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2,4-D and dicamba resistance mechanisms in wild radish: subtle, complex and population specific?

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• **Background and Aims** Resistance to synthetic auxin herbicides such as 2,4-dichlorophenoxyacetic acid (2,4-D) is increasing in weed populations worldwide, which is of concern given the recent introduction of synthetic auxin-resistant transgenic crops. Due to the complex mode of action of the auxinic herbicides, the mechanisms of evolved resistance remain largely uncharacterized. The aims of this study were to assess the level of diversity in resistance mechanisms in 11 populations of the problem weed *Raphanus raphanistrum*, and to use a high-throughput, whole-genome transcriptomic analysis on one resistant and one susceptible population to identify important changes in gene expression in response to 2,4-D.
• **Methods** Levels of 2,4-D and dicamba (3,6-dichloro-2-methoxybenzoic acid) resistance were quantified in a dose–response study and the populations were further screened for auxin selectivity, 2,4-D translocation and metabolism, expression of key 2,4-D-responsive genes and activation of the mitogen-activated proein kinase (MAPK) pathway. Potential links between resistance levels and mechanisms were assessed using correlation analysis.
• **Key Results** The transcriptomic study revealed early deployment of the plant defence response in the 2,4-D-treated resistant population, and there was a corresponding positive relationship between auxinic herbicide resistance and constitutive MAPK phosphorylation across all populations. Populations with shoot-wide translocation of 2,4-D had similar resistance levels to those with restricted translocation, suggesting that reduced translocation may not be as strong a resistance mechanism as originally thought. Differences in auxin selectivity between populations point to the likelihood of different resistance-conferring alterations in auxin signalling and/or perception in the different populations.
• **Conclusions** 2,4-D resistance in wild radish appears to result from subtly different auxin signalling alterations in different populations, supplemented by an enhanced defence response and, in some cases, reduced 2,4-D translocation. This study highlights the dangers of applying knowledge generated from a few populations of a weed species to the species as a whole.

Key words: 2,4-Dichlorophenoxyacetic acid, auxin, dicamba, plant defence, *Raphanus raphanistrum*, reduced translocation, resistance, RNaseq, signalling, wild radish.

INTRODUCTION

The auxinic herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) has been used to control dicotyledonous weeds in the USA since 1945 (Peterson *et al.*, 2016) and in the grain belt of Australia since the late 1940s (Radcliffe, 2002). The rate of evolution of resistance to synthetic auxin herbicides in weeds has been slow in relation to other commonly used post-emergence herbicides, but has increased in recent years due to heavier reliance on the synthetic auxins in response to high levels of resistance to other herbicides (e.g. Bernards *et al.*, 2012; Owen *et al.*, 2015). The recent release of transgenic crops resistant to 2,4-D or the synthetic auxin dicamba (3,6-dichloro-2-methoxybenzoic acid) means that auxinic herbicide use is likely to increase further in the coming years, and could cause the rate of resistance evolution in weeds to accelerate unless the transgenic crops are carefully managed.

In parallel to the slow rate of resistance evolution to synthetic auxins, research on the mechanisms of weed resistance to these herbicides has also lagged, because their mode of action is complex and has only recently been satisfactorily explained (reviewed in Grossmann, 2010; Peterson *et al.*, 2016). As the 'target site' of auxinic herbicides can be considered as comprising a large complex of auxin-binding proteins, transcriptional repressors and activators, ubiquitin-related proteins and proteasomes (reviewed in Dinesh *et al.*, 2016), most research into evolved resistance has identified non-target site-based resistance mechanisms such as reduced foliar uptake (e.g. *Glechoma hederacea*, Kohler *et al.*, 2004) or translocation (e.g. *Lactuca serriola*, Riar *et al.*, 2011;

Raphanus raphanistrum, Goggin *et al.*, 2016) of the herbicide, or its metabolic detoxification (e.g. *Carduus nutans*, Harrington and Woolley, 2006; *Papaver rhoeas*, Torra *et al.*, 2017). Very recently, the first target site mutation conferring auxinic herbicide resistance in a weed species was identified in the conserved domain II region of the auxin co-receptor IAA16 in dicamba-resistant *Kochia scoparia* (LeClere *et al.*, 2018). There was also evidence that the mutation caused cross-resistance to 2,4-D but not to the natural auxin indole-3-acetic acid (IAA) or the synthetic 1-naphthyleneacetic acid (NAA) (LeClere *et al.*, 2018). Studies on arabidopsis mutants have illustrated how mutations in different components of the auxin receptor and signalling complex can cause different changes in auxin cross-reactivity. In contrast to the situation in *K. scoparia*, a mutation in domain II of arabidopsis IAA16 resulted in resistance to IAA, NAA and 2,4-D (Rinaldi *et al.*, 2012). A mutation in SMAP1, a protein which modulates the ubiquitin ligase complex, conferred resistance to 2,4-D but not to IAA or NAA (Rahman *et al.*, 2006), and the well-known work of Walsh *et al.* (2006) showed that a mutation in the auxin receptor AFB5 led to plants that were resistant to picloram but not to IAA, NAA, 2,4-D or dicamba. In tomato, overexpression of *IAA15* caused greater sensitivity to IAA but not to 2,4-D (Xu *et al.*, 2015). Overall, the variable affinity of receptor complex components for different classes of auxins (Lee *et al.*, 2014), the different auxin selectivity conferred by specific mutations and the likely differences between plant species add up to a very complex situation when attempting to characterize target site resistance to auxinic herbicides.

Raphanus raphanistrum (wild radish) is a major dicotyledonous weed of southern Australian cropping systems and poses a significant problem for crop production in terms of its competition with crops, its ability to host crop pests and pathogens, and its persistent soil seed bank (reviewed in Owen *et al.*, 2015). In the most recent (2010) large-scale random survey of 2,4-D resistance in Western Australian wild radish populations, 76 % of populations contained resistant plants, a substantial increase from the 60 % recorded in the 2003 survey (Owen *et al.*, 2015). Two of the populations identified in the 2010 survey were studied for their mechanism of 2,4-D resistance and displayed greatly reduced translocation of the herbicide out of the treated leaf, which was hypothesized to be due to the loss of function of an ABCB-type auxin efflux transporter (Goggin *et al.*, 2016). There were also indications that alterations in auxin perception and/or signalling contributed to resistance, but it is unknown whether this is due to the defect in auxin transport, or a separate mechanism.

In theory, a selection pressure such as repeated herbicide application will enable all possible resistance mechanisms to evolve in the populations subjected to that selection pressure. Different mechanisms often require different strategies for successful management of the resistant populations. Therefore, to gain knowledge of the level of diversity of evolved 2,4-D resistance mechanisms in wild radish, two studies were carried out in parallel: a genome-wide transcriptomic comparison of the response to 2,4-D in a susceptible and a resistant population; and a screening of a further nine resistant populations collected in the 2010 survey for their level of 2,4-D resistance, auxin cross-resistance, and biochemical and physiological mechanism(s) of resistance. Expression of certain genes of interest identified in the transcriptomic study was also evaluated in the additional populations.

MATERIALS AND METHODS

Plant material

One hundred individuals from each of nine wild radish populations identified as 2,4-D resistant in the 2010 survey of the Western Australian grain belt (Owen *et al.*, 2015) were twice selected at the recommended field rate of 2,4-D (500 g ha^{-1}) as described in Goggin *et al.* (2016), in order to increase the homogeneity of each population. These were designated as populations R3–R11. The 2,4-D-susceptible (S1) and -resistant (R1 and R2) populations characterized in Goggin *et al.* (2016) and an additional herbicide-susceptible population from Carnac Island, Western Australia (Ashworth *et al.*, 2014), designated here as S2, were also included in the study. Production of the twice-selected populations R3–R11 commenced in April 2014 and was completed in October 2016. The different populations were isolated from one another in time and space during flowering to avoid cross-pollination between populations. A third round of selection was also performed on population R1. Unless otherwise indicated, the twice-selected R populations were used for all experiments. The original location of each population is shown on the map in Fig. 1.

An additional six populations were produced in 2016 by making three paired crosses between (twice-selected) R3 and R5 individuals. The parents were chosen based on their 2,4-D translocation patterns (see the Results), with R3 retaining almost all applied 2,4-D in the treated leaf and R5 translocating the herbicide around the entire plant. The three pairs of parent plants were kept separate from each other in glasshouses, pollinated by hand using a small paint brush, and seeds were collected separately from each parent plant. The F_1 populations were designated as N1, N2 and N3 (maternal parent: Non-translocator), and T1, T2 and T3 (maternal parent: Translocator), and their 2,4-D resistance levels and translocation patterns were assessed.

Herbicide spray treatments for dose–response studies

Seedlings of all generations (three or six replicates, depending on seed numbers; ten seedlings per replicate) were grown outdoors in plastic seedling trays containing potting mix (50 % composted pine bark, 25 % peat moss, 25 % river sand) during May–June 2017. Seedlings at the two- to three-leaf stage were sprayed with formulated 2,4-D amine (Amicide Advance 700) or dicamba (Kamba 500) (Nufarm, Laverton, Australia) at rates of 0, 125, 250, 500, 1000, 2000 and 4000 g ae ha^{-1} using a cabinet sprayer (Owen *et al.*, 2015), and plants were grown outdoors for a further 21 d with regular watering and fertilization. Individuals were considered to have survived if they possessed new, symptomless growth at the time of assessment. Above-ground biomass was harvested from the surviving plants, dried at 70 °C for 3 d, and weighed. For this experiment, the different generations of each population are distinguished with the use of subscripts, e.g. $R1_1$, $R1_2$, $R1_3$ for the once-, twice- and thrice-selected generations of R1.

