Chen Jinyi (Orcid ID: 0000-0002-4594-254X)
Han Heping (Orcid ID: 0000-0002-0918-2598)
Yu Qin (Orcid ID: 0000-0001-6379-9444)

A Val-202-Phe α-tubulin mutation and enhanced metabolism confer dinitroaniline resistance in a single *Lolium rigidum* population

Running title: Dinitroaniline herbicide resistance mechanisms in *Lolium*

Jinyi Chen[1], Zhizhan Chu[2], Heping Han[1], Danica E Goggin[1], Qin Yu[1], Chad Sayer[3], Stephen B Powles[1]

[1]Australian Herbicide Resistance Initiative (AHRI)–School of Agriculture and Environment, University of Western Australia (UWA), Perth, Australia

[2]College of Life Sciences, South China Agricultural University, Guangzhou, China

[3]Nufarm Limited, Melbourne, Australia

Correspondence

Qin Yu, Australian Herbicide Resistance Initiative (AHRI)–School of Agriculture and Environment, University of Western Australia (UWA), Perth, Australia

Email: qin.yu@uwa.edu.au

Authorship

J Chen and Q Yu designed this work. Jinyi Chen conducted the experiments and analysed the data. Z Chu and H Han helped with the rice transgenic study and D Goggin with the metabolic work. C Sayer offered herbicides. All authors contributed to the revisions of the manuscript.

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Abstract:

BACKGROUND: A *Lolium rigidum* population collected from Western Australia was previously reported as highly resistant to dinitroaniline herbicides mainly due to a Val-202-Phe substitution in the target site α-tubulin protein. To further determine the contribution of the 202 mutation to resistance, two sub-populations, respectively comprising the 202 mutant and wild type (WT) individuals, were isolated from within the same resistant population and subject to dinitroaniline herbicide doses. A rice transgenic study was conducted to demonstrate whether the amino acid substitution at the 202 residue confers resistance. In addition, as indicated in the phenotyping and genotyping study, non-target enhanced trifluralin metabolism was further examined in the same population.

RESULTS: The 202 mutants were more resistant than the WT plants. Rice calli transformed with the *L. rigidum* mutant α-tubulin gene (Val-202-Phe) were more resistant to dinitroaniline herbicides relative to calli transformed with the wild type gene. Also, enhanced trifluralin metabolism was detected in the 202 mutants in comparison to the susceptible seedlings.

CONLCUSION: Both target-site Val-202-Phe α-tubulin mutation and non-target-site enhanced trifluralin metabolism co-exist in this dinitroaniline-resistant *L. rigidum* population.

Key words: dinitroaniline; resistance; tubulin; mutation; *Lolium rigidum*

1 Introduction

Dinitroanilines (e.g. trifluralin, pendimethalin, ethalfluralin or oryzalin) are important pre-emergence herbicides used for weed control in a range of crops including cotton (*Gossypium arboretum* L.), soybean (*Glycine max* (L.) Merr.), wheat (*Triticum aestivum* L.) and oilseed (*Brassica napus* L.). Trifluralin (α,α,α-trifluoro-2,6-dinitro-*N*,*N*-dipropyl-*p*-toluidine), one of the most prominent dinitroaniline herbicides, has been used extensively to control grass weeds and certain dicot weeds since its commercialization in the 1960s. Due to long-term, repeated application, field-evolved dinitroaniline resistance is known in weedy species including goosegrass (*Eleusine indica* (L.) Gaertn.), green foxtail (*Setaria viridis* (L.) P. Beauv.), Palmer amaranth (*Amaranthus palmeri* S. Wats.), annual ryegrass (*Lolium rigidum* Gaud.), annual bluegrass (*Poa annua* L.), blackgrass (*Alopecurus myosuroides* Huds.) and water foxtail (*Alopecurus aequalis* Sobol.)[1-7].

Dinitroaniline herbicides are microtubule inhibitors in plants and protists. Microtubules are a highly conserved component of the eukaryotic cytoskeleton, comprised of α- and β-tubulin heterodimers [8]. In mitosis, the microtubules form a bipolar spindle apparatus capable of correctly positioning chromosomes within the cell plane and guiding separated chromatids to opposite ends of each daughter cell. During interphase, the microtubules are critical for orchestrating cell wall synthesis in plant cells [9]. After the application of dinitroaniline herbicides, mitosis in emerging seedlings is disrupted and two daughter cells fail to separate. The seedlings display swollen roots and ultimately die because they cannot emerge from the soil [10].

Dinitroaniline herbicides inhibit microtubule formation by targeting plant tubulin proteins, which are encoded by a small multi-gene family. In dinitroaniline resistant weeds, target site resistance due to substitutions of single amino acids in one of the tubulin isoforms have been shown to endow dinitroaniline herbicide resistance. Specific amino acid substitutions in plant α-tubulin isoforms

known to endow dinitroaniline resistance include Leu-136-Phe [11], Thr-239-Ile [12], Arg-243-Lys/Met [13] and Met-268-Thr [14]. A Val-202-Phe substitution was reported in several dinitroaniline-resistant *A. aequalis* populations [15], and in *L. rigidum* populations [16, 17]. However, direct evidence is lacking on whether this Val-202-Phe substitution confers resistance, because all the other known resistance mutations are at conserved sites closer to dinitroaniline binding, whereas the 202 residue is not conserved nor involved in herbicide binding [13, 18, 19].

