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Selective disruption of wheat secondary metabolism by herbicide safeners

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Dedicated to Prof. Rodney Croteau on the occasion of his 60th birthday.

Abstract

In wheat (*Triticum aestivum* L.), treatment with herbicide safeners enhances the expression of enzymes involved in pesticide detoxification and reduces crop sensitivity to herbicides. Since these same enzymes are involved in plant secondary metabolism, it was of interest to determine whether or not the safener cloquintocet mexyl perturbed phenolic metabolism in wheat seedlings. LC/ESI/MS analysis identified 14 phenolic substrates in the shoots of young wheat plants. Fragmentation imposed by collision induced dissociation identified specific *C*-glycosidic conjugates of 4′,5,7-trihydroxflavone (apigenin), 3′,4′,5,7-tetrahydroxyflavone (luteolin) and 3′-*O*-methylluteolin. Treatment of 7-day-old wheat shoots with cloquintocet mexyl resulted in an accelerated depletion of the conjugates of all three flavones, most notably with the glycosides of luteolin. In contrast, safener treatment caused the selective accumulation of 4′,5,7-trihydroxy-3′,5′-dimethoxyflavone (tricin) and the phenylpropanoid ferulic acid. Changes in phenolic content were associated with an increase in *O*-methyltransferase and *C*-glucosyltransferase activity toward flavonoid substrates as well as the classic enhancement of detoxifying glutathione transferases. Our results suggest that in addition to altering the capacity of wheat to metabolise herbicides and other xenobiotics, safeners can also cause a selective shift in the metabolism of endogenous phenolics.

Keywords: Gramineae; *Triticum aestivum*; Flavonoids; *C*-Glucosyltransferase; *C*-Glycosides; Glutathione transferases; Herbicide metabolism; *O*-Methyltransferases; Phenylpropanoids

1. Introduction

Phenylpropanoid and derived flavonoid metabolites are ubiquitous phenolic secondary products in higher plants with diverse roles which include functioning as components of cell walls, antioxidants, antimicrobial agents, antifeedants and insect attractants (Schijlen et al., 2004). As such, the branches of phenylpropanoid and flavonoid metabolism are under complex regulation mediated by plant development and biotic/abiotic stress. At the molecular level, chemicals known to elicit changes in phenolic metabolism include plant hormones, stress signalling compounds and carbohydrate components of plant and fungal cell walls (Zhao et al., 2005). Flavonoids are also known to accumulate when plants are exposed to synthetic compounds such as diphenyl ether herbicides (Landini et al., 2003). Thus, a number of diphenyl ethers, notably lactofen, were shown to elicit isoflavone accumulation in soybean leaves through the generation of reactive oxygen species (ROS) initiating

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hypersensitivity and priming the plants for phytoalexin synthesis. While these effects on flavonoid metabolism are very interesting, the activity of these synthetic compounds on plant secondary metabolism is a secondary effect resulting from photo-oxidative damage and general ROS generation.

With a long-term interest in studying the effects of crop protection agents upon plant biotransformation reactions, we have become interested in the effect non-phytotoxic herbicide safeners have on secondary and primary metabolism. Safeners are used to enhance herbicide selectivity when applied to cereal crops due to their ability to increase the rates of herbicide detoxification in the crop following a co-ordinated up-regulation of xenobiotic detoxifying enzymes (Davies and Caseley, 1999). Safener-induced enzymes encompass all four phases of xenobiotic detoxification and include cytochrome P450 mixed function oxidases (CYPs), glutathione transferases (GSTs), type 1 glucosyltransferases (UGTs) and ATP-binding cassette (ABC) transporter proteins (Coleman et al., 1997). Intriguingly, since these many of these proteins have been recruited from endogenous secondary metabolism, perturbations in their expression may give rise to altered profiles of natural products, such as phenylpropanoids and flavonoids.

To examine this possibility, we have treated germinating wheat seedlings with and without the wheat safener cloquintocet mexyl (compound **15** see Fig. 1), which is used to reduce the toxicity of aryloxyphenoxypropionate (AOPP) herbicides in this crop (Kreuz et al., 1991). Young wheat seedlings were used as they are particularly sensitive to safener-treatment (Zhang and Riechers, 2004), with cloquintocet mexyl (**15**) known to increase the rates of hydroxylation, ether cleavage and glucosylation of the AOPP herbicide clodinafop propargyl in this crop (Kreuz et al., 1991). Profiles of phenolic metabolites have then been monitored following treatment with cloquintocet mexyl and individual phenylpropanoids and flavonoids identified and quantified. In addition, we have monitored changes in the activity of representative enzymes of flavonoid metabolism to rationalise the changes in phenolic metabolism observed.

2. Results

2.1. Identification of phenolic metabolites in wheat

Using a combination of two LC/MS systems coupled with photodiode array detection and electrospray ionisation and quadrupole or time-of flight analysers (systems 1 and 2, respectively) operating in positive and negative ion mode, a total of 14 phenolic metabolites could be identified in 7-day-old wheat shoots (Compounds **1**–**14**), together with 5 unknown UV absorbing metabolites (Fig. 1). On resolution the compounds were identified as luteolin