Root elongation assays

Root elongation assays were performed on agar as described in Goggin *et al.* (2016), using 2,4-D and dicamba concentrations

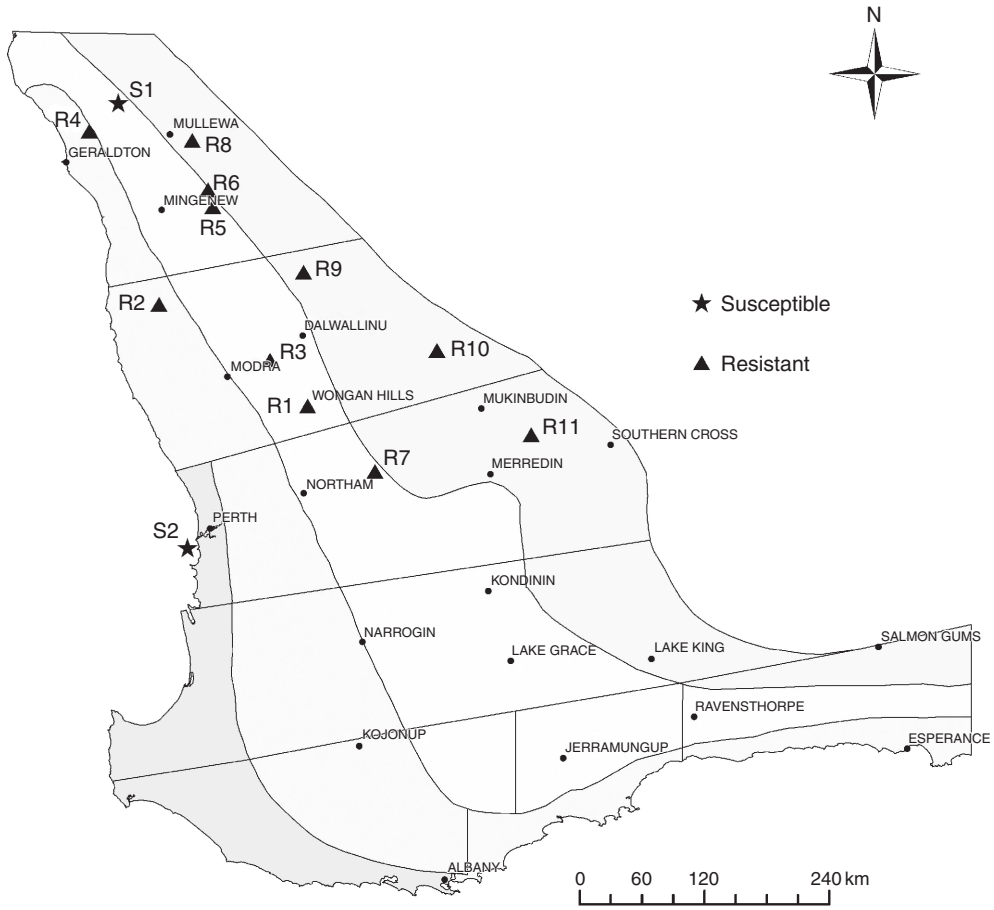

FIG. 1. Map of the Western Australian grain belt showing the locations from which the original 2,4-D-susceptible and -resistant wild radish populations were collected.

of 0, 0.01, 0.1, 0.5, 1, 10 and 50 μM, and IAA and NAA concentrations of 0, 0.01, 0.1, 0.5 and 1 μM. Three replicates of 3–5 seedlings were measured for each population at each auxin dose. Populations S1, R1 and R2, assayed in Goggin *et al.* (2016), were re-assayed alongside the other populations in the current study to allow direct comparisons between dose–response curves. Due to low seed numbers, the paired-cross populations were not included in this study.

2,4-D uptake, translocation and metabolism assays

Seedlings at the two- to three-leaf stage were used to assay uptake, translocation and metabolism of leaf-applied ^{14}C-labelled 2,4-D (ring ^{14}C [U] with a specific activity of 2.035 GBq mmol^{-1}; American Rabiolabeled Chemicals, St Louis, MO, USA) as described in Goggin *et al.* (2016). Twelve plants from each population were used for each of the translocation and metabolism assays, with the leaf and root washes from the translocation experiments used to assess leaf uptake and root secretion of [^{14}C]2,4-D.

Transcriptomics study

S1 and R1 plants were grown in seedling trays and maintained in a growth cabinet at 20/15 °C [12 h photoperiod of 200 μmol m^{-2} s^{-1} white light-emitting diode (LED) and incandescent light]. Seedlings at the three- to four-leaf stage were sprayed with 500 g ae ha^{-1} of formulated 2,4-D amine and then returned to the growth cabinet. The formulated herbicide, which could potentially elicit transcriptional changes due to its surfactants and adjuvants, was used in this study to reflect the selection pressure experienced by plants in the field (Duhoux *et al.*, 2015). Similarly, control plants were left untreated, representative of field conditions. Leaf tissue was harvested from treated and untreated plants at 2, 8 and 24 h after spraying, snap-frozen in liquid N_2 and stored at –80 °C. Two biological replicates of the two first leaves on individual plants were collected at each time point from the treated and control plants (Supplementary Data Fig. S1). To ensure that the sampled individuals were truly S or R, as appropriate, the plants were grown on under natural light in a glasshouse (22 °C, 13 h day length) for a further 21 d after leaf excision and then assessed for mortality or survival. After collection of the 24 h sample, the untreated control plants were also sprayed with formulated 2,4-D amine to confirm their resistance status.

Full details of the transcriptomics experiment are provided in the Supplementary Data Methods. Briefly, 24 sequencing libraries were constructed from 500 ng of total RNA and pair-end sequenced using the HiSeq 2000 platform (Illumina Inc., San Diego, CA, USA). Filtered reads were mapped to the *R. raphanistrum* reference genome (Moghe *et al.*, 2014),

and read counts were calculated for each gene. Supplementary Data Table S1 provides a summary of the sequencing data and mapping information. Differential gene expression analysis was performed on the read counts, and differentially expressed genes (DEGs) were functionally annotated using Blast2GO (Conesa *et al.*, 2005). A total of 91 % of all the DEGs were assigned GO terms (Supplementary Data Table S2) and subjected to GO enrichment analysis.

Screening of expression of 2,4-D-responsive genes

Expression of two of the genes identified in the transcriptomic study as being potentially important for 2,4-D resistance, *IAA30* and *MEKK1* (see the Results), was measured in 2,4-D-treated and -untreated plants from each S and R population using quantitative reverse transcription–PCR (qPCR) based on the methods of Taylor *et al.* (2016). Seedlings were grown and treated as for the transcriptomics study and harvested at 8 h after treatment (three biological replicates of two plants per replicate; one first leaf sampled per plant). Full details of the qPCR procedure, including primer sequences, are given in the Supplementary Data Methods. Expression of two of the *Raphanus sativus* qPCR reference genes recommended by Xu *et al.* (2012), *RPII* and *TEF2*, was also analysed, using the primer sequences from Xu *et al.* (2012). The RNA from the 8 h time point in the transcriptomic experiment was also subjected to qPCR. The paired-cross populations were not included in the qPCR study.

MAPK activation assay

Activation of the mitogen-activated protein kinase (MAPK) pathway in wild radish leaves was assayed using western blot-based immunodetection of phosphorylated MAPK proteins (Keinath *et al.*, 2010). Pilot studies on leaves harvested from 5 to 160 min after 2,4-D treatment indicated that the strongest signal was present at 5 and 10 min (data not shown), so a 10 min post-treatment time was selected for the main study. Leaves of seedlings (three replicates of three plants, one first leaf per plant) at the two- to three-leaf stage were sprayed with 500 g ha^{-1} 2,4-D amine and harvested into liquid N_2 10 min later, along with untreated control leaves sprayed with water (to account for any MAPK response to spray droplets hitting the leaves). The protein extraction, western blotting and signal quantification procedures are described in full in the Supplementary Data Methods.

Statistical analysis

The herbicide or auxin dose causing 50 % plant mortality (LD_{50}) or reduction in growth (GR_{50}) was calculated from the dose–response data from the foliar spray and root elongation experiments using the 'drc' package in R version 3.2.3 (R Core Team, 2015) or SigmaPlot version 12.0 (Systat Software Inc., San Jose, CA, USA). Overall differences in biomass of surviving plants were analysed using GenStat, 18th edition (VSN International, Oxford, UK). Differences between ^{14}C signals in the uptake, translocation and metabolism studies, between normalized qPCR-based gene expression levels and between phosphorylated MAPK bands, were analysed by one-factor analysis of variance (ANOVA) and the least significant difference (LSD) test at the 5 % level of significance. A principal components analysis was also performed according to Xia *et al.* (2009), but there was no significant separation between the S and R populations when the survival and biomass data were not included in the data set (Supplementary Data Fig. S2). Therefore, a Pearson correlation matrix between all measurements was constructed using Excel and used to assess the potential contribution of each parameter to 2,4-D resistance.

