Chemically induced herbicide tolerance in rice by the safener metcamifen is associated with a phased stress response

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Abstract

The closely related sulphonamide safeners, metcamifen and cyprosulfamide, were tested for their ability to protect rice from clodinafop-propargyl, a herbicide normally used in wheat. While demonstrating that both compounds were equally bioavailable \textit{in planta}, only metcamifen prevented clodinafop from damaging seedlings, and this was associated with the enhanced detoxification of the herbicide. Transcriptome studies in rice cultures demonstrated that whereas cyprosulfamide had a negligible effect on gene expression over a 4 h exposure, metcamifen perturbed the abundance of 590 transcripts. Changes in gene expression with metcamifen could be divided into three phases, corresponding to inductions occurring over 30 min, 1.5 h and 4 h. The first phase of gene induction was dominated by transcription factors and proteins of unknown function, the second by genes involved in herbicide detoxification, while the third was linked to cellular homeostasis. Analysis of the inducible genes suggested that safening elicited similar gene families to those associated with specific biotic and abiotic stresses, notably those elicited by abscisic acid, salicylic acid, and methyl jasmonate. Subsequent experiments with safener biomarker genes induced in phase 1 and 2 in rice cell cultures provided further evidence of similarities in signalling processes elicited by metcamifen and salicylic acid.

Keywords: Aromatic sulphonamide, cyprosulfamide, detoxification systems, next-generation sequencing, \textit{Oryza sativa}, salicylic acid, transcription factors, xenome.

Introduction

Safeners are a chemically diverse group of agrochemicals that enhance the selectivity of weed control in cereals by protecting the crop, but not the competing weeds, from herbicide injury (Riechers \textit{et al.}, 2010). Each safener is typically used in combination with specific post-emergence herbicides for each cereal crop application, either as a seed treatment or through co-application (Davies and Casely, 1999; Kraehmer \textit{et al.}, 2014). By partnering new safeners with existing herbicides, a crop which would normally not tolerate the active ingredient can be protected, allowing for new chemistries to be brought into play for weed control (Kraehmer \textit{et al.}, 2014). As a recent example, the aromatic sulphonamides cyprosulfamide and metcamifen (Fig. 1) are currently used in maize with herbicides acting on branched chain amino acid synthesis and tyrosine degradation,
herbicides (Riechers et al., 2010; Kraehmer et al., 2014). Despite their importance in modern weed control, we know remarkably little about how safeners activate the xenome in cereals. Their chemical diversity and requirement to be paired with specific herbicides for crop-specific applications suggest that their recognition, associated signalling, and elicitation of gene expression are complex and unlikely to proceed through a single point of recognition. Using metcamifen and the closely related cyprosulfamide as a test case of chemistry specificity, we have explored the ability of the two compounds to protect rice against clodinafop-propargyl, correlating any observed effects with changes in global gene expression, and explored the relationships between xenome gene induction elicited by safeners as compared with endogenous stress signalling agents.

Key safener-inducible xenome enzymes in cereals include both glutathione transferases (GSTs) and cytochrome P450s (CYPs) that are known to play major roles in herbicide detoxification (Riechers et al., 2010; Kraehmer et al., 2014).

Materials and methods

Plant material

Rice suspension cultures of Oryza sativa L. Tsukinohikari were maintained on N1 medium composed of 4.4 g l⁻¹ ChuN6 salts and vitamins (Duchefa Biochemie, The Netherlands), 0.3 g l⁻¹ casamino acids, 1 mg l⁻¹ 2,4-dichlorophenoxyacetic acid, and 20 g l⁻¹ sucrose (pH 5.6). Cells were maintained on a shaking incubator and subcultured at 7 d intervals. Black Mexican Sweetcorn (BMS) suspension cell cultures were maintained on growth medium containing 4.71 g l⁻¹ Murashige and Skoog medium including vitamins (M0222; Duchefa Biochemie), 1 mg l⁻¹ 2,4-dichlorophenoxyacetic acid, and 20 g l⁻¹ sucrose (pH 5.6), and maintained as above.

Rice (Oryza sativa L. ssp. Indica) cv. IR-64 seeds were chitted in sand at 28 °C for 3 d. Germinating seeds were sown in a mixture of John Innes compost (no. 2) and sand, and maintained under a 16 h photoperiod using sodium lighting at an intensity of 290 µEm⁻² S⁻¹ (28 °C day, 22 °C night). After 7 d, rice seedlings at the three-leaf stage were gently extracted from the compost and transferred into hydroponic growth units containing half-strength Hoaglands (Sigma-Aldrich) solution (pH 5.0) and 28 mg l⁻¹ Fe(III) EDTA complex. Seedlings were allowed to acclimate for 24 h prior to chemical treatments. Maize plants (Zea mays L. cv. Claxson) were grown to the three- to four-leaf stage (7 d after germination) and then transferred into hydroponic growth units containing half-strength Hoaglands.