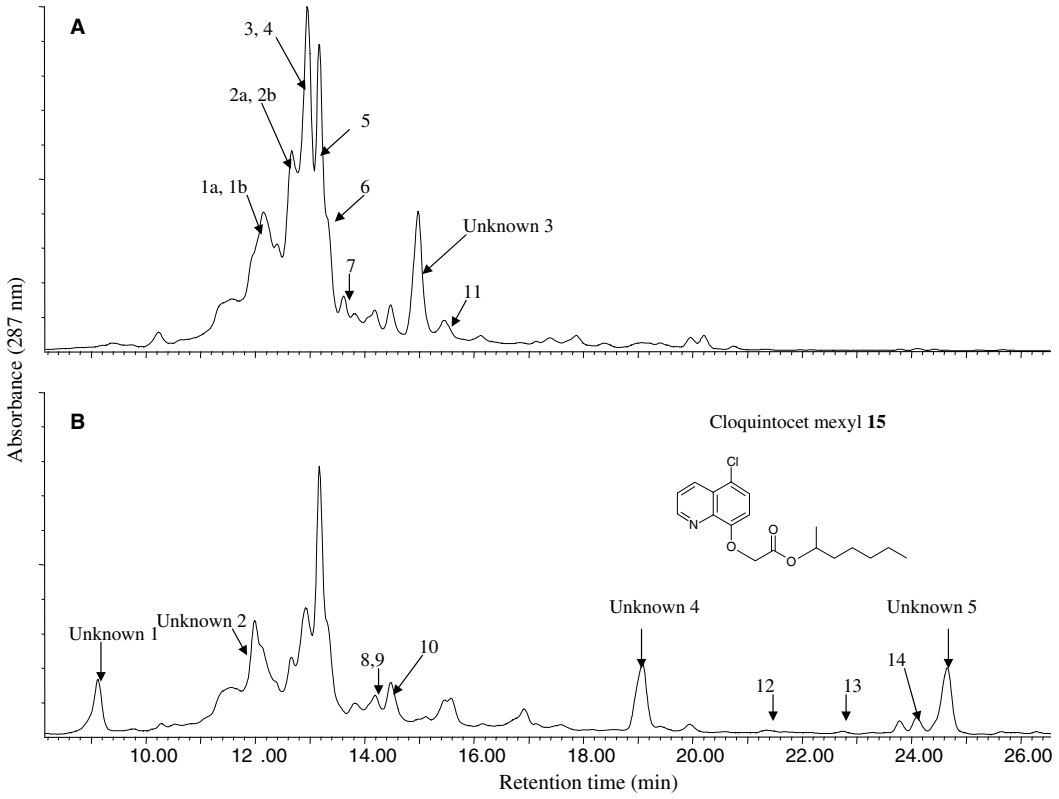


Fig. 1. HPLC (system 2) of UV-absorbing ($\lambda = 287$ nm) metabolites in wheat seedlings treated with (B) and without (A) cloquintocet mexyl for 4 days. Distinct metabolites were identified from their respective mass ions as detailed in Table 1. The structure of cloquintocet mexyl (compound **15**) is given for reference.

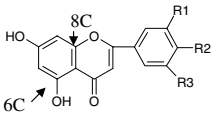
C-glucoside *C*-xyloside (cpd **1a**, **1b**), luteolin *C*-6-(2″-*O*-rhamnosyl) glucoside (**2a**), luteolin 6-*C*-glucosylglucoside (**2b**), apigenin 6-*C*-glucoside 8-*C*-xyloside (**3**), luteolin 6-*C*-glucoside (**4** and **7**), apigenin *C*-glucoside *C*-xyloside (**5** and **6)**, apigenin 6-*C* glycoside (**8)**, 3′-*O*-methylluteolin 6-*C*-glucosylglucoside (**9**), 3′-*O*-methylluteolin 6-*C*-(2″-*O*-rhamnosyl)-glucoside (**10**), 3′-*O*-methylluteolin 6-*C*-glucoside (**11**), ferulic acid (**12**) and a related derivative (**13**) and tricin (**14**). The MS of system 1 operating in both positive and negative ion mode was used to identify the flavonoid conjugates after imposing conditions favouring collision induced dissociation (CID) during ionisation (cone voltage increased up to 45 V) and comparing the fragmented ions obtained with those characterised previously (Li et al., 1991, 1992). For reference, these fragmented ions are listed in Table 1. Application of acid hydrolysis in methanol (methanolysis) to selectively cleave *O*-glycosidic bonds and CID-imposed fragmentation was found to be useful in distinguishing between *O*-linked and *C*-linked glycosides as well as determining the site of sugar conjugation (Li et al., 1991, 1992).

In the case of the flavonoids, although a large number of conjugates could be identified, these were based on a restricted range of flavones, namely 4′5,7-trihydroxflavone (apigenin), 3′,4′,5,7-tetrahydroxyflavone (luteolin) and 3′-*O*-methylluteolin (Table 1). In wheat, these flavones accumulate as their respective 6-*C* and/ or 8-*C* glycosidic conjugates. The 8-*C*-glucosides of apigenin, luteolin and 3′-*O*-methylluteolin are historically named as vitexin, orientin and scoparin, respectively, while the respective 6-*C*-glucosides are known as isovitexin, isoorientin and isoscoparin, respectively (Julian et al., 1971;Harborne et al., 1986). Several variations in the sugar substitutions of the three invariant flavones have been described for wheat in the literature (Julian et al., 1971; Michael et al., 1998; Estiarte et al., 1999). In many cases, differences in the analytical methods, or the lack of published spectra, have made direct comparisons with the flavonoids identified in the current study difficult. In common with previous studies, isovitexin, isoorientin and isoscoparin were identified. Perhaps the most interesting break with older studies is that the respective monoglucosylated 8-*C*-glucosides were not identified, nor were 6,8-*C*-diglucosyl-apigenin (vicenin) or 6,8-*C*-diglucosyl-luteolin (lucenin). Instead our data suggests that the 6,8-*C*-diglycoside conjugates contained glucose and xylose (Table 1). Using CID-imposed fragmentation and MS it was possible to identify the sites of conjugation of the respective sugars in several, but not all, cases. In the