RESULTS

Quantification of whole-plant resistance to 2,4-D and dicamba

As expected, all R populations showed greater ($P < 0.05$) survival of a foliar spray with 125–4000 g ha^{-1} 2,4-D or dicamba than the S1 and S2 populations. The overall mean LD_{50} values for the pooled S vs. R populations were 75 vs. 3400 g ha^{-1} for 2,4-D, and 275 vs. 6400 g ha^{-1} for dicamba (Fig. 2A). The resistance level of the individual R populations (expressed as the ratio of R:S LD_{50} values) ranged from 7- to 60-fold for 2,4-D and from 2- to 32-fold for dicamba (Supplementary Data Table S3), and there were no discrete groups of populations that were consistently different from each other except that population $R1_2$ had a lower LD_{50} than all (2,4-D) or most (dicamba) of the other R populations (Fig. 2B, C; Supplementary Data Table S3). In most populations, there was no difference in resistance levels between generations 1 and 2 for both 2,4-D and dicamba (Supplementary Data Table S3). There were no significant differences between the six paired crosses in terms of their survival or growth under either 2,4-D or dicamba treatment, so the data for these populations were pooled. The LD_{50} values for 2,4-D were not significantly different between the paired crosses and their parents (populations $R3_2$ and $R5_2$) (Supplementary Data Table S3). The same result was obtained for dicamba, but in this case a reliable LD_{50} could not be calculated for the crosses. One-factor ANOVA at each dicamba rate confirmed that there were no significant differences between populations in terms of overall mean survival, but that the crosses had higher survival than parent $R5_2$ at 500 and 2000 g ha^{-1} (Supplementary Data Fig. S3).

Although 2,4-D and dicamba had similar effects on the biomass of the R survivors, S survivors were less affected by dicamba than by 2,4-D at doses of 125–500 g ha^{-1} (Fig. 3A). The effects of 2,4-D and dicamba on the biomass of the surviving plants were not subjected to a GR_{50} analysis, because the biomass of some R populations was not greatly affected at any herbicide dose (Fig. 3B, C; Supplementary Data Table S4); instead, the mean biomass of survivors across all herbicide doses was compared. Almost all of the R populations had a significantly ($P < 0.05$) higher biomass than the S populations, but again there were no consistent patterns between R populations (Fig. 3B, C; Supplementary Data Table S4). There were significant positive correlations between plant survival (LD_{50}) and survivor biomass under both 2,4-D and dicamba treatment (Supplementary Data Table S5).

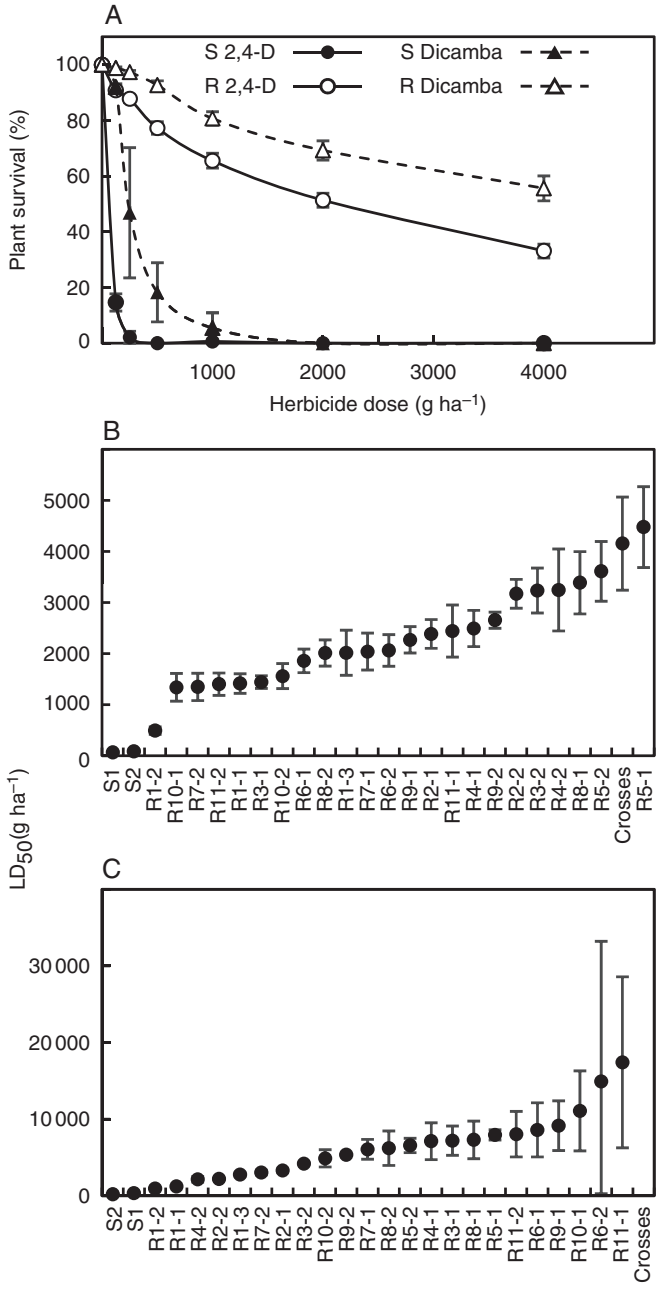

FIG. 2. Survival of wild radish populations at 21 d after treatment with 0–4000 g ha^{-1} 2,4-D or dicamba. (A) Mean survival of both S vs. all R populations at each herbicide dose; (B) resistance level of each population to 2,4-D, expressed as the dose required to kill 50 % of the population (LD_{50}); (C) resistance level of each population to dicamba, expressed as the LD_{50}. For (B) and (C), populations are plotted in ascending order of LD_{50}. In each plot, bars represent the standard error. Significant differences between each pair of populations are presented in Supplementary Data Table S3.

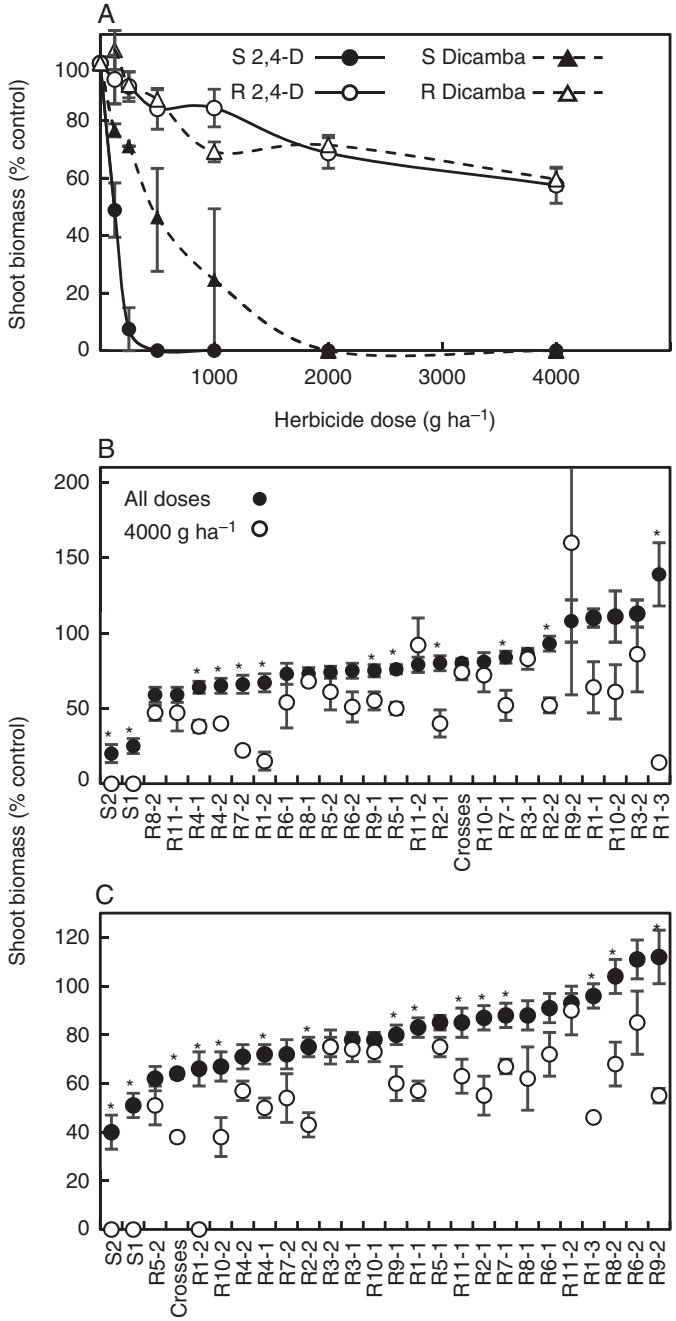

FIG. 3. Biomass of surviving plants at 21 d after treatment with 0–4000 g ha^{-1} 2,4-D or dicamba. (A) Mean biomass (expressed as a percentage of untreated plant biomass) of both S vs. all R populations at each herbicide dose; (B) biomass of 2,4-D survivors averaged over all doses or at 4000 g ha^{-1}; (C) biomass of dicamba survivors averaged over all doses or at 4000 g ha^{-1}. For (B) and (C), populations are plotted in ascending order of overall biomass. In each plot, bars represent the standard error. Asterisks denote a significant ($P < 0.05$) difference between overall biomass and biomass at 4000 g ha^{-1} within populations; significant differences between each pair of populations are presented in Supplementary Data Table S4.