Chemical treatments

Cell suspension cultures (5 d old; n=3) were dosed with 5 mM stocks of metcamifen or cyprosulfamide, prepared in DMSO, to give a final concentration of 5 µM, with DMSO only used as the control treatment. This concentration of safener was selected following dose–response studies with metcamifen (see Supplementary Fig. S1 at JXB online). Abscisic acid (ABA), methyl jasmonate (MeJA), and salicylic acid (SA) were added to a final concentration of 100 µM, and hydrogen peroxide (H₂O₂) to 1 mM. Upon harvest, cells were separated from the growth medium by filtration, then immediately frozen in liquid nitrogen, and stored at −80 °C.

Hydroponically grown seedlings were treated with metcamifen, or cyprosulfamide prepared in DMSO as 10 mg ml⁻¹ stocks by transferring the plants into root growth medium containing 10 ppm safener (26.8 µM metcamifen; 27.5 µM cyprosulfamide), or the equivalent volume of DMSO (control). After a 1 h exposure, seedlings were removed from the treatment solution and the roots were washed and transferred into fresh medium. To determine the level of safener-derived protection against phytotoxic injury, the hydroponically grown seedlings were foliar-sprayed with 100 g l⁻¹ clodinafop-propargyl (Valiant) at rates equivalent...
to 20, 40, and 80 g ha\(^{-1}\) by diluting in deionized water containing 0.5% (w/v) of the oil-based adjuvant Adigor\(^{\circledR}\). The track-sprayer was set up with a flat fan even spray 8002 nozzle at a height of 30 cm above two-thirds of the total plant height. The sprayer speed was 85 cm s\(^{-1}\), giving an application volume equivalent to 300 l ha\(^{-1}\). Seedlings were sprayed in groups of three plants and assessed visually for phytotoxicity 21 d after application. Herbicide injury was scored on a scale from 0% to 100%, based on symptom severity assessed on degree of cessation of plant growth, stunting, and necrosis of both old and newly emerging foliage. At either end of the scale, 0% was assessed as no effect on plant development or growth as compared with untreated controls, while 100% damage corresponded to completely dead plants with no visible green or growing tissues (Hall et al., 2000).

For metabolism studies with clodinafop-propargyl, metcamifen was applied as a pre-treatment to the hydroponic solution of maize seedlings at the two- and three-leaf stage, 24 h before the application of clodinafop-propargyl. Clodinafop-propargyl was applied onto the leaf surface in 200×0.2 µl droplets. At time points over 48 h, seedlings were divided into shoots and roots (n=4), and the shoots extracted in 3× (w/v) acetonitrile:water (4:1) and analysed for the combined disappearance of clodinafop-propargyl and clodinafop acid using LC-MS.

**Safener uptake in cell cultures**

Five-day-old rice N1 cell cultures were pH adjusted using 10% (v/v) 5 mM succinate (pH 5.0) and allowed to recover for 1 h, before transferring to shake flasks. Cells previously killed by freezing at –80°C, were re-suspended in buffered N1 culture medium as controls. Cells were then dosed with 5 µM metcamifen, or cyprosulfamide, and the medium sampled at timed intervals to monitor the uptake of the safeners into the cells. To provide calibration for quantitative LC-MS, a standard curve was prepared by diluting the safeners in buffered growth medium. Cell viability was determined using a vital dye, by measuring the formation of red formazan after incubating 1 ml of the cultures with an equal volume of 0.5% (w/v) 2,3,5-triphenyltetrazolium chloride solution in 50 mM KH\(_2\)PO\(_4\) (pH 7.5) for 4 h in the dark.

**Uptake and translocation with radiolabelled metcamifen**

Hydroponically grown seedlings were dosed with 100 kBq of [benzoyl]-U-ring-\(^{14}\)Cmetcamifen (specific activity: 1934 MBq mmol \(^{-1}\); Selcia Limited, Ongar, UK) via the growth medium. At 1, 3, 6, 24, and 48 h after treatment, plants were removed from vials, pressed flat onto cardboard, and then freeze-dried. Phosphoimages of the samples were acquired overnight using a beta scanner (GE Healthcare Typhoon FLA3000).

**LC-MS analysis**

Plant tissue samples were homogenized in 3× (w/v) acetonitrile:water (4:1) prior to analysis on a Waters Acquity 1-class ultraperformance liquid chromatograph linked to a Waters Xevo TQ mass spectrometer. Herbicides and safeners were resolved on an Acquity C18 BEH (2.1×50 mm; 1.7 µm) column, at a flow rate of 0.7 ml min\(^{-1}\) using a gradient eluent system of (A) water plus 0.2% (v/v) formic acid and (B) acetonitrile. Initial conditions were 5% solvent B at 0 min, followed by a gradient of 5–95% solvent B between 1.1 min and 1.5 min, held at 95% B until 1.6 min, and then returned to initial conditions until 2.0 min. For MS detection, a collision energy of 30 V was used. The parent and transition ions were m/z 306.0 and m/z 206.9 for metcamifen, m/z 375.1 and m/z 254.1 for cyprosulfamide, and m/z 350.1 and m/z 90.9 for clodinafop-propargyl. In each case, the concentrations of the parent mass ions were determined by calibration using authentic reference standards.