Table 1
Metabolite identification in untreated wheat seedlings and following a 4 day treatment with cloquintocet mexyl **15**

Metabolite	Retention time (min) System 1	Retention time (min) System 2	Identity	λ max (nm)	$[M+H]^+$	Fragmentation 45V (System 1)
Unknown 1	–	9.1	–	267	–	–
Unknown 2	–	11.9	–	323	188.1	–
la	16.4	12.2	Luteolin *C*-glucoside *C*-xyloside	329	581.2	563, 545, 527, 497,
1b		12.4		329	581.2	485, 473, 461, 443, 425
2a	21.2	12.6	Luteolin *C*-6-(2″*O*-rhamnosyl) glucoside	330	595.2	–
2b	19.07	12.7	Luteolin 6-*C*-glucosylglucoside	335	611	449, 431, 329
3	20.4	12.85	Apigenin 6-*C*-glucoside 8-*C*-xyloside	335	565.2	547, 529, 511, 481, 469, 457, 445, 427, 409
4	21.2	13.0	Luteolin 6-*C*-glucoside	336	449	431, 413, 383, 353, 329, 299
5	–	13.06	Apigenin *C*-glucoside *C*-xyloside	336	565.2	–
6	–	13.2	Apigenin *C*-glucoside *C*-xyloside	334	565.2	–
7	–	13.6	Luteolin 6-*C*-glucoside	336	449	–
8	26.7	14.1	Apigenin 6-*C* glycoside	336	433.1	415, 397, 367, 337, 313, 283
9	25.34	14.2	3′-*O*-methylluteolin 6-*C*-glucosylglucoside	344	625	463, 445, 427, 343
10	27.36	14.5	3′-*O*-methylluteolin 6-*C*-(2″*O*-rhamnosyl)-glucoside	347	609	463, 445, 427, 343
11	28.2	15.5	3′-*O*-methylluteolin 6-*C*-glucoside	342	463	445, 427, 409, 367, 343,313
Unknown 3	–	14.9	–	266	207.1	–
Unknown 4	–	18.7	–	288	207.1	–
12	–	21.2	Ferulic acid	322	195.2	–
13	–	22.6	Ferulic acid derivative	322	195.2	–
14	–	24.0	Tricin	351	330.1	–
Unknown 5	–	24.6	–	265	241.2	–

The metabolites referred to correspond to the peak labelling in Fig. 1. For reference, the numbering system for the substitution of the flavones is shown in the footnote. If the site of sugar conjugation could not be defined by MS the metabolites are described as the respective un-numbered C-glycosides.



	R1	R2	R3	Compound
Apigenin	H	OH	H	**16**
Luteolin	OH	OH	H	**17**
3′ *O*-methyl-luteolin	OH	OCH_3	H	**18**
Tricin	OCH_3	OH	OCH_3	**14**

case of apigenin, the presence of two resolvable conjugates (compounds **5** and **6**) each with mass ions corresponding to the flavone *C*-linked to both xylose and glucose suggested that each sugar could substitute at either 6-*C*, or 8-*C* positions. In addition to single sugars being C-conjugated in each position, selective hydrolysis studies also demonstrated the presence of the 6-*C*-rhamnosyl–glucosides (compound **2a**) and 6-*C*-glucosyl-glucosides of luteolin (**2b**) and 3′-*O*-methylluteolin (**9**), respectively.

2.2. Changes in phenolic metabolite profiles on safener treatment

To study quantitative changes in secondary metabolite profiles, LC/MS system 2 was utilised. Wheat seedlings (7-day-old) were exposed to either 23 μM cloquintocet mexyl (compound **15**, see Fig. 1), or solvent carrier and the phenolic content determined at 0, 2, 4 and 7 days after treatment. As determined by monitoring the eluant for UV-absorbing aromatic compounds, safener treatment caused a major change in the content of phenolic compounds within 4 days of administration (Fig. 1). Due to the fact that several flavonoid conjugates co-chromatographed with one another, absolute quantification of individual metabolites was limited to those which migrated as defined UV-absorbing peaks containing a single entity as determined by MS. However, it was also possible to determine the relative abundance of co-eluting flavone-glycosides based on single ion monitoring (SIM) of the respective parent compounds. Based on a combination of quantification by UV absorbance and SIM, changes in the individual phenolic metabolites could be quantified over the course of the 7-day treatment (Table 2).

Analysis of the perturbation in flavones was much simplified by considering the fate of the aglycones (see Table 1), rather than that of the multiple conjugates of apigenin (compound **16**), luteolin (**17**) and 3′-*O*-methylluteolin (**18**) present. As the changes seen for the conjugates of each aglycone followed the same patterns over the course of the treatment, changes in the overall patterns of flavonoid metabolism were best considered by summing the total contents of each flavone (Table 2). In the control plants, the concentrations of the conjugates of apigenin (**16**) and 3′-*O*-methylluteolin (**18**) underwent a slight decline over the treatment period. In contrast, two days into the experiment, levels of the luteolin (**17**) metabolites dropped by approximately a third. Treatment with cloquintocet mexyl (**15**) drastically accelerated the disappearance of luteolin and an unknown compound, metabolite **3**. As

Table 2
Changes in the concentrations of phenolic metabolites in wheat shoots 0, 2, 4 and 7 days after ± treatment with the safener cloquintocet mexyl (**15**)