Auxin cross-resistance in young seedlings

In terms of the root elongation response of the second generation of R populations, there were no correlations between their relative levels of resistance (GR_{50}) to 2,4-D, dicamba, IAA and NAA (Supplementary Data Table S5). Overall, the seedlings were less sensitive to dicamba than to the other auxins, with the GR_{50} of the S populations being one to two orders of magnitude higher for dicamba (Table 1). Populations R1, R2, R3 and R4 were the most resistant to 2,4-D (GR_{50} >200 μM), followed by R5, R6 and R8 (>100 μM) (Table 1). There were

fewer significant differences between R populations in terms of dicamba resistance, and populations R4, R7, R10 and R11 were statistically no more resistant than S1 and S2 (Table 1). Populations R2, R4, R6 and R7 were more resistant to IAA than all of the other populations, and R1, R7 and R10 were the most resistant to NAA (GR_{50} >100 μM in both cases) (Table 1). Populations R5 and R6 were the most susceptible to IAA and NAA, respectively. There were moderate positive correlations between the foliar-spray 2,4-D LD_{50} of a population and its root GR_{50} on 2,4-D- or dicamba-containing agar, but not between the other auxins, or between the dicamba foliar spray and root elongation data (Supplementary Data Table S5).

Uptake, translocation and metabolism of [^{14}C]2,4-D

In all populations, >90 % of applied [^{14}C]2,4-D was absorbed by the leaves in 24 h, with the amount of ^{14}C recovered in the leaf washes ranging from 1 to 9 % (Supplementary Data Table S6). Less than 1 % of applied ^{14}C was recovered in root washes from each population (Supplementary Data Table S6). The extent of translocation of [^{14}C]2,4-D out of the treated leaf differed widely between populations, but most of the translocated ^{14}C was found in the stem tissue, followed by the roots, with little observed in the untreated leaves (Fig. 4A). Based on the mean proportion of recovered ^{14}C residing in the treated leaf, the 13 populations were placed into four significantly ($P < 0.05$) different groups, i.e. (*a*) >85 % of recovered ^{14}C in the treated leaf: R1, R2, R3, T2, N1 and N2; (*b*) 70–83 %: R6, R7, T1 and N3; (*c*) 50–68 %: R8, R9 and T3; and (*d*) <30 %: S1, S2, R4, R5, R10 and R11 (Fig. 4B–E). There was a weak positive correlation between the proportion of ^{14}C retained in the treated leaf and survivor biomass following the 2,4-D foliar spray (Supplementary Data Table S5). When the 12 individuals assayed per population were examined separately, there were also clear intrapopulation differences in translocation. Some populations had uniformly high or low translocation levels across all 12 individuals (e.g. R3, R4); some were uniform except for a few dramatically different individuals (e.g. R10, N1); and some showed a gradient of treated-leaf ^{14}C levels across the population (e.g. R7, R9) (Supplementary Data Fig. S4).

To check whether the observed higher level of ^{14}C in the roots than in the treated leaves of population R5 (Fig. 4A) was due to sequestration in the root apoplast, R5 plants were grown hydroponically (Goggin *et al.*, 2016) along with S2 (susceptible translocator), R3 (resistant non-translocator) and R4 (resistant translocator), and root apoplasts from plants with leaf-applied [^{14}C]2,4-D were isolated according to Zhang *et al.* (2009) (Supplementary Data Methods). The negligible recovery of ^{14}C from both the apoplast and symplast in all populations, including R5 (Supplementary Data Table S7), indicates that apoplastic sequestration of [^{14}C-2,4[D is unlikely to occur in the roots of population R5.

All of the studied populations except R9 showed the same pattern of 2,4-D metabolism, with three metabolite bands separating from parent 2,4-D on thin-layer chromatography (TLC) chromatograms (metabolite 1 was not detected in population R9; representative chromatograms are shown in Fig. 5A). The proportion of total ^{14}C signal migrating identically to standard [^{14}C]2,4-D ranged from 75 % in population R9 to 25 % in paired-cross N1, with the other populations spread relatively evenly between these values (Fig. 5B). There were also some differences between populations in the proportion of ^{14}C present in each metabolite band. There was no correlation between the extent of [^{14}C]2,4-D metabolism and survival of a foliar spray of 2,4-D or dicamba, but there were weak to moderate correlations between the amount of ^{14}C retained in the treated leaf and the proportion of ^{14}C present as parent 2,4-D (negative correlation) and metabolite 3 (positive correlation) (Supplementary Data Table S5).

Changes in gene expression in populations S1 and R1 in response to 2,4-D

Due to the different genetic backgrounds of populations S1 and R1, this transcriptomics study focused on comparing 2,4-D-induced changes in gene expression in the two

TABLE 1. *Root elongation response of seedlings grown on agar containing auxins*

	2,4–D			Dicamba			IAA			NAA		
	GR_{50} (nM)	R:S1	R:S2	GR_{50} (nM)	R:S1	R:S2	GR_{50} (nM)	R:S1	R:S2	GR_{50} (nM)	R:S1	R:S2
S1	12 ± 1^{f}	–	1	181 ± 56^{d}	–	2	3 ± 2^{f}	–	3	13 ± 6^{de}	–	1
S2	10 ± 2^{f}	1	–	113 ± 53^{d}	1	–	1 ± 1^{g}	0	–	25 ± 10^{cd}	2	–
R1	264 ± 50^{a}	22	26	1809 ± 379^{ac}	10	16	69 ± 29^{b}	23	69	109 ± 47^{ab}	8	4
R2	308 ± 23^{a}	26	31	2308 ± 517^{ac}	13	20	159 ± 43^{a}	53	159	34 ± 7^{c}	3	1
R3	374 ± 24^{a}	31	37	7619 ± 2400^{a}	42	67	44 ± 12^{cd}	15	44	28 ± 7^{c}	2	1
R4	314 ± 31^{ab}	26	31	1827 ± 511^{cd}	10	16	206 ± 27^{a}	69	206	77 ± 20^{b}	6	3
R5	109 ± 11^{cd}	9	11	3760 ± 899^{bc}	21	33	5 ± 2^{f}	2	5	24 ± 8^{cd}	2	1
R6	110 ± 16^{cd}	9	11	2808 ± 913^{ab}	16	25	175 ± 35^{a}	58	175	7 ± 2^{e}	1	0
R7	32 ± 8^{e}	3	3	2035 ± 840^{acd}	11	18	141 ± 32^{a}	47	141	102 ± 16^{ab}	8	4
R8	166 ± 30^{bc}	14	17	6158 ± 1105^{ab}	34	54	56 ± 17^{bc}	19	56	13 ± 4^{de}	1	1
R9	79 ± 10^{de}	7	8	3323 ± 656^{ac}	18	29	28 ± 7^{de}	9	28	36 ± 8^{c}	3	1
R10	53 ± 9^{e}	4	5	1023 ± 335^{acd}	6	9	59 ± 15^{bcd}	20	59	154 ± 33^{a}	12	6
R11	60 ± 8^{e}	5	6	1213 ± 559^{acd}	7	11	17 ± 5^{e}	6	17	93 ± 17^{b}	7	4

GR_{50} values were calculated from dose–response curves, and the R:S1 or R:S2 ratio represents the ratio of GR_{50} values between the R and S1 or S2 biotypes. Within columns, different letters denote significant ($P < 0.05$) differences between values.