**Real-time quantitative PCR analysis**

Total RNA was extracted using TRI-reagent (Sigma-Aldrich) and first-strand cDNA was synthesized using an oligo(dT) primer Og2 (Supplementary Table S1) and a Superscript II reverse transcriptase kit from Invitrogen according to the manufacturer’s instructions. Based on the next-generation sequencing data, three transcripts were selected as representing genes induced by metcamifen, namely OsGSTL1 (LOC_Os07g23570), safener-responsive protein 1 (LOC_Os06g32000), and CYP709C9 (LOC_Os03g17480), with the reference gene OsEF-1a (LOC_Os03g08020) used for normalization. Primer sets for each amplification product are given in Supplementary Table S1. Real-time quantitative PCR (qPCR) was performed using Fast SYBR® Green Master Mix (Applied Biosystems), on the StepOnePlus™ Real-time PCR System (Applied Biosystems). Biological replicates for cell cultures (n=3) or seedlings (n=4) were each assayed in technical triplicates, with data analysis performed using StepOne v2.2.2 software (Applied Biosystems).

**Next-generation sequencing**

Rice N1 cell cultures (n=3) were dosed with 5 µM metcamifen, 5 µM cyprosulfamide, or DMSO alone, the latter serving as a control. For each treatment, cells were sampled at 30, 90, and 240 min. Total RNA was extracted using a Ribopure™ RNA Purification Kit (Ambion Inc.), and the integrity of the RNA was determined using a 2100 Bioanalyzer (Agilent). Samples [RNA integrity number (RIN) >9.00] were then subjected to RNA sequencing (RNA-seq) analysis on a HiSeq 2000 system (Illumina) using the manufacturer’s standard library preparation protocol (National Center for Genome Resources, Santa Fe, NM, USA). Nine samples per lane were sequenced to generate single-end 100 bp reads which were aligned to the rice (O. sativa cv. japonica) v7.0 nuclear and organelle genomes (Kawahara et al., 2013). Alignment was performed using GSNAP version 2011_03_38 (Wu and Nacu, 2010). Count data were generated at gene level, using annotations for nuclear, plastid, and mitochondrial genomes, for 46 103 gene models. Count data were then analysed with R libraries EDaseq and edgeR to normalize the data and determine differential expression. No significant GC bias was detected, but significant bias was detected based on differences in gene length. In each case, data were normalized for length bias using full quantile within-lane normalization and then subjected to full quantile normalization for between-lane normalization. Data were then separated into groups, based on the type and duration of treatment, and analysed for differential expression. A statistical model was built from these groupings and the data were fitted to a negative binomial distribution, using a generalized linear model (glmFit function from edgeR), followed by likelihood ratio tests. False discovery rates (FDRs) were calculated, and genes were considered to be differentially expressed if fold change between means of sample replicates were >2 and the FDR was <0.05. Gene set enrichment analyses were performed on gene sets built from Gene Ontology (GO) terms assigned to rice genes based on InterPro scan results and manual annotation using data from Syngenta’s GreenPhyl orthologue database. Separate gene sets were built for biological processes, molecular function, and cellular compartment GO terms. In addition, gene sets were built based on their roles in defined molecular pathways as defined by Gramene (http://pathway.gramene.org/gramene/ricecyc.shtml).

**Data deposition**

The replicated (n=3) transcriptome data from each time point in the rice culture studies are registered with NCBI in the GEO repository as GSE124666. https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE124666

**Results**

**Safening of clodinafop-propargyl in rice and maize seedlings**

Rice and maize plants were grown hydroponically, and transiently (1 h) exposed to safeners via the root media to mimic a seed treatment, as an alternative to spray application. The plants were then sprayed with clodinafop-propargyl at 33% above the field rate commonly used for weed control in wheat (60 g
Mean phytotoxicity (%) 21 d after spraying the 20 g ha⁻¹ clodinafop treatment rate, with almost total differential safening effect in rice was more pronounced against the herbicide at this field rate (Fig. 2B; Table 1). The metcamifen and cyprosulfamide provided 70% protection shown near 100% damage (Fig. 2A; Table 1). In maize, both corresponding non-safened and cyprosulfamide-treated plants showed near 100% damage (Fig. 2B; Table 1). The differential safening effect in rice was more pronounced at the 20 g ha⁻¹ clodinafop treatment rate, with almost total protection afforded by metcamifen, while the root exposure to cyprosulfamide resulted in similar levels of damage to those seen in non-safened plants (Table 1).