Metabolite	0	Untreated metabolite concentration (nmol g^{-1} fresh weight ± s.e. $n = 5$)			Safener-treated metabolite concentration (nmol g^{-1} fresh weight ± s.e. $n = 5$)		
		2 day	4 day	7 day	2 day	4 day	7 day
Unknown 1	Not detected	Not detected	Not detected	Not detected	36%	100%	41%
Unknown 2	0	12%	27%	14%	29%	100%	64%
1a,**b** Luteolin 6-*C* glycoside 8-*C* xyloside	128 ± 5	88 ± 4	78 ± 3	75 ± 3	95 ± 6	33 ± 2	17 ± 2
2a Luteolin *C*-6-(2″-*O*-rhamnosyl) glucoside + 2b Luteolin 6-*C*-glucosylglucoside	158 ± 9	150 ± 9	87 ± 4	79 ± 6	119 ± 12	29 ± 3	12 ± 2
3 + **5** + **6** Apigenin xylosyl glycoside	395 ± 9	362 ± 11	316 ± 15	258 ± 14	331 ± 10	252 ± 9	96 ± 4
4 Luteolin 6-*C*-glucoside	53 ± 3	47 ± 4	46 ± 4	40 ± 2	21 ± 3	6 ± 1	2 ± 0
7 Luteolin 6-*C*-glucoside	23 ± 4	14 ± 3	14 ± 3	14 ± 3	13 ± 2	Not detected	Not detected
8 Apigenin 6-*C* glycoside	14 ± 2	14 ± 2	11 ± 2	11 ± 1	11 ± 2	7 ± 2	3 ± 0
9 3′-*O*-methylluteolin 6-*C*-glucosylglucoside	17 ± 2	17 ± 1	17 ± 2	12 ± 2	17 ± 1	17 ± 2	8 ± 1
10 3′-*O*-methylluteolin 6-*C*—(2″-*O*-rhamnosyl)-glucoside	12 ± 2	12 ± 2	12 ± 2	12 ± 2	12 ± 2	12 ± 2	6 ± 1
11 3′-*O*-methylluteolin 6-*C*-glucoside	12 ± 1	19 ± 1	15 ± 3	12 ± 2	16 ± 1	15 ± 1	9 ± 1
Unknown 3	100%	47%	12%	21%	15%	Not detected	Not detected
Unknown 4	100%	24%	Not detected	Not detected	43%	47%	26%
12 + **13** Ferulic acid metabolites	2.0 ± 0	2.1 ± 0	2.1 ± 0.3	2.0 ± 0.1	6.1 ± 0.3	11.4 ± 0.4	6.0 ± 0
14 Tricin	1.1 ± 0.3	1.5 ± 0.3	1.1 ± 0.3	1.2 ± 0.2	1.4 ± 0.2	8.8 ± 0.5	7 ± 0.2
Unknown 5	Not detected	Not detected	Not detected	Not detected	9%	100%	42%
Total luteolin metabolites	362 ± 9	300 ± 9	225 ± 4	208 ± 6	247 ± 12	68 ± 3	31 ± 2
Total apigenin metabolites	409 ± 9	376 ± 11	327 ± 15	269 ± 14	342 ± 10	259 ± 9	99 ± 4
Total 3′-*O*-methylluteolin metabolites	41 ± 2	48 ± 2	44 ± 3	36 ± 2	45 ± 2	44 ± 2	23 ± 1

Total concentrations for each metabolite class (aglyca) are given at the foot of the table. Metabolite identities refer to those given in Table 1. Unknowns with no reference standard are shown as % of maximum peak area for that metabolite.

compared with the controls, within seven days of safener treatment, a significant depletion of conjugates of apigenin (**16**) and 3′-*O*-methylluteolin (**18**) was also observed. While the original major flavone conjugates were disappearing in response to safener treatment, the levels of 4,5,7-trihydoxy-3,5-dimethoxyflavone (tricin, compound **14**) and ferulic acid (**12**) increased (Table 2). In addition, three unknown (UN) compounds (metabolites UN1, UN2, and UN5) also accumulated. The total absence of UN1 and UN5 in the untreated wheat seedlings, combined with their simple UV spectra, suggested that these two compounds were most likely intermediates derived from the degradation of cloquintocet mexyl (**15**). In contrast, UN2 was most likely a simple natural phenolic, but could not be identified by reference to any available standard.

2.3. Changes in enzymes of secondary metabolism on safener-treatment

Treatment of wheat with cloquintocet mexyl (**15**) has previously been shown to enhance the expression of GSTs as well as elevate *O*-glucosyltransferase (OGT) activity toward the flavonol quercetin and the flavone luteolin (Brazier et al., 2002). The results of the flavonoid profiling studies suggested that the effects of safeners on enzymes of plant secondary metabolism might be more widespread. Western blotting studies using antisera raised to phi class GST polypeptides active in xenobiotic detoxification (data not shown), demonstrated an enhancement in GST expression after exposure to cloquintocet mexyl (**15**) essentially as reported previously (Brazier et al., 2002). GST activity toward the model substrate 1-chloro-2,4-dinitrobenzene was increased 2.5-fold within 2 days of cloquintocet mexyl (**15**) treatment (Table 3). Enzymes of phenylpropanoid and flavonoid biosynthesis selected for assay were phenylalanine ammonium lyase (PAL), *O*-methyltransferases (OMTs) active toward caffeic acid and luteolin and *C*-glucosyltransferase (CGT) and OGT activity directed toward 2-hydroxyflavanone and flavone substrates, respectively. While CGT and OGT assays were carried out using a published radioassay (Brazier et al., 2002), OMT activities had to be determined without radioisotopes using a novel HPLC-based method (Fig. 2). In each case, the identity of the methoxylated product was confirmed by ESI-ToF MS. The results of these studies showed that 4 days after safener treatment CGT, OGT and OMT activities all underwent a significant enhancement, while PAL activity was unaffected (Table 3).