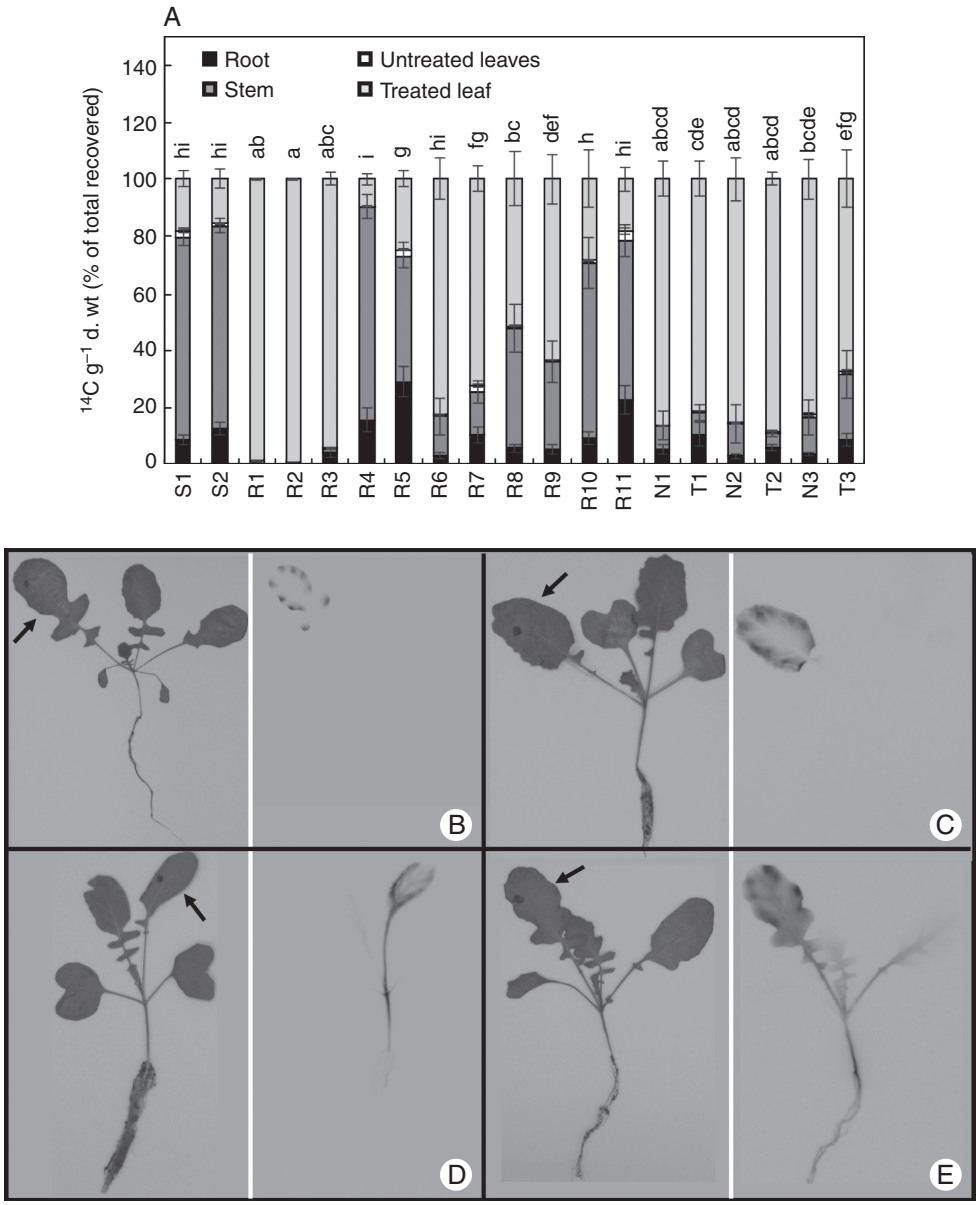

FIG. 4. Location of ^{14}C-labelled 2,4-D at 24 h after application to a single leaf of wild radish seedlings. (A) Quantification of ^{14}C in dried plants separated into treated leaf and untreated leaves, stem and roots. Different letters above bars denote significant ($P < 0.05$) differences between the proportion of recovered ^{14}C in the treated leaf of each population. Values are means ± s.e. ($n = 12$ individuals). For comparative purposes, the data for populations S1, R1 and R2, generated in Goggin *et al.* (2016), are included. (B–E) Representative translocation patterns as illustrated by photographs of seedlings (left panels; arrow indicates treated leaf) and their corresponding phosphor images (right panels). (B) Group 1 (>85 % of ^{14}C in treated leaf), represented by population R3; (C) group 2 (70–83 %), represented by population R7; (D) group 3 (50–68 %), represented by population R8; (E) group 4 (<30 %), represented by population R5.

populations across three time points, rather than on constitutive differences. In response to 2,4-D, 5180 genes out of 39 384 were significantly differentially expressed based on a $\log_2$ fold change (logFC) of >2 and a false discovery rate (FDR) of <0.05 (Supplementary Data Table S2), which were filtered through to 748 genes based on a logFC of >8. Overall, population R1 exhibited a more rapid, extensive and positive response to 2,4-D than did S1. By 2 h after treatment, 124 genes had been upregulated in the R1 population compared with 54 genes in the S population (Fig. 6A, B; Supplementary Data Table S8). Across all time points, more genes were up-regulated in R1 and more were down-regulated in S1 (Fig. 6A, B; Supplementary Data Table S8).

Gene Ontology (GO) enrichment analysis identified the top ten enriched terms for biological process, cellular component and molecular function for each treatment and population at each time point (i.e. 12 sample types in total). Overall, the magnitude of enrichment was greater in R1 than in S1 for upregulated genes, and the opposite was true for downregulated genes (Supplementary Data Table S9). In the cellular component category, most samples were enriched in terms related to intracellular parts and organelles (Supplementary Data Table S9),

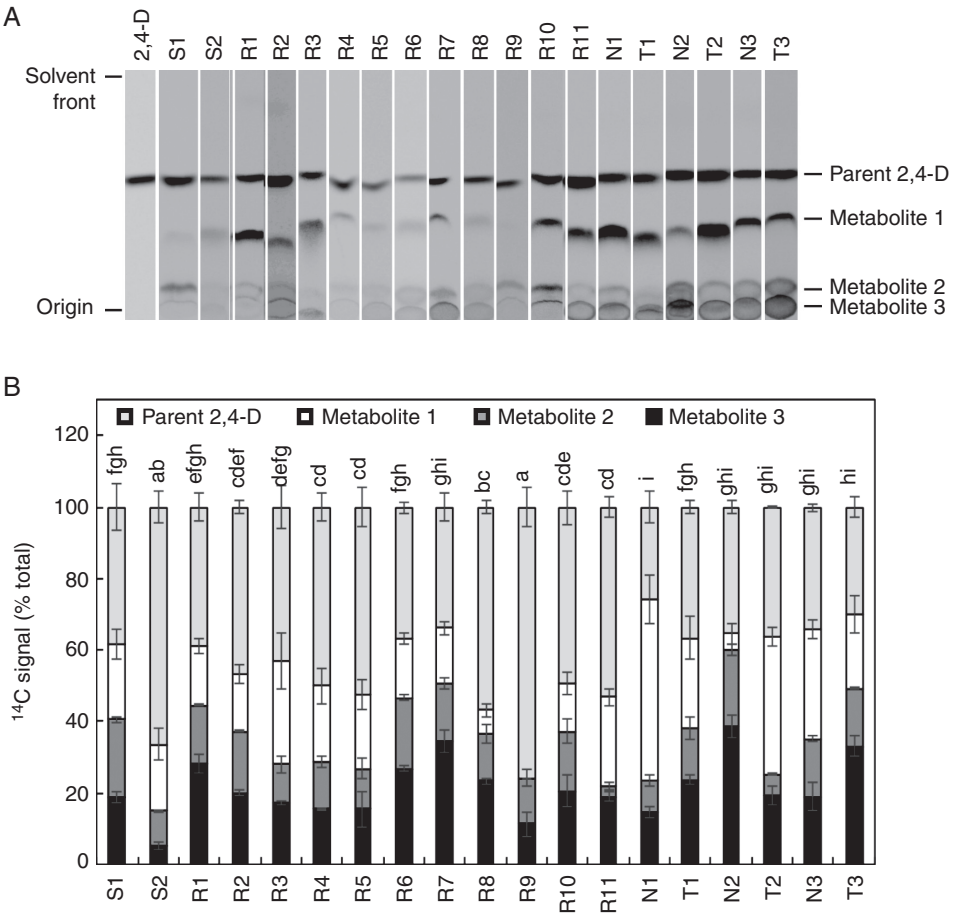

FIG. 5. Metabolism of ^{14}C-labelled 2,4-D in wild radish. Plants were treated with [^{14}C]2,4-D applied to a single leaf and extracted 96 h later; metabolites were separated from parent 2,4-D by thin-layer chromatography and detected by phosphor imaging. (A) Representative chromatograms for each population, run alongside authentic [^{14}C]2,4-D. (B) Densitometric quantification of ^{14}C signals in each TLC lane. Values are means ± s.e. (n = 3 replicates of four plants). Different letters above bars denote significant ($P < 0.05$) differences between populations in the relative amount of ^{14}C migrating with parent 2,4-D.

but the plasma membrane term was uniquely enriched in the upregulated R1 genes at 2 h after treatment, and in the downregulated S1 genes at 2 and 24 h (Fig. 6C). There was less overlap between S1 and R1 samples in the biological process and molecular function terms. Some of the biological process terms that were uniquely or more highly enriched in the upregulated R1 genes at 2 h after treatment (e.g. response to chemical, response to stimulus and defence response) became enriched in the S1 genes only at the later time points (Fig. 6C). The greatest difference in enrichment of biological process terms for downregulated genes came at 24 h after treatment, with the S1 population being more strongly enriched in terms related to response to stimuli and defence response (Fig. 6C). The enrichment level of molecular function terms was lower than in the other categories, and the least overlap between R1 and S1 occurred in the upregulated genes at 8 and 24 h after treatment: the R1 terms were related to signalling and activation (e.g. kinase binding, calmodulin binding and adenylyltransferase activity), whilst the S1 terms were related to glucosyltransferase and ligase activity (Supplementary Data Table S9).

