'-dichloro-phenyl)-4-(3,3,6-trimethyl-9-oxa-1,5-diazabicyclo[3.4.0]nonanealkyl)-5-methylisoxazolecarboxamide), could enhance GST activity in corn plants by 1.13–1.48 times [44]. Induced GSTs activities of cotton treated with potential safeners (diazabicyclo derivatives) were

increased to 21~88% in shoots and 33~90% in roots [47]. The study reported by Scarponi et al. showed that GSTs metabolic activity in wheat plants was increased by 77.4%, 56.4%, and 93.9% after treatments with safeners cloquintocet-mexyl, fenflufen, and fenchlorazole-ethyl, respectively [53]. In corn seedlings treated with safener MG-191, increased levels of GSH and GSTs were observed, revealing the possibility that MG-191 protects corn by increasing those plants' ability to detoxify herbicides [25]. Tolerance of herbicides in rice was enhanced because safeners can induce a specific GST isozyme; for example, GST I, II, and III were first found in corn, which has affinities to herbicides [54]. Safener treatments also induce transcriptional activation of specific GST genes and enhance expression of respective enzymes of GSTs [55]. For example, *OSGSTF3*, *OSGSTF5*, and *OSGSTU39* in rice can be enhanced via treatment of fenclorim derivatives [42], which catalyze conjugation of herbicides with GSH in crops to detoxify them. For chiral safener, significant differences of GST activities could be observed from treatments of R-29148 and its two isomers in chloroacetanilide herbicide acetochlor-treated corn. The *R*-isomer of R-29148 increased the highest expression level of GSTs, about 1.87 folds and 13.6 folds, compared with that of the *S*-isomer and racemic R-29148, respectively [56]. It is almost certain that GSTs of crop plants, such as rice, corn, and wheat, can be induced by safeners with various degrees so as to enhance crop tolerances of herbicide. Enhancement of crop-plant tolerances of herbicide is mainly evidenced by the fact that safeners can induce specific GST isoenzymes that are compatible with the metabolites of herbicides [26]. In addition, efficiencies of safeners are closely associated with their ability to induce GST activity.

**Table 1.** Recent commercial safeners and some safener candidates induced enhancement of GST activity.

Commercial Safeners	References	Commercial Safeners	References
1,8-naphthalic anhydride (NA)	[13,14]	dichlormid	[15,16]
R-28725	[17,18]	R-29148	[19]
furilazole	[20]	AD-67	[21]
benoxacor	[22,23]	MG-191	[24]
cyometrinil	[25]	oxabetrinil	[26]
fluoxfenim	[27]	acetamate	[28]
fenclorim	[29,30]	fenchlorazole-ethyl	[31]
isoxadifen-ethyl	[32]	mefenpyr-diethyl	[33]
cluquintocet-mexyl	[34]	cyprosulfamide	[35]
Natural safeners	References	Natural safeners	References
melatonin	[36]	gibberellin	[37]
sanshools	[38]	isopimpinellin	[39]
5-methoxypsoralen	[39]	Z-ligustilide	[40]
brassinolide	[41]		
Safener candidates	References	Safener candidates	References
( <i>E</i> )-4-(2-substituted hydrazinyl)-6-chloro-2-phenylpyrimidines	[42]	<i>N</i> -alkyl amides	[43]
diazabicyclo derivatives	[44]	1,3-disubstituted imidazolidine or hexahydropyrimidine derivatives	[45]
phenyl isoxazole analogues	[46]	diazabicyclo derivatives	[47]
ester-substituted pyrazole derivatives	[48]	substituted phenyl oxazole derivatives	[49]
substituted dichloroacetylphenyl sulfonamide derivatives	[50]	quinoxaline derivatives	[51]
substituted oxazole isoxazole carboxamides	[52]	<i>N</i> -tosyloxazolidine-3-carboxamide	[53]

## 2.2. Induction of UGTs

Both UGTs and GSTs are detoxification enzymes in stage ii of the herbicide metabolism process. UGTs in plants play an important role in detoxification of pathogenic toxins, hormone balance, and detoxification of pathogenic toxins. In stage i, resulting reaction

products mediated by carboxylesterase or P450s can be catalyzed by UGTs to bind with glucose and form *O*-glucoside, *N*-glucoside, or glucose esters (glycosylation). At present, only a few studies have shown that safeners can enhance glycosylation of herbicides, thereby protecting crops from herbicide injury. For example, Kreuz et al. reported that cloquintocet mexyl could enhance wheat glycosylation of the metabolite of clonafop propar-gyl [56]. Brazier et al. found that cloquintocet-mexyl could selectively enhance effects of *O*-glucosyltransferase (OGT) on herbicide molecules in wheat plants [34]. Edwards et al. studied many different types of GSTs and UGTs via treatments of different safeners in wheat and corn plants [57]. That study showed that activity of GSTs was induced only by cloquintocet-mexyl in wheat plants, whereas other safeners mainly induced activity of UGTs. In corn plants, however, several safeners mainly induced activity of GSTs. Therefore, safener induction of detoxifying enzymes in crops may be species-specific. The role of UGTs in the present study of function of herbicide metabolism and the influence of safener on it are still unclear. Therefore, more studies are needed.