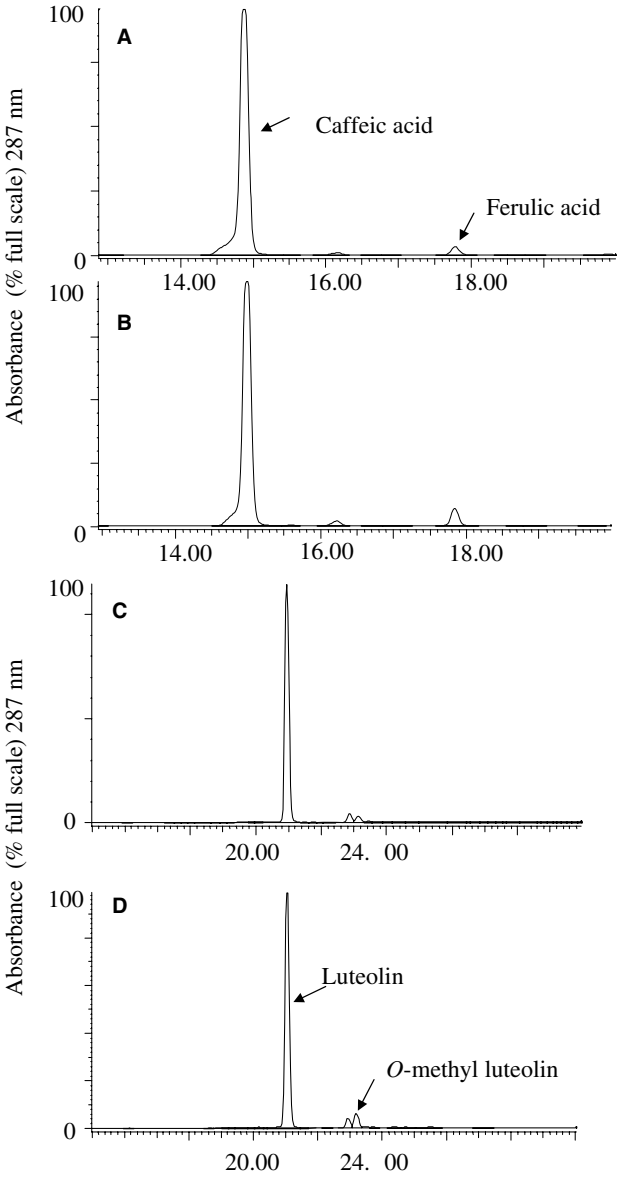


Fig. 2. HPLC analysis of reaction products formed from incubating *S*-adenosyl methionine with caffeic acid (A, B) and luteolin (C, D) using protein extracts derived from untreated wheat plants (A, C) or seedlings treated for 4 days with the safener cloquintocet mexyl (B, D). Reaction products (ferulic acid, **12**, and methylluteolin) were identified by ESI-ToF MS.

Table 3
Enzyme activity in extracts from untreated and cloquintocet mexyl (**15**)-treated wheat following a 4 day exposure

	Control shoot	Safener-treated shoot
GST activity toward 1-chloro-2,4-dinitrobenzene (nkat mg^{-1} protein)	0.6 ± 0.02	1.5 ± 0.02
Caffeic acid *O*-methyltransferase activity (pkat mg^{-1} protein)	1.0 ± 0.04	2.1 ± 0.01
Luteolin *O*-methyltransferase activity (pkat mg^{-1} protein)	0.42 ± 0.02	0.71 ± 0.03
O-glucosyltransferase activity toward luteolin (fkat mg^{-1} protein)	0.88 ± 0.05	4.00 ± 0.56
C-glucosyltransferase activity toward 2-hydroxynaringenin (fkat mg^{-1} protein)	3.64 ± 0.03	6.76 ± 0.39
Phenylalanine ammonia lyase activity (pkat mg^{-1} protein)	14.5 ± 0.5	12.0 ± 0.03

Results refer to mean ± s.e.($n = 3$).