All of the genes that changed expression (logFC >8) in response to 2,4-D at each time point are listed in Supplementary Data Table S8. Based on the GO enrichment analysis, genes that were upregulated in R1 but not in S1 were considered the most important for 2,4-D resistance. There were 12 genes consistently upregulated in the R1 population. One of these (*MAP KINASE KINASE KINASE 1* or *MEKK1*) was consistently downregulated in the S1 population, and five were downregulated at one or two time points in the S1 population; these encoded three receptor-like protein kinases of the plasma membrane, cyclic nucleotide-gated channel 12 (CNGC12) and spermidine synthase 1 (Table 2). The genes encoding a SAUR-like auxin-responsive protein and the non-canonical Aux/IAA transcription factor IAA30, consistently upregulated in R1, were only upregulated in S1 at 24 h after 2,4-D treatment, and with a lower logFC (Table 2). The top transiently upregulated genes in R that were not upregulated in S were a TIR-NBS-LRR-class disease resistance protein gene (2 and 24 h), the canonical Aux/IAA repressor *IAA29* (8 and 24 h) and the auxin efflux transporter *ABCB11* (24 h) (Table 2). The IAA30 protein is stable under high auxin concentrations and involved in dampening the general auxin response (Sato and Yamamoto, 2008); MEKK1 is implicated in a range of defence responses; and expression of both was consistently upregulated in R1 but not in S1. Therefore, these two genes were selected for further investigation in the other R populations.

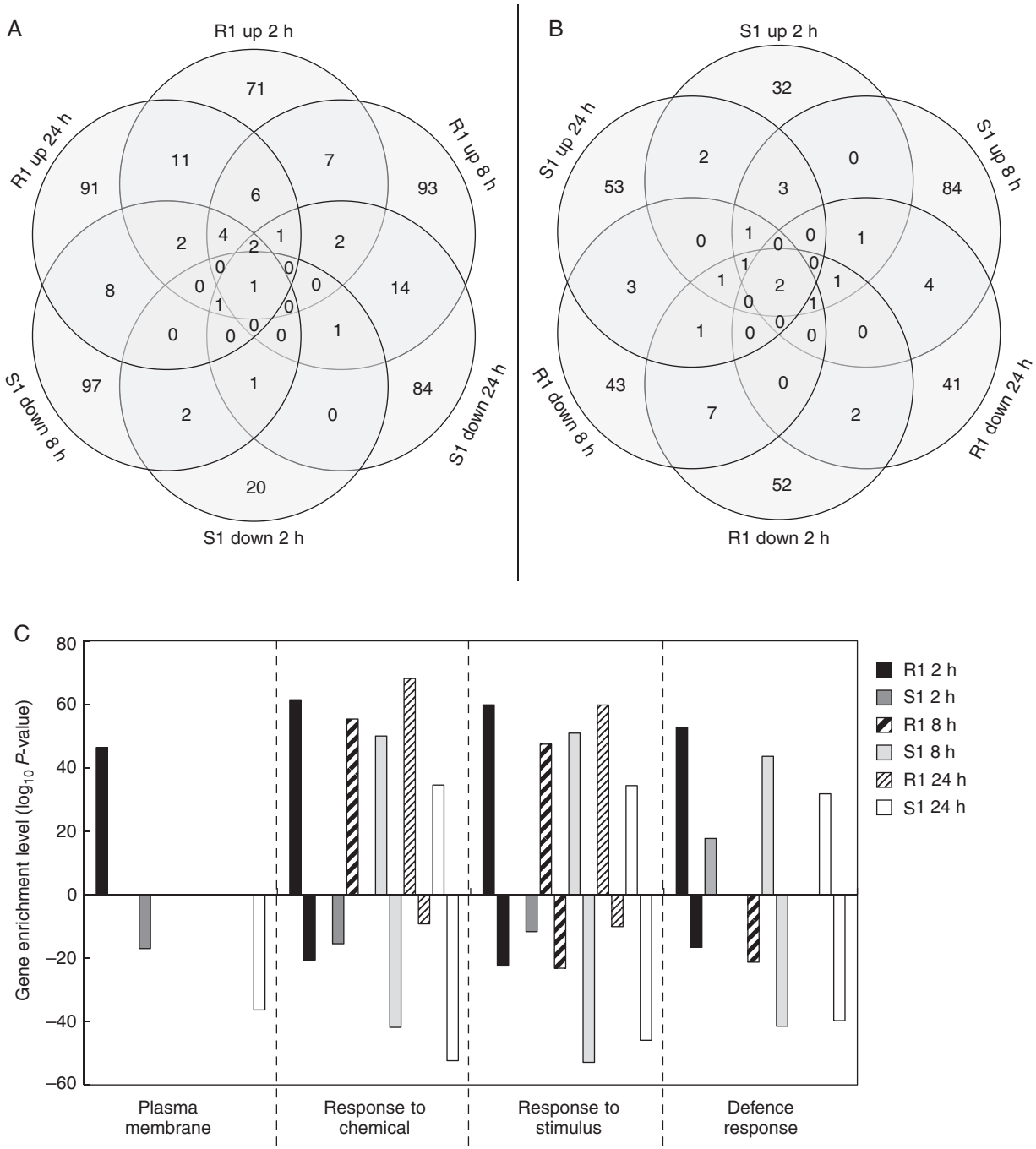

FIG. 6. Gene expression differences in 2,4-D-susceptible (S1) and -resistant (R1) *Raphanus raphanistrum* populations in response to 2,4-D. The number of genes that were up- or downregulated (logFC >8; FDR <0.05) at 2, 8 and 24 h after 2,4-D treatment are shown in Venn diagrams, with the overlapping regions indicating the number of common genes differentially expressed at each time point; namely (A) genes upregulated in R1 and downregulated in S1; and (B) genes upregulated in S1 and downregulated in R1. (C) Comparison of the level of gene enrichment in selected GO terms in the R1 and S1 populations, expressed as the $\log_{10}$ *P*-values generated by REVIGO. Upregulated genes were given a positive sign and downregulated genes a negative sign, to aid in visual interpretation.

Screening of gene expression and MAPK phosphorylation in response to 2,4-D

Relative expression of *IAA30*, as measured by qPCR, increased 5- to 80-fold in response to 2,4-D in all populations, but the increase was only statistically significant ($P < 0.05$) in populations S1, S2, R3, R5 and R6. There were no significant differences between populations in their relative expression of *IAA30* either under control conditions or following 2,4-D treatment (Fig. 7A). However, there was a moderate positive correlation between relative expression of *IAA30* in the presence of 2,4-D and root elongation on 2,4-D-agar (Supplementary Data Table S5). Constitutive relative expression of *MEKK1* and its response to 2,4-D was highly variable, with transcripts being undetectable in some populations. Treatment with 2,4-D

TABLE 2. *Differentially expressed genes (logFC >8) showing upregulation in the R1 but not the S1 population, either at individual time points (2, 8 or 24 h after 2,4-D treatment) or across the entire experiment*

Gene ID	Description	logFC in R			logFC in S		
		2 h	8 h	24 h	2 h	8 h	24 h
RrC12279_p1	MAP kinase kinase kinase 1 (MEKK1)	8.3	8.6	8.1	–9.3	–8.9	–8.7
RrC43619_p1	Leaf rust 10 disease-resistance locus receptor-like protein kinase-like 2.8 (LRK10L2.8)	10.4	9.9	9.3	–	–	–11.6
RrC2386_p2	Wall-associated receptor kinase-like 10 (WAKL10)	11.6	9.0	9.2	–	–10.2	–9.2
RrC1873_p1	Spermidine synthase 1 (SPDS1)	10.7	8.3	10.2	–	–10.2	–
RrC3222_p2	Cyclic nucleotide-gated ion channel 12 (CNGC12)	10.7	8.2	11.0	–	–8.6	–
RrC12206_p1	Probable receptor-like protein kinase At2g42960	9.4	9.1	11.1	–	–8.6	–
RrC5519_p2	ICE1 transcriptional activator	9.8	9.3	10.0	–	–	–
RrC4756_p4	Clathrin interactor EPSIN2	9.3	9.2	9.3	–	–	–
RrC12897_p1	IAA30	9.4	9.3	10.0	–	–	9.4
RrC1785_p1	SAUR-like auxin-responsive protein	8.6	9.0	9.3	–	–	8.4
RrC25837_p1	Disease resistance protein TAO1=like	12.2	–	12.9	–	–	–
RrC18958_p1	IAA29	–	14.5	8.6	–	–	–
RrC1066_p4	Auxin efflux transporter ABCB11	–	–	11.8	–	–	–

For both the R1 and S1 populations, levels of gene expression were compared in treated vs. untreated plants.

caused variable changes in expression in different populations, but none of the changes was statistically significant (Fig. 7B). There were no differences between populations in relative *MEKK1* expression under control conditions; however, following 2,4-D treatment, population R2 had significantly higher *MEKK1* expression than all the other populations, and expression in R8 was higher than in R1, R3, R4, R5, R6, R10 and R11 (Fig. 7B).

A comparison of the relative expression of *IAA30* and *MEKK1* in the RNA isolated from S1 and R1 in the population screening study vs. the transcriptomic study revealed that the responses to 2,4-D were not entirely consistent between studies, with the change in *IAA30* expression being more pronounced in S1 (Fig. 7A inset), and expression of *MEKK1* not being significantly affected by 2,4-D (Fig. 7B inset), in the screening study.