### 2.3. Induction of P450s

In addition to UGTs and GSTs, P450s are widely found in plants and represent the largest enzyme family. P450s—which can catalyze aryl hydroxylation, cyclomethyl hydroxylation, *N*-demethylation, and *O*-demethylation of herbicide molecules—play a very important role in stage i of herbicide metabolism in plants [58,59]. Early research showed that the relationship between P450s and herbicide metabolism mainly comes from in vitro experiments of plant microsomes. Related P450 inhibitor experiments on plants supported this finding [60]. In 1969, Frear et al. found that cotton could metabolize monuron, which was the first evidence that P450s were involved in herbicide metabolism [61]. At present, safeners can induce enhancement of metabolism of herbicides—such as oxopropionic acid esters, sulfonylureas, imidazolinone, sulfonamides, chloroacetamide, and aryloxybenzene—mediated by P450s (Table 2) [16].

**Table 2.** Recent commercial safeners and some safener candidates induced enhancement of P450 activities.

Commercial Safeners	References	Commercial Safeners	References
dichlormid	[61]	NA	[62–67]
R-28725	[68]	R-29148	[69]
furilazole	[20]	AD-67	[70]
benoxacor	[71]	MG-191	[72]
cyometrinil	[73]	fluoxfenim	[74]
fenclorim	[69]	fenchlorazole-ethyl	[75]
isoxadifen-ethy	[76]	mefenpyr-diethyl	[32]
Natural safener	References	Safener candidates	References
melatonin	[39]	quinoxaline derivatives	[51]
diazabicyclo derivatives	[47]		

From the perspective of enzymology, many studies have demonstrated that activity of P450s can be induced and enhanced by safeners. In 1979, Robert et al. found that safener dichlorimid could enhance the safener effect to protect corn from herbicide injury caused by thiocarbamate herbicide *S*-ethyl dipropyl thiocarbamate (EPTC) [61]. Persans et al. reported that NA and its analogues could induce activity of P450s in corn, then stimulate that corn to tolerant herbicide triasulfuron [62]. Deng et al. found that NA could enhance the detoxification effect (*O*-demethylation) in rice by 4.5 times [63], and Liu et al. found that content of P450s in corn plants after treatment with NA and fenclorim was increased by 5.63 and 3.30 times, respectively, from that of the control [64,65]; content of internal P450s was 8.54 and 2.20 times higher than that of the control.

From the gene transcription level, there is sufficient evidence that suggests safeners can induce P450 gene expression. For example, NA can induce expression of *CYP81C1*, *CYP81C2*, and *CYP71A11* in tobacco plants. Expression of genes was enhanced more than two times [66]. NA can also induce expression of *CYP71C1*, *CYP71C3*, *CYP72A5*, *CYP73A7*, and *CYP92A1* genes in wheat [67]. However, little is known about the upstream and downstream molecular mechanisms of P450 gene transcription induced by safeners. This is likely due to technical reasons that hinder the identification of coding gene P450.

#### 2.4. Induction of ABC Transporters and Glutathione Transporters

Accumulation of toxins in the cell wall leads to activity of detoxification enzymes in stage ii. Some GSH conjugates can inhibit activities of GSTs and glutathione reductase (GR) [77]. Therefore, these conjugates need to be transported further. ABC transporters are a superfamily in plants. ABC transporter multidrug resistance-associated protein (MRP) plays an important role in transport of glutathione–herbicide and glucose–herbicide conjugates to vacuoles [78,79]. Martinoia et al. found that metolachlor in barley, as well as the conjugate of metolachlor with GSH, could be transferred to vacuoles by ATP-binding transporters [80]. MRP genes were identified and found in a variety of plants; for instance, the ZMMRP1 gene, an ABC transporter gene in corn, plays an important role in transport of alachlor conjugates [81].