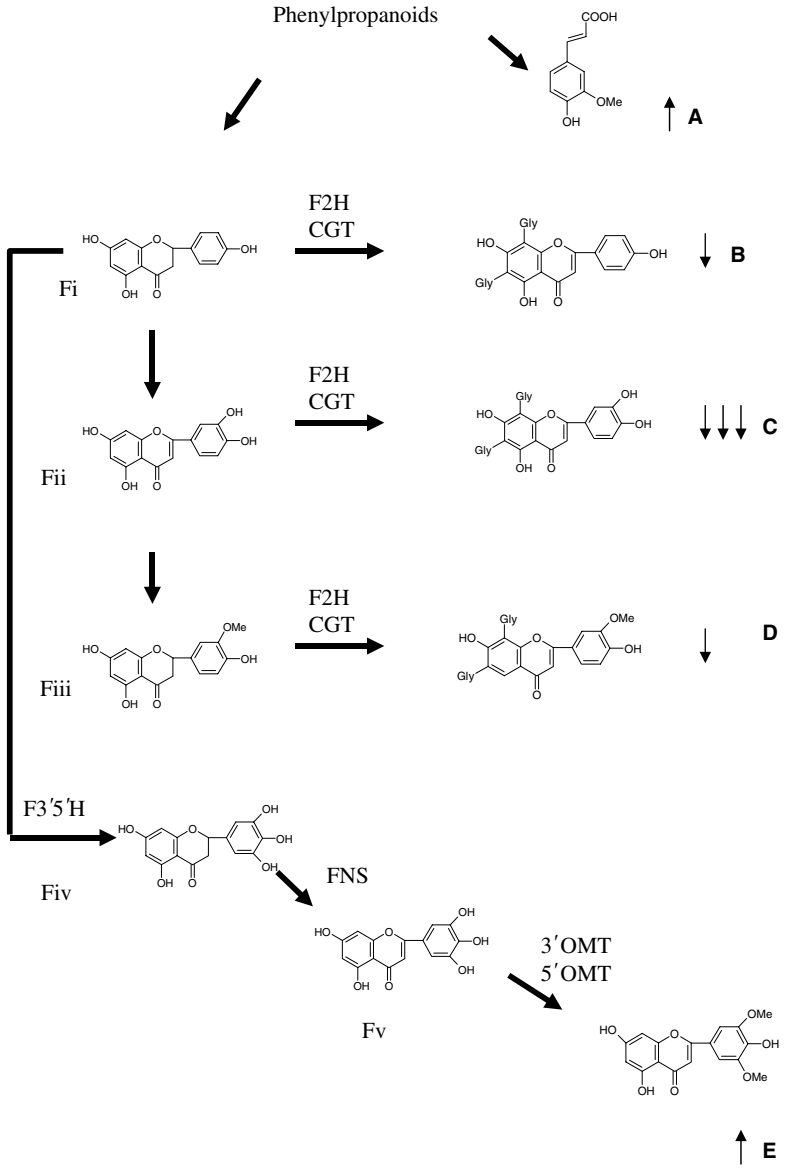


Fig. 3. Proposed biosynthesis of major phenylpropanoid (A = ferulic acid, compound **12**) and flavonoid (B = *C*-glycosides of apigenin, compound **16**; C = *C*-glycosides of luteolin, compound **17**; D = *C*-glycosides of 3′-*O*-methylluteolin, compound **18**; E = tricin, compound **14**) metabolites identified in wheat with the effect on relative concentrations following treatment with the safener cloquintocet mexyl (**15**) shown using arrows. The pathway is based on known biosynthetic activities in higher plants and is modelled on the observed changes seen on safener treatment. Synthesis of the *C*-glycosylated flavones proceeds via the flavanone intermediates Fi (4′,6,8-trihydroxyflavanone), Fii (3′,4′,6,8-tetrahydroxyflavanone) and Fiii (4′,6,8-trihydroxy-3′-methoxyflavanone) through the action of flavanone 3′hydroxylase (F3′H) and the 3′-*O*-methyltransferase (3′-OMT), respectively. The respective flavanone (Fi, Fii, Fiii) intermediates are then acted on by flavanone 2-hydroxylase (F2H) followed by *C*-glycosyltransferases (CGTs) acting on positions 6 and 8, with dehydration of the glycosylated 2-hydroxyflavanone intermediates resulting in the formation of the flavone *C*-glycosides described in Table 1 . Biosynthesis of tricin (E) is proposed to involve the formation of Fiv (3′,4′,5′,5,7-pentahydroxyflavanone) following the action of flavanone 3′,5′-hydroxylase (F3′,5′H) on Fi. Fiv is then converted to the 3′,4′,5′,5,7-pentahydroxyflavone by flavone synthase (FNS) which is then sequentially methylated by a 3-OMT and a 5-OMT to give tricin (E, compound **14**).

3. Discussion

Our results demonstrate that post-germination applications of the safener cloquintocet mexyl cause a depletion in the content of flavone *C*-glycosides in growing wheat seedlings, especially in the case of the conjugates of luteolin. In contrast, safener treatment caused a selective accumulation of ferulic acid (**12**) and tricin (**14**). At a simplistic level, the safener appears to be effecting the same accelerated degradation of endogenous flavone *C*-glycosides as is seen with herbicides and their metabolites (Davies and Caseley, 1999). However, it is also clear that cloquintocet mexyl (**15**) is also causing an enhanced production of ferulic acid (**12**) and tricin (**1**), suggesting that in addition to enhancing turnover, the safener is also redirecting phenolic metabolism. To understand further what

was happening, it was necessary to examine the enzymology of the flavonoid pathway in wheat. Based on the published literature from other plants, it is possible to propose how flavonoid synthesis in wheat is organised (Fig. 3). The central precursor to the pathway would be 4′,5,7-trihydroxyflavanone, with the derived major flavonoids apigenin (**16**), luteolin (**17**) and 3′-*O*-methylluteolin (**18**) accumulating only as their respective *C*-glycosides. By inference from what is known in buck-wheat (*Fagopyrum esculentum*), all these *C*-glycosides are derived from the respective activated 2-hydroxyflavanone intermediates (Kerscher and Franz, 1987). Interestingly, our results suggest that whereas *C*-glucosylation predominates at the 6 position, 8-*C* conjugation utilises xylose. Based on this differential substitution with sugars, this suggests that either a single CGT with dual glycosylating activities, or two distinct CGTs must be responsible for synthesising the wheat di-*C*-glycosides. By analogy with what occurs in licorice, following *C*-glycosylation, the respective flavones are then formed from the dehydration of the 2-hydroxyflavanone (Akashi et al., 1998). In the reaction scheme shown, it is assumed that for each *C*-glycosylated flavone, hydroxylations and methylations proceed at the level of the flavanone precursor by inference from what is known of the respective enzyme activities in other crop plants (Schijlen et al., 2004). Flavanone 3′-hydroxylase (F3′H) acts upon 4′,5,7-trihydroxyflavanone to produce the luteolin precursor, which would then undergo 3′-*O*-methylation to prime 3′*O*-methylluteolin (**18**) synthesis. While these structural elaborations have been described for flavanone substrates (Schijlen et al., 2004), the respective reactions would be unlikely to occur following *C*-glycosylation. The presence of tricin (**19**), suggests that in addition to F3′H, wheat probably also contains the CYP flavanone 3′,5′ hydoxylase (F3′,5′H). Intriguingly, wheat does not accumulate *C*-glycosides of tricin (**19**) and so its formation as the flavone presumably proceeds via a conventional flavone synthase. In the case of tricin (**19**), methylation is more likely to proceed after conversion to the flavones, since OMTs which methylate flavones at the 3′ and 5′ positions in plants have been described (Gauthier et al., 1998).