As activation of the MAPK pathway can be more dependent on protein phosphorylation than gene expression (e.g. Zhang and Klessig, 1997), immunoblotting was used to detect MAPK phosphorylation in each population before and after 2,4-D treatment (Fig. 7C). Two bands of 42 and 44 kDa were detected by the primary antibody (see Fig. 7C inset for a representative blot). Constitutive levels of phosphorylated MAPK were highly variable between populations, but populations R6, R8 and R9 had higher levels than S1, S2, R1, R4, R5 and R11 (Fig. 7C). The only statistically significant ($P < 0.05$) increase in phosphorylated MAPK in response to 2,4-D occurred in population R5. Again, there was large variation between populations in the level of post-treatment phosphorylated MAPK, with R3, R5 and R8 having higher levels than S1, S2, R1 and R11 (Fig. 7C). There may be a relationship between MAPK phosphorylation and resistance to 2,4-D, and particularly dicamba, with moderate to strong positive correlations existing between constitutive levels of phosphorylated MAPK and (1) survival of a dicamba foliar spray; (2) biomass of survivors of a 2,4-D or dicamba spray; and (3) root elongation on dicamba-containing agar (Supplementary Data Table S5). The level of MAPK phosphorylation following 2,4-D treatment was also moderately to strongly correlated with survival of a 2,4-D spray and root elongation in the presence of dicamba (Supplementary Data Table S5).

DISCUSSION

An overarching finding of this study is that when exploring mechanisms of evolved auxinic herbicide resistance in a weed species, the conclusions drawn from one or two populations cannot be extended to all the populations in a region. An initial study on two 2,4-D-resistant wild radish populations (R1 and R2) from the Western Australian grain belt (Goggin *et al.*, 2016) identified greatly reduced translocation of 2,4-D out of the treated leaf as the major mechanism of resistance, possibly coupled with a second mechanism at the level of auxin perception or signal transduction. The second mechanism, investigated here in a transcriptomics study on population R1, appeared to involve an enhanced defence response at the plasma membrane and in the MAPK pathway, and a dampening of the auxin signal by increased expression of the IAA30 and IAA29 transcriptional repressors. However, extending the study of resistance mechanisms to other populations has demonstrated that the situation is much more complex.

Auxin resistance and cross-resistance

The overall similarity in the 2,4-D resistance level (LD_{50}) between R populations, and the observations that (1) resistance of a population was usually not increased by a second round of selection with 2,4-D; (2) it is unknown how many times each population was selected with 2,4-D in the field before it was collected; and (3) the progeny of the paired crosses were no more resistant than their parents suggests that there may be a genetic or physiological factor limiting the level of resistance that can be achieved (e.g. partial redundancy in the affected part of the auxin signalling complex: Calderón-Villalobos *et al.*, 2012).

There was evidence of differential auxin selectivity in the different populations. All of the 2,4-D-resistant populations were also resistant to a dicamba foliar spray, but there was no correlation between the levels of resistance to 2,4-D and to dicamba as measured by survival or biomass increase following foliar spraying. Similarly, whilst all of the populations were

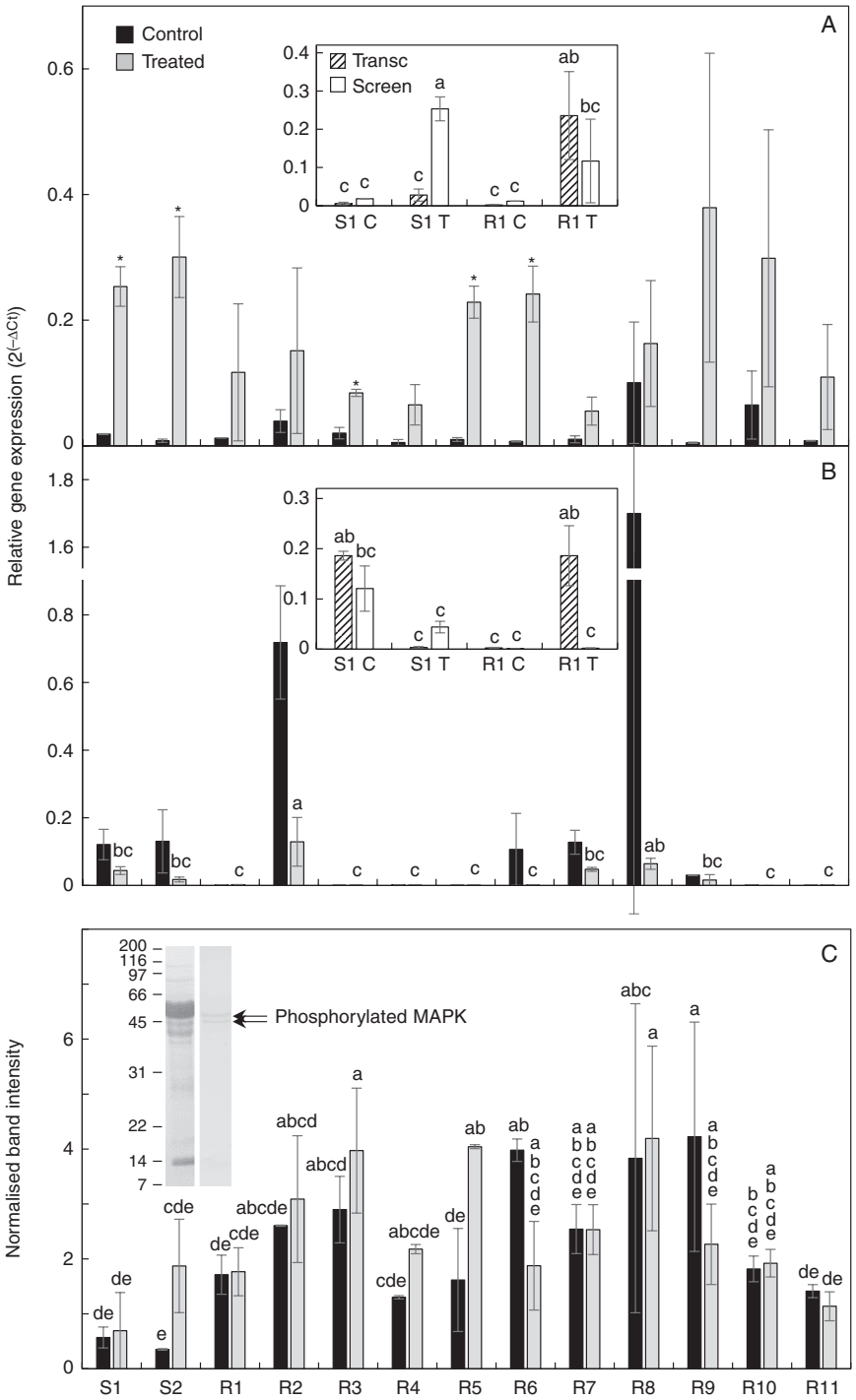

FIG. 7. Gene expression and MAPK activation in response to 2,4-D. Relative expression of (A) *IAA30* and (B) *MEKK1* was quantified by qPCR at 8 h after 2,4-D treatment and in corresponding untreated controls for each population. Data are presented as expression of the gene of interest relative to the reference genes *RPII* and *TEF2* using the $2^{-\Delta Ct}$ method. (C) Immunodetection of MAPK phosphorylation at 10 min after 2,4-D or mock treatment, using western blots probed with phosphor-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antiserum. Signal intensity of phosphorylated MAPK was normalized against the protein staining intensity of the small subunit of Rubisco. Values are means ± s.e. ($n = 3$ replicates of two plants for qPCR and three plants for western blots). In (A), where there were no significant differences between populations, asterisks denote a significant ($P < 0.05$) difference between treated and control expression within a population; in (B), where there were no significant differences between treatments, different letters above bars denote significant differences between 2,4-D-treated populations; in (C), different letters above bars denote significant differences between populations and treatments. Insets show (A) a comparison of *IAA30* and (B) a comparison of *MEKK1* expression in the RNA samples from the transcriptomics (Transc) and screening (Screen) studies on populations S1 and R1 (control and treated samples denoted by C and T, respectively); and (C) a representative western blot with Ponceau-stained total protein and standard molecular masses (kDa) on the left and the signal from phosphorylated MAPK on the right. Different letters above bars in the insets in (A) and (B) denote significant differences between populations and treatments.

2,4-D resistant in the root elongation assays, some were not dicamba resistant and other (different) populations were not resistant to NAA. It is well known that different auxins interact with components of the auxin signalling and transport machinery in distinct ways (e.g. Walsh *et al.*, 2006; Yang and Murphy, 2009; Simon *et al.*, 2013), so the observed differences in auxin selectivity between the 2,4-D-resistant wild radish populations, and in their relative resistance levels, strengthen the hypothesis that 2,4-D resistance is conferred by different alterations in auxin signalling in different populations (Chandler, 2016). In view of the recent discovery of a mutation conferring evolved dicamba and 2,4-D resistance in the IAA16 protein of *Kochia scoparia* (LeClere *et al.*, 2018), it is tempting to speculate that these signalling alterations may be due to mutations in various Aux/IAA proteins in the different populations. The sequencing data from the transcriptomic study on S1 and R1 is currently being analysed for population-specific variations as the first step in resolving this question.

Does reduced 2,4-D translocation actually contribute to resistance?