The results with clodinafop-propargyl applied at a full field rate to maize and a partial field rate to rice demonstrated that both safeners must have been absorbed from the root medium to exert their protective effect in planta. One explanation for the weaker safening seen with cyprosulfamide was that this compound might have been less bioavailable in the seedlings than metcamifen. The primary effect of clodinafop is to cause the rapid arrest of growth at the meristems and leaf bases by inhibiting the plastidial form of acetyl-CoA carboxylase which is most active and abundant in rapidly growing tissues (Podkowski et al., 2003). It is therefore logical that if differential bioavailability caused the differences in response to the two safeners in rice, then the accumulation of metcamifen in the leaf base (meristem) of rice would be greater than that of cyprosulfamide following an identical exposure. To test this hypothesis, rice plants were fed with the two compounds, and the concentrations of the parent safeners were determined in the foliage and stems over a time course using LC-MS (Table 2A). Based on this analysis, cyprosulfamide actually accumulated more rapidly than metcamifen in both leaves and meristems over the first 6 h, though by 22 h uptake was effectively equivalent. This demonstrated that differences in gross translocation to the meristems were unlikely to account for the differential safening seen with the two safeners.

It was thus of interest to investigate whether or not differences in the physical properties of the two safeners could affect their uptake into rice cells, thereby affecting their intracellular bioavailability. For this study, rN1 cell suspension cultures were used as a simple proxy for dividing rice cells. Rice cultures were exposed to 5 µM metcamifen or cyprosulfamide, and the disappearance of the safeners from the medium was monitored by HPLC (Fig. 3). This treatment concentration was arrived at as being physiologically relevant from gene induction studies carried out with OsGSTL1 (Supplementary Fig. S1), a member of the Lambda (L) class of GSTs previously shown to be responsive to the safener fenclorim (Brazier-Hicks et al., 2018). In live cells, the rate of uptake of the two safeners was very similar, with the compounds both having equilibrated between the medium and cells 30 min after dosing (Fig. 3A). Neither compound accumulated in the dead cell controls (Fig. 3B). This experiment demonstrated that both safeners, which are very similar structurally (Fig. 1) and possess similar physical properties (cyprosulfamide, pKa 3.8 and logP 2.0; and metcamifen, pKa 4.5 and logP 2.1), essentially behave identically with respect to their bioavailability in rice tissue.

**Table 1.** Hydroponically grown rice and maize seedlings were exposed ± a 1 h root pre-treatment with metcamifen, cyprosulfamide, or solvent control and sprayed with adjuvanted (but unsafened) clodinafop-propargyl, at rates equivalent to 20, 40, or 80 g active ingredient ha⁻¹ in three replicates, each containing three individual plants.

| Species g ha⁻¹ | Clodinafop-propargyl | Mean phytotoxicity (%) 21 d after spraying | No safener | Cyprosulfamide | Metcamifen |
|----------------|-----------------------|---------------------------------------------|-----------|----------------|-----------|
| Rice 80        | 100 a                 | 100 a                                       | 38 d      |                 |           |
| 40             | 96 b                  | 86c                                         | 34 d      |                 |           |
| 20             | 39 d                  | 34 d                                        | 5 e       |                 |           |
| Maize 80       | 99 a                  | 35 d                                        | 33 d      |                 |           |
| 40             | 99 a                  | 38 d                                        | 33 d      |                 |           |

After 21 d, seedlings were assessed for phytotoxic injury using a percentage scoring system where 0% corresponded to no visible injury as compared with untreated plants and 100% corresponded to dead plants with no visibly green or growing tissue. Scores were averaged to the nearest whole integer across the treatments and data were analysed using ANOVA, with F-test and 5% least significant differences (LSDs) calculated. Mean values showing statistically different toxicity scores are shown using letters, ranging from ‘a’ the highest level of damage through ‘b, c, and d’ to ‘e’, the latter referring to no perceptible injury. SD=0.07; F-test probability <0.0001; 5% LSDs minimum 0.12499; maximum 0.13974.

**Fig. 2.** Safening of clodinafop-propargyl in hydroponically grown rice and maize. Seedlings of (A) rice and (B) maize were treated for 1 h with metcamifen (MCF) or cyprosulfamide (CSM) dosed at 10 ppm via the medium and then transferred to fresh solutions to mimic a seed treatment with the compounds.
Metcamifen promotes the accelerated detoxification of clodinafop in rice