Specificity and transport characteristics have not yet been clarified. In *Beta vulgaris*, chlorsulfuron occurred after saccharification via the proton's reverse transport mechanism to the vacuolar membrane microcapsules [82], while glycosylated products of primisulfuron were transferred by the ABC transporter that was transferred to the vacuoles [83]. The mechanism by which plants isolate the conjugates of herbicide glucose may be species-specific and relate to the chemical properties of glucoside conjugates [78]. Studies have shown that MRP can be induced by safeners. Gaillard et al. found that cloquintocet-mexyl treatment could affect vacuolar membrane transporters of glutathione conjugates and glucose in wheat plants [79]; transportation activity of glucose conjugates was nearly doubled. Theodoulou et al. found that cloquintocet-mexyl could simultaneously induce transcription of five GST genes and one MRP transporter gene [84]. Zhang et al. found that cloquintocet-mexyl could induce expression of the *TtMRP1* gene in wheat leaves [85]. The expression level was enhanced to 13 times that of the control. Cloquintocet-mexyl could enhance the expression level of the *TtMRP1* gene in wheat coleoptile by 9.5 times that of the control. In addition, it enhanced the expression level of the *TtMRP2* gene by 2.3 times that of the control. Pang et al. also found that enhanced expression of the *ZmMRP1* gene could be significantly induced by dichlorimid [86].

Recent studies have found that the GSH transporter, which is located on the plant cell membrane, can mediate the transport of GSH and glutathione–herbicide conjugates, indicating that the GSH transporter is an important component of the plant-detoxification system for exogenous compounds [86]. Pang et al. found that induction of *ZMGT1* genes in corn was related to alachlor tolerance in corn varieties [87]. In addition, that study also found that expression of the *ZmGT1*, *ZmGST27*, and *ZmRP1* genes in corn could be induced and enhanced by dichlorimid, indicating that the GSH transporter might be involved in the foreign-substance detoxification process related to GSH binding [86]. In corn and *Arabidopsis thaliana*, the GSH transporter could be metabolized by flusulfuron methyl (which is metabolized by glycosylation) [86]. This may mean that GSH transporters are also involved in other detoxification pathways. In sum, the role of GSH transport in herbicide metabolism should be studied further.

### 3. Effect of Safeners on Herbicide Absorption and Transport

Evidence supported that safeners might work by inhibiting absorption and transport of herbicides. Han et al. found that fenclorim reduced rice-root absorption of pretilachlor [88], which was thought to be a potential mechanism by which pretilachlor acts. However, other studies have indicated that safeners have no effect on absorption of herbicides. Wu

et al. recognized that the protective effect of fenclorim on rice is mainly due to enhanced metabolism of alachlor in rice rather than a change in absorption or transport of pretilachlor in rice [30]. Scarponi et al. found that fenclorim did not affect absorption or accumulation of pretilachlor by rice, but retention of pretilachlor in rice seedlings was accelerated [89]; the detoxification metabolic rate of rice seedlings with alachlor was increased. Bunting et al. studied the isoxadifen ethyl using inhalation of formamide sulfuron, a  $^{14}\text{C}$  tracing technology [90]. Those results showed that absorption of formamide sulfuron methyl was not directly related to the safener. Köcher et al. also found that mefenpyr diethyl has been widely used in treatment of sulfonylureas mesosulfuron and iodosulfuron sodium salt (iodosulfuron methyl sodium) [90]. However, it is unknown whether that safener will interfere with absorption of herbicides by crops. In addition, information is lacking about whether safeners directly interfere with transport of herbicides in crops.

#### 4. Target Site Competition (Structure–Activity Theory)

Owing to the structural similarity of some herbicides and their safeners, some scholars have proposed that safeners may compete for the same binding sites as pesticides, allowing them to play a protective role against herbicides (structure–activity theory) [91]. This means safeners can compete with herbicide molecules. Yenne et al. [92] used a computer-aided molecular model program. The chemical structures of several groups of commonly used safeners and herbicides were compared, and results showed that the most successful safener–herbicide pairs were those in which both components were highly similar at the molecular level. Ezra et al. showed that eradican (EPTC) and its structurally similar safener [93], dichloramide, presented competitive action at target sites. Walton et al. reported that safener R-29148 and herbicide alachlor competed for a protein binding site in corn [94], supporting the structure–activity theory. With development of computer-aided drug molecular design (CADD) methods, numerous studies of molecular docking results of safeners bound to related herbicide targets suggested that safeners could compete with herbicides at the action sites of herbicide targets [44–46,48,49,52]. However, some research indicated that safeners do not directly affect the interaction between herbicide and target site. In vivo experiments showed that NA treatments did not affect the inhibition effect of chlorsulfuron on acetolactate synthase synthetase (ALS); in addition, fenchlorazole-ethyl did not reduce inhibition of fenoxaprop-ethyl on acetyl CoA carboxylase (ACCase) in wheat [93]. This might be due to the reason that these safeners did not have similar structures to those of the combined herbicides.