The ability of several R populations to translocate ^{14}C-labelled 2,4-D out of the treated leaf as efficiently as the S populations, whilst showing the same (or higher) levels of resistance as non-translocating populations, demonstrates that reduced translocation is not necessary, and possibly not sufficient, for resistance to 2,4-D in wild radish. This is particularly well illustrated by the paired crosses, which inherited reduced translocation from parent R3, but were not significantly more resistant than their translocating parent R5. Kaundun *et al.* (2012) proposed that in weed populations with a strong target site mutation conferring herbicide resistance, the subsequent accumulation of minor resistance genes may not necessarily increase plant survival. In that case, reduced 2,4-D translocation in wild radish would be considered a minor mechanism. The complexity of the situation is highlighted by the fact that 2,4-D resistance in F_1 and F_2 families generated from wild radish populations with field-evolved resistance was characterized as being due to either one or two major genes with dominant or incompletely dominant inheritance, depending on the population (Busi and Powles, 2017).

There were weak to moderate positive correlations between reduced 2,4-D translocation and (1) root growth on 2,4-D- or dicamba-containing agar and (2) biomass of survivors of the 2,4-D foliar spray. This implies that the reduced translocation phenomenon present in the leaves of some populations could also exist in the roots; and that reduced translocation contributes to the vigour of survivors, rather than to survival itself, by decreasing the amount of 2,4-D reaching the meristems. The contribution of reduced translocation to resistance could be quantified by assessing the 2,4-D resistance level and translocation ability of arabidopsis auxin efflux mutants (e.g. *abcb11*: see below) that have otherwise normal auxin signalling, but that is outside the scope of the current study.

Another complication with interpreting the relevance of reduced 2,4-D translocation to resistance is that whilst the phenomenon appears to be dominant in non-translocating population R3 (based on the reduced translocation of the paired crosses) and probably also in R1 and R2, whose populations were uniformly non-translocating after both one (data not shown) and two (Supplementary Data Fig. S3) rounds of selection with 2,4-D, the proportion of non-translocating individuals in populations R6, R7, R8 and R9 was not, or only slightly, increased between the first and second rounds of selection (data not shown). It is thus possible that there is more than one pathway towards reduced translocation in wild radish. The detailed study on populations S1, R1 and R2 in Goggin *et al.* (2016) demonstrated that there was no movement of 2,4-D into the leaf vacuoles (or apoplast) in these populations, but that does not necessarily exclude the possibility of vacuolar sequestration in other populations. It should be noted, however, that the polar metabolites of 2,4-D in wild radish are labile (see below) and the acidic conditions inside the vacuole would allow a significant proportion of unconjugated, protonated 2,4-D to diffuse back through the tonoplast membrane into the cytosol (Carrier *et al.*, 2008). The transcriptomics study identified the auxin efflux transporter ABCB11 as a likely 2,4-D transporter in R1 (Table 2), so it is possible that a dominant loss-of-function mutation in ABCB11 causes reduced translocation in R1, R2 and R3, whilst the weaker expression of reduced translocation in R6, R7, R8 and R9 is caused by, for example, a less effective or recessive mutation in ABCB11 or in a minor auxin efflux transporter (such as the ABCB4 bidirectional transporter: Yang and Murphy, 2009); or mislocalization of part of the ABCB population (Yang *et al.*, 2013). The status of ABCB11 in 2,4-D-resistant wild radish is currently under investigation.

Enhanced metabolism does not contribute to 2,4-D resistance in wild radish

There was no evidence that enhanced, irreversible metabolism of 2,4-D is a resistance mechanism in wild radish, with the pattern of metabolites in all populations matching that of the labile metabolites that were partially characterized in Goggin *et al.* (2016). The lability of the metabolites makes it likely that in the living plant, there is a constant turnover of metabolites back to parent 2,4-D, and indeed metabolites 2 and 3 possess auxin activity in root elongation experiments (unpublished data). However, the weak correlations observed between [^{14}C]2,4-D movement and 2,4-D metabolism (higher metabolite 2 corresponds to less ^{14}C in roots; higher metabolite 3 corresponds to more ^{14}C in treated leaves) suggest that these two highly polar metabolites may be less phloem mobile than parent 2,4-D.

Auxin signalling and plant defence

Although *IAA30* was identified as a potential key resistance gene in the transcriptomics study on population R1, there was no correlation between expression of this gene after a 2,4-D foliar spray and plant survival when all the populations were screened. There was also a much higher induction of *IAA30* in S1 in the screening study compared with the transcriptomics study, which could be due to the high genetic variability present in wild radish populations (Bhatti *et al.*, 2016). However, a noteworthy aspect of IAA30 is that it lacks the domain II degron sequence characteristic of the canonical Aux/IAA proteins

(Sato and Yamamoto, 2008). This domain triggers Aux/IAA degradation in the presence of high auxin concentrations and is the region in which the dicamba resistance-conferring mutation occurs in the IAA16 of *K. scoparia* (LeClere *et al.*, 2018). Therefore, although a conclusive link between *IAA30* overexpression (leading to a dampening of the auxin response) and 2,4-D resistance could not be established in the current study, further investigation is warranted.

In fact, there was a moderate positive correlation between the relative expression of *IAA30* after 2,4-D treatment and root elongation on 2,4-D-agar. *IAA30* may therefore contribute to the capacity of a population to grow in the presence of 2,4-D. The fact that the weak correlation between *IAA30* expression and 2,4-D resistance was detected in the root elongation study (controlled environment) and not the foliar spray study (outdoors) reinforces the influence that environmental conditions may have on the response of plants to herbicides (e.g. Ganie *et al.*, 2017). It also suggests that, as suspected from the differential auxin responses of the R populations, more than one auxin signalling protein is involved in 2,4-D resistance in wild radish. In arabidopsis, there are six auxin receptors and 29 Aux/IAA transcriptional repressors which form co-receptor complexes with different binding affinities for each other and for the different auxins (Calderón-Villalobos *et al.*, 2012). With the high genetic variability of wild radish, it is not beyond the bounds of possibility that repeated selection with 2,4-D has resulted in alterations in sequence or expression of different co-receptor components in different populations, which would affect not only their resistance to various auxins, but also the fitness of the survivors.

Expression of *MEKK1*, which was highly variable between populations, did not have significant correlations with any of the measures of 2,4-D or dicamba resistance, or any parameters contributing to resistance. However, there were indications of a positive relationship between MAPK phosphorylation (constitutive and 2,4-D-induced) and resistance to 2,4-D and, in particular, dicamba. Although the signals from the MAPK blots were relatively faint and the results need to be interpreted with caution, the possible link between constitutive MAPK phosphorylation and synthetic auxin resistance is in line with the observed early activation of defence gene expression in 2,4-D-treated R1 compared with S1. The question of whether this 'readiness' for defence against synthetic auxins is linked to the putative alterations in auxin signalling also present in the resistant populations remains open until the relevant auxin signalling component(s) can be identified.

Implications for management of 2,4-D-resistant wild radish

With the apparent diversity in auxin signalling alterations and the at-best moderate contribution of reduced translocation to 2,4-D resistance, it is unlikely that a universal chemical or genetic solution (e.g. compounds restoring translocation or bypassing the putative block in auxin signalling; use of the CRISPR/Cas9 gene drive) could be found to control 2,4-D-resistant wild radish. As most of these populations are also resistant to one or two other herbicide modes of action (Owen *et al.*, 2015), the best control options would involve strategic mixtures of still effective modes of action plus non-chemical measures, namely prevention of seed bank replenishment at harvest (Walsh *et al.*, 2017) and increased competition from the crop (Kelly *et al.*, 2013). Informal observations of the 11 resistant populations studied here have revealed that some appear to suffer a fitness penalty whilst others are highly vigorous. Concentration of research efforts on the biology of the resistant populations might help to inform decisions about the most suitable and economical control method(s) for each population.

In summary, this study demonstrates that evolved 2,4-D resistance in 11 Western Australian wild radish populations is likely to be conferred by a variety of auxin signalling defects that all result in a resistance level (LD_{50}) of around 2000–4000 g ha^{-1}, or 4–8 times the recommended field rate. The great variety and redundancy of auxin signalling genes, and the high intrapopulation variability of wild radish, makes identification of the resistance genes a challenging task indeed.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Supplementary Methods. Figure S1: diagram of experimental design for transcriptomics study. Figure S2: results of principal components analysis. Figure S3: dicamba dose–response curve for populations R3 and R5 and their progeny. Figure S4: quantification of [^{14}C]2,4-D translocation for 12 individuals in each population. Table S1: summary of sequence data and mapping information. Table S2: list of differentially expressed genes (log FC >2, FDR <0.05) in R1 and S1 populations at 2, 8 and 24 h after 2,4-D treatment. Table S3: plant survival (LD_{50}) after a foliar spray with 2,4-D or dicamba. Table S4: plant biomass after a foliar spray with 2,4-D or dicamba. Table S5: Pearson correlation matrix. Table S6: quantification of ^{14}C in leaf and root washes of seedlings treated with [^{14}C]2,4-D. Table S7: marker enzyme activities and ^{14}C in apoplast and symplast of seedling roots. Table S8: list of differentially expressed genes (log FC >8, FDR <0.05) used for constructing Venn diagrams in Fig. 6. Table S9: GO enrichment analysis of differentially-expressed genes.

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