Based on the selective safening of clodinafop in rice by metcamifen, work then concentrated on the respective mechanism of action of this compound. First, the uptake and translocation of hydroponically supplied $^{14}$C-metcamifen were monitored over a 48 h period using a beta imager (Fig. 4). After 1 h, radiolabel had been taken up into the roots and could be detected in the second and third leaf. By 3 h, radioactivity was present in all plant parts, reaching maximal levels at 24 h post-treatment, with accumulation being particularly intense in the meristems. The nature of the translocated radiolabel was not investigated, but, based on previous LC-MS studies with the unlabelled compound that had not detected any related metabolites over a 24 h period, it was assumed that the majority of the radioactivity observed was derived from the parent $^{14}$C-metcamifen. Having demonstrated that the root-dosed safener was present in the foliage 24 h after treatment, clodinafop-propargyl was applied to the leaves of each hydroponically grown rice plant after a 24 h exposure to metcamifen. Plants which had not been pre-treated were then used as unsafened controls. Plants at both the two- and three-leaf stage were employed, with the disappearance of clodinafop-propargyl and the phytotoxic clodinafop acid over a 48 h period monitored by LC-MS to determine the relative rates of herbicide degradation. Rates of the disappearance of total clodinafop (ester and acid combined) were noticeably faster in control plants than down-regulation of expressed genes (Supplementary Table S2). Metcamifen treatment resulted in the greatest induction of transcripts at 1.5 h (Fig. 5; Supplementary Table S2), with 170 genes showing only transient elevation (Fig. 5B). With respect to quantitative perturbation, the metcamifen treatment resulted in more pronounced up-regulation of expressed genes (Supplementary Table S2). In contrast, cyprosulfamide treatment only induced 20 genes and down-regulated a single transcript, with the greatest change observed at 0.5 h (Fig. 5). In terms of similarities in transcriptome induction, only five genes were up-regulated by both safener chemistries, with metcamifen treatment resulting in the greatest induction of transcripts at 1.5 h (Fig. 5B). With respect to quantitative perturbation, the metcamifen treatment resulted in more pronounced up-regulation of expressed genes (Supplementary Table S2). In contrast, cyprosulfamide treatment only induced 20 genes and down-regulated a single transcript, with the greatest change observed at 0.5 h (Fig. 5). 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metcamifen-inducible genes followed a linear increase in expression over the 4 h treatment period, revealing the transient nature of the early transcriptional events triggered by this safener (Supplementary Table S2). The first wave of transcripts accounted for 9% of the total metcamifen-inducible genes as compared with 73% affected in the second phase and 16% in the third (Fig. 6).

Having established an apparent phasing in the induction of the rice transcriptome following metcamifen treatment (Fig. 6), changes in gene expression were then linked to putative function. As a first step, the perturbed transcripts at each time point were subdivided into broad functions based on their GO terms, namely those involved in biological processes, molecular functions, and structural cellular components (Table 4). In each case, the expression kinetics of members of these three functional GO terms were determined. The Roast algorithm was then used to calculate the probability that a set of genes were over-represented and then to rank order the gene sets by FDR <0.05. Using this methodology, at 0.5 h, a significant number of biological processes (33 gene sets) and molecular functions (28 gene sets) were up-regulated by metcamifen treatment, with the number continuing to increase through 1.5 h to a maximum at 4 h. The increase in these two GO terms between 1.5 h and 4 h coincided with an overall decline in the numbers of induced genes during this period (Fig. 5). In contrast, down-regulation of gene sets did not occur in large numbers until 1.5 h after metcamifen treatment (Table 4). Examining functions in more detail showed that at 0.5 h, multiple biological processes related to sulfur metabolism were enriched, including upstream processes associated with S assimilation and serine biosynthesis, together with genes encoding enzymes involved in glutathione-linked metabolic processes such as protein S-glutathionylation (Supplementary Table S3). Transcription responses related to phytohormone signalling were observed at 0.5 h, including perturbation of genes known to be responsive to ABA, SA, JA, and cytokinins (Supplementary Table S3). At 0.5 h, xenome genes were noticeably enriched in the metabolic function GO terms, while transport (biological process) and binding (molecular function) of molecular oxygen were depressed. No cellular components were enriched at 0.5 h but, by 1.5 h, up-regulated gene sets included those encoding proteins involved in vacuolar processing, including those linked to xenobiotic detoxification. In addition, gene sets encoded in several organelles including the mitochondrion, chloroplast, and peroxisomes were also induced (Supplementary Table S3).

Pathway analysis provided further evidence of the coordinated induction of genes involved in glutathione biosynthesis and phytohormone regulation, along with those linked to amino acid degradation (Supplementary Table S4).