#### 5. Effecting the Target Enzyme Activity

Some studies have indicated that safeners can improve enzyme activities of herbicide targets inhibited by herbicides. ALS is an important herbicide target enzyme in plants, involved as the first enzyme in the synthetic process of branched chain amino acids such as valine, leucine, and isoleucine. Some herbicides (for example, sulfonylureas herbicides) are thought to act via inhibition to produce ALS in weeds, indirectly blocking the production of branched-chain amino acids, which in turn affects protein synthesis and ultimately causes weeds to die [94]. It has been reported that safeners can improve activity of ALS in crops, thus protecting crops from injury caused by herbicides. Rubin et al. found that activity of ALS in root and stem tissues after treatment with allyl chloride increased by 30% and 24%, respectively [95]. Milhomme et al. found that activity of ALS in the corn plant increased by more than 40% and by about 20% after treatment with NA and oxabetrinil, respectively [19]. Research conducted by Zhao et al. on corn showed that safener R-28727 could alleviate injuries caused by chlorimuron ethyl [20]. Differences in mechanisms of chiral safeners could also be found in the aspect of affecting target enzyme activity. Chiral R-28727 analogue (*R*)-3-dichloroacetyl-2,2-dimethyl-4-ethyl-1,3-oxazolidine (*R*-enantiomer) increased ALS activity inhibited by chlorimuron-ethyl from 45 to 97% compared with the control (only treated with chlorimuron ethyl), exhibiting better activity than that of *S*-enantiomer [18]. In addition, R-28725 can significantly improve ALS activity

in corn plants inhibited by imazethapyr in vivo [96,97]. Natural safeners showed similar mechanisms of action [41]. However, opposed to the results above, Barrett found that treatments of NA, acetonitrile, flurazole, and dichlorimid in corn and sorghum seedlings did not cause significant enhancement of ALS activity [98]. Therefore, safener effect on ALS activity is still not clearly understood, and further research should be carried out to clarify why safeners can enhance the ALS activity of herbicide-treated crop plants.

## 6. Induction of Signaling Pathway

Safeners can induce expressions of defense and detoxification genes in monocotyledonous crops to protect crops from herbicides, rendering them non-toxic to crop plants, which indicates that those safeners might open detoxification signal pathways for foreign or endogenous substances in the form of herbicides that have not been identified. However, the main target of safener signaling remains unknown. Currently proposed mechanisms for safener detoxification are described as follows: (i) Safeners might be able to regulate activity and abundance of transcriptional activators (or repressors) that interact with regulatory elements of the defense gene promoter, regulating expressions of herbicide metabolism-related genes [5]. (ii) Safeners might adopt a mechanism similar to that of auxin to regulate gene expressions [5]. For example, Xu et al. found that in wheat, pheromone response element gene *TTGSTU1* could be promoted simultaneously by safener and auxin induction [81], indicating that safener and plant auxin could regulate GSTs expression with a similar mechanism. The results of a study by Zhang et al. also supported this finding [86]. (iii) Safeners might take advantage of the signal pathways mediated by oxylipins or cyclopentenone, thus enhancing expressions of plant detoxification and defense-related proteins [1]. Gene expression assays on *Arabidopsis thaliana* confirmed that when combined with oxidative stress, there were similarities between the related lipoxygenase pathway and the safener signal [1]. In response to oxidative stress, plants accumulate  $\alpha$ -oxidized lipids of linolenic acid, including oxidized lipids, cyclopentenone, and phytoprostanes [11]. Jasmonic acid is the most important plant oxylipin, so safeners may use jasmonic-acid signaling pathways to induce detoxification and defense enzymes [27]. Loeffler et al. found a substance with a structure similar to that of jasmonic acid; it triggered plant defense and detoxification reactions in research of *Arabidopsis thaliana* cell cultures [82]. (iv) Safeners can be induced by salicylic-acid signaling-pathway detoxification and defense enzymes [5,25]. Increased gene expression induced by safeners may overlap with salicylic-acid-related plant stress-defense signaling pathways, and there is evidence that many genes regulated by safeners can be induced by salicylic acid [25]. Therefore, multiple signaling pathways could be related to the reaction of safeners in plants. However, further studies are needed to identify the main targets of the safener signaling pathways.