Having proposed a metabolic pathway for the wheat flavonoids, it is then possible to explain the observed results with the safener. In untreated plants, over the 7 day period, the total concentration of luteolin (**17**) was reduced by almost 50%, while apigenin (**16**) and 3′-*O*-methylluteolin (**18**) underwent a more modest decline. This suggests that synthesis of the *C*-glycosides during this time is limited and that turnover of these compounds to produce either insoluble, or non-UV absorbing, metabolites is occurring in the order luteolin (**17**)> apigenin (**16**)= 3′-*O*-methylluteolin (**18**). The effect of the safener is to accelerate this metabolism and hasten the disappearance of all three flavones. It is also possible that the safener is acting to suppress the formation of the *C*-glycosides, though the up-regulation of the respective CGT activity would argue against this. As well as enhancing the endogenous catabolism of the *C*-glycosides, the safener causes an accumulation of ferulic acid (**12**) and tricin (**19**) and this is associated with an enhanced expression of OMT activity toward both caffeic acid and luteolin. This methylation is presumably occurring at the 3-position and significantly, a 3-OMT from *Chrysosplenium americanum* has been shown to catalyse the methylation of both caffeic acid and luteolin (Gauthier et al., 1998). This suggests that the induction in OMT activity observed may be due to a single enzyme and this would help explain why both the methylated flavone tricin (**14**) and ferulic acid (**12**) co-accumulated in the cloquintocet mexyl-treated shoots. Consistent with this model, in a recent study with the wheat progenitor *Triticum tauschii*, a protein tentatively identified by proteomics as caffeic acid 3-*O*-methyltransferase was selectively induced in the coleoptiles following treatment with the safener fluxofenim (Zhang and Riechers, 2004). Based on the proposed pathway (Fig. 3), we might also expect F3′,5′H and the flavone 5′OMT to be similarly induced. Safeners have been shown to induce several CYPs of unknown endogenous function in wheat (Davies and Caseley, 1999). Perhaps one of these safener-inducible enzymes is an F3′,5′H.

This study has thrown new light on the synthesis of flavonoids in wheat and the role of safeners in regulating their synthesis and degradation. In our studies, treatment with the safener did not cause any identifiable stress and in fact we have even noted a growth promoting effect with these compounds in young wheat seedlings (unpublished data). This would suggest that the enhanced disappearance of flavonoids following treatment with cloquintocet mexyl (**15**) is not due to cell death or premature senescence, which is known to cause a decline in flavone *C*-glycosides in wheat (Penuelas et al., 1999). The catabolic pathways responsible for depleting these natural products are unknown and it is intriguing that they respond to safeners in a similar manner to that determined for the enhanced conjugation and vacuolar deposition pathways involved in pesticide metabolism (Coleman et al., 1997). Of similar significance, the selective safener-induced accumulation of a methylated phenylpropanoid and flavone also demonstrates that these chemicals can enhance specific branches of phenolic metabolism. With the exception of synthetic analogues of plant hormones and signalling agents, there are few reports of xenobiotics selectively perturbing flavonoid metabolism in plants. The herbicide lactofen invokes a major increase in the accumulation of antifungal isoflavones in soybean (Landini et al., 2003). However, this appears to be a result of the herbicide-generating ROS which mimic the early stages of the hypersensitive response and prime the plant for phytoalexin production. In contrast, modern safeners such as cloquintocet mexyl (**15**) cause no discernable oxidative damage to plants (Davies and Caseley, 1999). Current studies are now directed toward understanding the significance of selective changes in plant phenolic metabolism invoked by herbicide safeners in cereals.

4. Experimental

4.1. Plant treatments

Cloquintocet mexyl (**15**) was obtained from Riedel de Haën and prepared as a 23 mM solution in acetone. Wheat (*Triticum aestivum* cv. Hunter) was obtained and grown in moist vermiculite as described previously (Cummins et al., 1997). Plants were grown for 7 days (shoot length 8 cm) and then watered with cloquintocet mexyl (23 μM in 0.1% v/v acetone) or with 0.1% v/v acetone alone. Application rates for the safener were chosen based on those used in earlier studies, where 10 μM cloquintocet mexyl (**15**) was administered directly into the cut stems of wheat plants (Kreuz et al., 1991). Shoot tissue samples (2 g, $n = 5$) were harvested by cutting at ground level up to 7 day post-treatment. Plant tissue was frozen in liquid nitrogen and stored at −80 °C until required.

4.2. Secondary metabolite extraction and analysis

Frozen plant tissue (2 g) was homogenised with a mortar and pestle using acid washed sand as an abrasive and extracted with ice-cold MeOH (6 ml). The slurry was centrifuged (4000*g* 10 min, 4 °C) and the pellet then re-extracted with cold MeOH (6 ml) and re-centrifuged. Methanol extracts were combined, dried under reduced pressure at 40 °C and re-suspended in 2 ml MeOH prior to storage at −20 °C.