Postulating that gene induction associated with metcamifen treatment at the earliest time (0.5 h) point would be linked to signalling events and that changes at 1.5 h reflected primary defence responses toward herbicides, we refined our analysis of the safener transcriptome to regulatory and xenome genes, respectively. Analysis of the genes induced (>2-fold) at 0.5 h showed that 20% of them were annotated as transcription factors (TFs; Table 5), comprising 39 TFs from 10 different gene families, with NAC and C2H2 orthologues being the most numerous. The other major ‘functional’ category represented in the first wave of responsive genes was annotated as proteins of unknown function, with these comprising a third of induced transcripts at 0.5 h (Table 5). Among these was the most highly metacamifen-inducible transcript, a gene of unknown function, LOC_Os09g32000, that we termed Safener-Responsive Protein 1 (SRP1) (Supplementary Table S2). Further analysis of the first-wave transcripts was performed to determine whether they were linked to common regulatory processes. The nine most highly induced (>5-fold) transcripts at 0.5 h were subjected to bi-clustering analysis using the Genevestigator tool (Hruz et al., 2008). Of these, six genes were found to be co-regulated over a total of 19 conditions (Supplementary Fig. S2), including responses to cold stress, dehydration, iron deficiency, and Rice blast fungus infection. Intriguingly, the six most repressed genes at 0.5 h clustered with expression

**Fig. 3.** The uptake of 5 µM metcamifen (circles), or cyprosulfamide (squares) from culture medium into live (A) or dead (B) suspension-cultured rice cells over a 2 h period. Results show means, with the error bars representing the respective SDs (n=3).
Phased gene induction in herbicide safening

Changes seen in plants suddenly exposed to warmer temperatures. Examining the early response genes induced at 1.5 h identified a rich selection (13% of all induced transcripts) of xenome genes, including CYPs, 12-oxophytodienoate reductases (OPRs), GSTs, glucosyltransferases (UGTs), ATP-binding cassette (ABC) transporters, and MATE efflux family proteins. In contrast, only five xenome genes were induced at 0.5 h (Table 5). In total, 22 of the 50 most highly induced genes determined after metcamifen treatment were linked to xenome function, including CYPs and UGTs potentially involved in the hydroxylation and glucosylation of the safened clodinafop as previously described in wheat (Kreuz et al., 1991).

Safener and plant stress hormone signalling in rice suspension cultures

The transcriptome studies demonstrated that metcamifen induced many genes also known to be responsive to abiotic and biotic stress. By focusing on the early gene transcriptional events, it was clear that many of these were involved in plant hormone biosynthesis and related signalling. It was therefore of interest to explore whether there was a relationship between plant hormone signalling and the early responses to metcamifen in rice. For this study, three biomarker genes were selected to represent the first and second waves of expression. SRP1 was identified as a ‘first-wave’ biomarker gene. The xenome biomarkers of the second wave of induction included the highly induced OsGSTL1 and CYP709C9.

Biomarker gene expression was monitored by qPCR using rice cell cultures treated for 1 h with ABA, MeJA, and SA at a concentration of 100 µM in the presence and absence of 5 µM metcamifen. As SA at concentrations >100 µM can cause the production of reactive oxygen species (Miura and Tada, 2014), an additional treatment of 1 mM H2O2 was also included. Exposure to ABA, SA, and H2O2 enhanced all three biomarker transcripts (Fig. 7). In contrast, MeJA only induced CYP709C9 transcripts (Fig. 7C). When metcamifen was co-applied with ABA, the induction was additive with all three biomarker genes, suggesting that they were exerting their signalling via different pathways. A similar additive effect was observed.

Changes in overall numbers of distinct genes expressed in rice cultures following treatment with metcamifen or cyprosulfamide (both at 5 µM) over a 4 h time course as compared with untreated controls at three time points (>2-fold change; FDR <0.05)

| Treatment          | No. of genes |          |          |          |          |          |          |          |          |          |
|--------------------|--------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
|                    |              | Up-regulated |          | Down-regulated |          |          |          |          |          |          |
| Metcamifen         |              | 159      | 373      | 177      | 26       | 77       | 61       |          |          |          |
| Cyprosulfamide     |              | 17       | 1        | 2        | 0        | 0        | 1        |          |          |          |

![Fig. 4. Uptake and translocation of radiolabelled metcamifen in hydroponically grown rice seedlings. Seedlings were incubated in growth medium containing 20 ppm [14C]-benzoyl-metcamifen (equivalent to 100 000 Bq) and the translocation of radiolabel determined using phosphoimaging over 48 h.](image)

![Table 3. Changes in overall numbers of distinct genes expressed in rice cultures following treatment with metcamifen or cyprosulfamide (both at 5 µM) over a 4 h time course as compared with untreated controls at three time points (>2-fold change; FDR <0.05)](table)

![Fig. 5. Co-incidence of transcripts perturbed by metcamifen and cyprosulfamide treatment (5 µM) in rice cell cultures at 0, 1.5, and 4 h as represented by a Venn diagram showing the coordinated response to metcamifen treatment resulting in either (left) up- or (right) down-regulation of genes.](image)
with CYP709C9 following co-treatment with MeJA and metcamifen (Fig. 7C). Notably co-treatment with metcamifen and SA did not result in any additional induction of the expressed protein and CYP709C9 beyond that determined with SA alone. A similar effect was also observed with metcamifen and H₂O₂ co-treatment. This strongly indicated coincidence in the signalling pathways responsible for metcamifen- and SA/H₂O₂-mediated gene induction.