## 7. Effects of Safeners on Weeds

Current research indicates that safeners hardly enhance metabolic capacity of weeds to detoxify herbicides. Yun et al. treated rice and *Juncellus serotinus* with NA respectively, finding that *O*-dealkylation activity of P450s in *Juncellus serotinus* was not effectively induced, but its activity in rice plants was doubled [99]. Brazier et al. also found that activity of *O*-glucosyltransferase in black grass (*Alopecurus myosuroides*) was not affected after treatments with cloquintocet-mexyl and dichlormid, but its activity in wheat was significantly induced [34]. Hu et al. studied differences in physiology, biochemistry, and gene transcription when rice and barnyard grass responses to safener fenclorim were compared. Fenclorim reduced oxidative damage in rice but not in barnyard grass. Transcriptome analysis also revealed that fenclorim induced more genes related to herbicide metabolism in rice than in barnyard grass, especially GSTs genes [100]. After study of black grasses with different levels of non-target resistance, obvious changes in the weed herbicide dose-response curve was not seen. This proved that mefenpyr-diethyl has no significant effect on enhancement of non-target resistance of black grass [101]. Cummins et al. also revealed that mefenpyr-diethyl could slightly enhance fenoxaprop-ethyl detoxification ability of

black grass [102]. However, this research also indicated that enhancement of metabolic detoxification ability induced by mefenpyr-diethyl black grass was insufficient to explain enhancement of weed resistance. Significantly, this was the only report that safeners could induce and enhance metabolism of herbicides in weeds.

## 8. Conclusions and Perspectives

The molecular mechanism by which safeners act may involve complex interactions between multiple signals and detoxification pathways, which can protect plants from herbicide damage. In recent years, researchers have focused on mechanisms of action of herbicide safeners, and a great deal of research has been carried out regarding safeners' efficacy of protecting crops from some detoxification enzymes involved in damage from herbicides, such as P450s. Some consensus has been reached on the role of GSTs and UGTs, but exact mechanisms of action of safeners are still unclear and need further study. However, many studies have shown that at various stages of herbicide detoxification in crops, all enzymes may be induced by safeners (limited by technical conditions). Because studies conducted on detoxification enzymes of safeners have been conducted on different plants, some results are difficult to compare; therefore, more studies should focus on detoxification of herbicides using one or a few plant species. Expression of stage-related genes at the protein level and the transcription level changes whether safeners can enhance weed control metabolism of herbicides and evolution of non-target resistance of safeners in weeds. In addition, although it is known that many signaling pathways are involved in mechanisms of action of safeners, details of action and those pathways are still unclear. Finally, few studies have been conducted on detoxification and metabolism of safeners for herbicides in plants in stage iii.

Clarifying mechanisms of action of safeners is important for developing new high-efficiency and selective safeners. In the future, it is urgent to make full use of molecular biology and biochemistry methods in order to study mechanisms of action of herbicides at the molecular level and explore interaction mechanisms of herbicides and safeners in crop systems. In view of deficiencies in the current research of mechanisms of action of herbicide safeners, future research should focus on the following: (i) utilizing gene-transcription technologies, such as genomics and proteomics, to study types and functions of enzymes involved in herbicide metabolism in plants, as well as effects of safeners on various enzymes involved in herbicide metabolism; (ii) furthering study of the effect of safeners on herbicide metabolism in weeds and clarifying the relationship between safeners and evolution of non-target herbicide resistance; (iii) conducting in-depth research of signaling pathways of safeners and clarifying how safeners regulate expression of detoxification genes; and (iv) determining concentration of herbicide metabolites in plants.

From the perspective of safener design, all commercial safeners and as-prepared safener candidates used in this experiment were discovered by a random-screening strategy, which occupied large amounts of time, manpower, and funding resources; due to this, reasons for actions of those safeners are unclear and targets of those safeners are still uncertain. However, according to the summarized mechanisms in our paper, several novel safener design approaches could be applied. Easy-to-obtain structures or compounds with simple structures (for instance, salicylic acid), which can enhance metabolism of herbicides in crops, could be used as active fragments in discovery of new safeners to enhance the success rate of random screening. As safeners might compete with herbicides to bind to targets in crops, protein-crystallization techniques such as cryoelectron microscopy could be used to obtain crystal structures of targets in crop plants or crystal complexes of safener and herbicide targets. On this basis, research of virtual screening and structure-based safener design can be carried out. When crystal structure is difficult to obtain, software such as Alpha Fold could be used to predict it. Deep learning algorithms could also be applied to improve efficiency and accuracy of kinetic optimization of structure models and corresponding virtual screening. We believe that with deepening research of mechanisms of safeners, novel safeners could be designed in a more efficient and quicker way in the future.

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