Two LC-MS procedures were employed to characterise phenolic wheat metabolites, with system 1 being used to induce fragmentation of the flavone conjugates for structural elucidation before and after methanolysis and system 2 being used for quantification of the intact flavone conjugates.

4.2.1. System 1

Wheat extracts (5 μl) were injected onto a Supersphere C18 column (250 × 2 mm i.d.; 4 μm particle size. Merck, Darmstadt, Germany) at a flow rate of 0.2 ml min^{-1} using a Waters 2690 HPLC pump. The column was eluted with a mixture of solvent A (H_2O:MeCN:AcOH 95:4.5:0.5 v/v) and solvent B (MeCN:H_2O:AcOH 95:4.5:0.5 v/v) using a series of linear gradients 0–5 min 10% B, 5–40 min 10%–30% B, 40–48 min 30%–100% B. The eluant was passed through a Waters 996 diode array detector prior to entry into a Micromass (Manchester UK) ZQ mass spectrometer after electrospray ionisation (ESI). The ESI source potentials were set at capillary 3 kV, lens 0.5 kV, extractor 4 V. Under standard ESI conditions the cone voltage was set at 30 kV, while for low energy collision-induced dissociation (CID) studies the voltage was increased to 45 kV. The source temperature was maintained at 120 °C with desolvation at 300 °C, using nitrogen as the nebulizing and desolvation gas. As required, flavonoid glycosides were methanolysed with anhydrous methanol (1 ml) containing 85 μl of acetyl chloride at 100 °C for 60 min prior to analysis. Peaks of interest were identified by mass ion data and spectral scan analysis before and after methanolysis as required.

4.2.2. System 2

Wheat extracts (50 μl) were injected onto a Synergi Polar column (250 mm × 2 mm i.d., 5 μm particle size. Phenonemex, UK) in a mobile phase comprising solvent A (H_2O:HCO_2H 99.5:0.5 v/v) and solvent B (MeCN:HCO_2H 99.5:0.5 v/v) in a ratio of 9:1 at 0.2 ml min^{-1}. After 2 min at 10% B linear gradients of 2–7 min 10–20% B, 7–27 min 20–60% and 27–42 min 60–100% B. The column eluate was equally divided with one path passing through a Waters 996 diode array detector ($\lambda = 220$–600 nm) and the other into a Micromass LCT time-of-flight (ToF) mass spectrometer (Micromass, Manchester) after ESI. Samples were analysed in both positive and negative ion modes at a cone voltage of 30 V. For quantification, chromatograms were displayed at 287 nm and peaks of interest integrated using apigenin as the reference flavone and ferulic acid for the phenylpropanoids. Where it was not possible to identify unknown compounds, changes in their relative abundance were recorded as the % of the absorbance at 287 nm, relative to the maximum determined for each component over the course of the experiment.

4.2.3. Enzyme analysis

Frozen shoot tissue was ground to powder using a mortar and pestle and extracted in 5 v/w 100 mM Tris, pH 7.2 containing 2 mM dithiothreitol (DTT). Proteins were precipitated by the addition of $(NH_4)_2SO_4$ to 80% saturation and desalted by gel filtration using Sephadex G25 columns prior to assay and protein content determined using dye-binding reagent (BioRad) with γ-globulin as a reference standard. GST activity toward 1-chloro-2,4-dinitrobenezene (CDNB) was measured spectrophotometrically (Brazier et al., 2002). Phenylalanine ammonium lyase (PAL) activity was determined using L-phenylalanine as substrate, and D-phenylalanine as a control as described (Edwards and Kessman, 1992).

CGT and OGT activity were determined using 2-hydroxynaringenin (2,5,7-trihydroxyflavanone) and luteolin, respectively. The 2-hydroxynaringenin was prepared from the flavone chrysin (Kerscher and Franz, 1987), while luteolin (**17**) was purchased from Apin Chemicals, Oxon. Crude protein (125 μg) in 20 mM Tris–HCl, pH 8.0, 2 mM DTT was incubated with flavonoid substrate (66 μM) at 30 °C. The reaction was initiated by the addition of UDP-[^{14}C-glucose] (100,000 dpm, 304 mCi $mmol^{-1}$, Amersham Biosciences) and incubated for 20 min. Reactions were stopped with 250 μl 0.3 M HCl and partitioned with 200 μl of water-saturated EtOAc. The organic phase was radioassayed directly by liquid scintillation counting and OGT activity determined after correcting for controls (Brazier et al., 2002). To determine CGT activity the organic phase was dried down under nitrogen, re-suspended in 500 μl of 0.7 M HCl and heated at 100 °C for 1 h and then re-partitioned with EtOAc and radioassayed as for OGT activity. For OMT determinations,

protein (500 μg) was incubated with 1 mM luteolin or 1 mM caffeic acid and 1 mM *S*-adenosylmethionine in extraction buffer for 30 min at 30 °C. The reaction was stopped by the addition of 1 vol of MeOH and reaction products analysed by LC-MS using a Synergi Polar column (Phenonemex, 250×4.5 mm) eluted at 1 ml min^{-1} using the mobile phase conditions described for system 2. Retention times for substrates and products with mass ions were caffeic acid, 14.9 min, $[M+H]^+$ 181.2; ferulic acid, 17.8 min, $[M+H]^+$ 195.2; luteolin, 21.0 min, $[M+H]^+$ 287.4; *O*-methylluteolin, 23.1 min, $[M+H]^+$ 301.4. Control reactions used heat-denatured protein in place of active enzyme.

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