**Perturbation of biomarker transcripts in rice seedlings over 24 h**

To confirm that the gene induction determined in cell cultures accurately reflected safener responses in whole plants, hydroponically grown rice seedlings were dosed with metcamifen and cyprosulfamide via the roots, and changes in the expression of the three biomarker genes were determined in the stems and the leaves (Fig. 8). All biomarkers were induced by metcamifen in the stems by 1 h, with SRP1 being similarly responsive in whole plants to that determined in cell cultures. The levels of transcripts then declined between 3 h and 6 h, to be followed by a second induction at 24 h, demonstrating that metcamifen invoked a two-phase induction of the biomarkers. In the remaining foliage, while all three biomarkers were enhanced, this biphasic induction was not observed. Treatment with cyprosulfamide did not significantly induce OsGSTL1 transcripts in either tissue type, or at any time point.

**Discussion**

**Specificity of safening in rice is underpinned by phased changes in gene expression**

This study provides new insight into the chemical specificity of herbicide safener action. While metcamifen safens clodinafop-propargyl in both rice and maize, the closely related compound cyprosulfamide only provided protection in maize, clearly demonstrating the species specificity of safener chemistries. Plant and cell uptake experiments demonstrated that both safeners behaved identically in rice and were equally bioavailable. These transcriptome studies demonstrated that whereas metcamifen caused the phased induction of hundreds of genes within 0.5 h of treatment, cyprosulfamide had a negligible effect on gene transcription over the 4 h study period.

The selective functional safening by metcamifen in rice was associated with a rapid and phased induction of genes. In previous studies with a chemical series based on the safener fenclorim (Skipsey et al., 2011), we had discovered evidence that safening was associated with the fast (within 20 min) induction of detoxifying enzymes such as GSTs, which we termed a rapid xenome response (RXR). This was in contrast to chemicals that induced a slow xenome response (SXR) determined over several hours that did not promote functional safening (Skipsey et al., 2011). Using RNA-seq analysis in rice cultures over a much shorter treatment window, we have now revealed the complexity of the RXR and its association with three ‘waves’ of gene induction to deliver effective safening with metcamifen. In contrast, cyprosulfamide was unable to elicit an RXR and did not protect the plants from injury by clodinafop.

The first wave of transcription in this study was both very rapid (<30 min) and mostly transient, which is consistent with the general properties of immediate–early genes (IEGs). IEGs

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**Table 4.** Perturbation of cellular function as defined by changes in transcripts clustered by Gene Ontology (GO) terms in rice cell cultures treated with 5 µM metcamifen over a 4 h period

| GO term category | Time point (h) | No. of gene sets |
|------------------|---------------|-----------------|
|                  | Up-regulated  | Down-regulated  |
| Biological processes | 0.5 | 33 | 1 |
|                   | 1.5 | 174 | 142 |
|                   | 4.0 | 255 | 114 |
| Molecular functions | 0.5 | 28 | 1 |
|                   | 1.5 | 128 | 87 |
|                   | 4.0 | 175 | 92 |
| Cellular components | 0.5 | 0 | 0 |
|                   | 1.5 | 16 | 43 |
|                   | 4.0 | 37 | 35 |

FDR Mixed <0.05

| No. of genes up-regulated by metcamifen |
|----------------------------------------|
| First wave | Second wave | Third wave | Other | Total |
| All genes  | 38 | 32 | 68 | 14 | 441 |
| Transcription factors | 8 | 25 | 3 | 3 | 39 |
| Xenome | 5 | 50 | 8 | 0 | 63 |
| Unknown function | 13 | 49 | 12 | 2 | 76 |

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**Table 5.** Summary of the number of transcription factors, xenome genes, and genes of unknown functions up-regulated by metcamifen during each of the induction waves shown in Fig. 6.
Phased gene induction in herbicide safening do not require protein synthesis and so their expression is not affected by translational inhibitors (Bahrami and Drabløs, 2016). Genes encoding TFs and proteins of unknown function were strongly represented in these IEGs. Representatives from all the major stress-inducible TFs were present, including members of...
the NAC, bZIP, MYB, and AP2/EREBP gene families (Shao et al., 2015). In contrast to the eight WRKYs perturbed by safener treatment in Arabidopsis (Behringer et al., 2011), this class of TFs were not induced by metcamifen in rice. Only one of the first-wave TFs, SNAC1 (STRESS-RESPONSIVE NAC1), has been characterized with respect to its function in rice as being linked to drought tolerance (Hu et al., 2006). A third of the early genes induced by metcamifen have not been ascribed any function in rice to date. A study of >1000 genes of unknown function in Arabidopsis revealed that the majority were responsive to at least one abiotic stress (Luihua et al., 2013), suggesting that many of the respective proteins must have protective roles. Other first-wave genes of interest included the tau GSTs OsGSTU21 and OsGSTU31, that would usually be classified as part of the xenome response. These two GSTUs are orthologues of the Arabidopsis AtGSTU17/ERD9, which is rapidly induced by dehydration (Kiyosue et al., 1994) and acts as a negative regulator of drought and salt tolerance by modulating glutathione levels (Chen et al., 2012).

Xenome genes encoding proteins involved in xenobiotic detoxification were highly represented in the second phase of induction (0.5–1.5 h). Comparison of the xenome genes induced in rice cells by fenclorim (Brazier-Hicks et al., 2018) revealed that while similar numbers of GSTs and UGTs were induced during the RXR, by both safeners, only half as many CYPs were up-regulated by metcamifen. While the most highly up-regulated GST, UGT, and CYP genes were induced by both chemistries, many of the wider responses within these xenome gene classes were chemistry specific. Such variation may help explain why some safener–herbicide partnerships are more successful than others based on their ability to enhance specific types of herbicide detoxification. Most of the induced xenome enzymes are of unknown function with regard to detoxification capability. One notable exception was the inducible CYP81A9, whose overexpression confers tolerance to the herbicides bentazon and bensulfuron in rice (Pan et al., 2006). As well as xenome genes, transcripts linked to glutathione metabolism/homeostasis and sulfur assimilation pathways were coordinately induced in this second wave. In addition to GSTs and glutaredoxins, glutathione reductase expression was also elevated, suggesting a need to regulate perturbations in the cell redox environment. Examination of the genes involved in S assimilation revealed that metcamifen treatment did not induce the classic sulfur starvation biomarkers, such as high-affinity sulfate transporters, seen with fenclorim treatment (Skipsy et al., 2011). Instead, metcamifen treatment resulted in up-regulation of a putative low-affinity transporter (LOC_03g09940), whose orthologues in Arabidopsis are responsible for long-distance translocation of sulfate in planta (Gigolashvilli and Kopriva, 2014). Additionally, an ATP sulfurylase (LOC_Os04g02050) that is responsive to a range of plant stresses and involved in the first committed step in S assimilation was also induced (Anjum et al., 2015), strongly suggesting a central role for this pathway in the safening response.

The final RXR wave of induction (at ~4 h) included two gene families, late embryogenesis abundant proteins (LEA) and peroxidoxins. LEAs are highly hydrophilic, intrinsically disordered proteins which have a stabilizing intracellular space-filling function (Candat et al., 2014), also functioning as molecular chaperones during oxidative stress (Kima et al., 2011). This suggests that this late-induced response to safeners is related to cellular stabilization.

Safening shows links to endogenous stress signalling

The results of this study also provide new insights into the relationship between the safener response and endogenous signalling. Gene sets related to phytohormone biosynthesis and related GO terms were up-regulated during the early stages of the RXR, suggesting that phytohormone signalling is involved in the cellular response to safeners. Similarly, several safener-inducible genes are also up-regulated by phytohormones. For example, in the current study, CYP709C9 was induced by both metcamifen and MeJA, with the orthologue CYP709C1 being similarly responsive to safeners and jasmonate in wheat (Kandel et al., 2005). This suggests a conserved response for this gene function across species. When examining other potential synergies between metcamifen and plant hormones, co-treatment with either ABA or MeJA resulted in an additive induction of safener biomarker genes, suggesting that the respective signalling events were exerted via different pathways. In contrast, metcamifen treatment gave no additive induction on the biomarkers above that seen with SA and H2O2 treatment, suggesting that the respective signalling pathways are shared. Studies in Arabidopsis using mutants found that the majority of safener-inducible genes were dependent on TGA TFs and/or SA (Behringer et al., 2011). Understanding how synthetic agrochemicals interact with multiple phytohormone signalling pathways offers a potentially powerful route to regulate important traits, such as herbicide sensitivity, which as illustrated in this study can then extend the applications for existing crop protection agents.

Supplementary data

Supplementary data are available at JXB online.

Table S1. Oligonucleotides used in real-time qPCR studies.

Table S2. Next-generation sequencing data derived from metcamifen-treated rice cultures in Excel spreadsheet format with genes identified by the LOC identifiers as referenced to http://rice.plantbiology.msu.edu/.

Table S3. Changes in cellular function following treatment of rice cell cultures with metcamifen as determined through the relative abundance of transcripts defined by their Gene Ontology terms.

Table S4. Changes in transcript abundance in rice cell cultures, 0.5, 1.5, and 4 h after treatment with metcamifen as clustered on the basis of their association with specific metabolic pathways as presented in Excel spreadsheet format.

Fig. S1. Time and dose dependence of gene induction in rice cell cultures following treatment with metcamifen.

Fig. S2. Bi-clustering of gene expression of transcripts identified in the first wave of induction following metcamifen treatment of rice cultures as compared with changes in these genes observed in 754 conditions detailed in the Genevestigator database.
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