Lolium rigidum (annual ryegrass) is an obligate cross-pollinated diploid grass weed which has become ubiquitous throughout Australia since its initial introduction as a pasture plant in the 1800's [20]. For decades, most of the *L. rigidum* infesting cropping fields was well controlled by trifluralin. However, recently, trifluralin resistance in *L. rigidum* has been increasingly documented across Australia [21-24]. Target-site α-tubulin mutations resulting in amino acid substitutions (e.g. Val-202-Phe, Thr-239-Ile and Arg-243-Met/Lys) and non-target-site enhanced trifluralin metabolism have been reported in dinitroaniline-resistant *L. rigidum* [13, 16, 17, 25]. As the Val-202-Phe mutation was identified with a higher frequency than other tubulin mutations in resistant *L. rigidum* populations [16, 17], it is necessary to provide further evidence as to its contribution to target-site dinitroaniline resistance. Therefore, this study aimed 1) To characterise resistance to dinitroaniline herbicides in a purified *L. rigidum* sub-population homozygous for the Val-202-Phe substitution, 2) To confirm whether the Val-202-Phe substitution endows dinitroaniline resistance by using rice genetic transformation, and 3) To clarify whether non-target-site metabolic resistance is also present in this trifluralin-resistant *L. rigidum* population.

2 Materials and methods

2.1 Plant material

A trifluralin resistant *L. rigidum* population (M4/16) was originally identified in a random weed resistance survey of the Western Australian cropping region in 2010 [24]. About 60% plants in this population survived 960 g a.i. ha^{-1} trifluralin (the recommended field rate), and 90% of the survivors contained the Val-202-Phe substitution in α-tubulin [17]. Plants (more than 20 individuals) homozygous for the Val-202-Phe mutation were identified and allowed to cross-pollinate with each other to produce the purified sub population (the 202-phenylalanine-type, hereafter referred to as 202FT). Meanwhile, M4/16 plants (more than 10 individuals) without any known resistance-conferring α-tubulin mutations were used to generate the purified wild type (Val-202) sub-population (hereafter referred to as WT). In addition, an herbicide susceptible population, SVLR1 (hereafter referred to as S), [26] was included as a control in some experiments.

2.2 Response of the resistant 202FT vs WT sub-populations to dinitroaniline herbicides

It is possible that population M4/16 possesses non-target-site-based resistance to dinitroaniline herbicides which could confound analyses of the contribution of the Val-202-Phe target site mutation to resistance. In order to offset possible contributions from non-target-site mechanisms, the purified 202FT sub-population was compared with the WT (but not S) sub-population in a dose-response experiment with four dinitroaniline herbicides. The two sub-populations were sprayed with various doses of trifluralin, pendimethalin, ethalfluralin and oryzalin. There were 20 germinating seeds in each pot, three replicate pots per herbicide rate, and six rates for each herbicide (Table 1). Herbicide treatment methods and spray facilities were the same as described in Chen et al. [17]. After treatment, the pots were kept in a glasshouse under natural light at a day/night temperature of 25/15 °C, watered and fertilized regularly. This experiment was conducted in April 2017, the natural

growing season for *L. rigidum*. The number of survivors, classified as emerged seedlings with healthy new growth, was assessed 21 days after treatment.

2.3 Val-202-Phe mutation validation by rice genetic transformation

The full coding sequence of α-tubulin containing the Val-202-Phe mutation was amplified and cloned using the published primer A4F2/A4R2 [17]. DNA cassette construction, *Agrobacterium tumefaciens* transformation and rice callus transformation protocols were the same as described in Chu et al. (2018). Transformed rice calli were grown on NB-medium (3% sucrose, 0.4% N6 salts, 0.03% casamino acids, 0.2% 500×N6 vitamin, 0.29% proline, 0.03% 10 g L^{-1} 2,4-D and 0.4% gelrite, pH= 5.8) containing various rates of dinitroaniline herbicides as follows: trifluralin at 0, 5, 10, 50, 250 µg L^{-1}, pendimethalin at 0, 2.5, 7.5, 10, 20 µg L^{-1} and ethalfluralin at 0, 10, 50, 75, 200 µg L^{-1}. Petri dishes were sealed with self-sealing thermoplastic (Parafilm M, Bemis Company, Neenah WI), and kept in an incubator at 28 °C in the dark. Herbicidal symptoms in the rice calli became obvious at 21 days after treatment. DNA was isolated from randomly selected WT and 202-Phe rice calli according to Yu et al. [27]. The introduction of the transgene into rice callus DNA was confirmed by PCR using the primer pair HygF/HygR (HygF: GACCTGCCTGAAACCGAACTG; HygR: CCCAAGCTGCATCATCGAAA), followed by agarose gel electrophoresis to visualise the 562 bp PCR product. The PCR cycling was run as follows: 94°C 5 min, 35 cycles of 94°C 30 s, 55 °C 30 s, 72°C 60 s, and a final extension step of 10 min at 72°C.

2.4 Phenotype and genotype correlation analysis

Phenotype-genotype correlation analysis was conducted to determine if resistance mechanisms other than target-site tubulin mutations coexist in the 202FT sub-population. Two S×202FT parental

pair-crosses were used, and F1 seeds from the two crosses were harvested separately: one from the S parent of one pair, and one from the resistant 202FT parent of another pair. Their corresponding ψ-F2 seeds were named as F2S1 and F2R2, respectively.

The ψ-F2 seeds were imbibed on moistened filter paper at 24 °C in the dark for 48 h until just germinating (radical length approximately 5 mm), and then transplanted onto Petri dishes containing 1 µM trifluralin dissolved in 0.6% agar-water. This trifluralin concentration represents the discriminating rate for susceptible and resistant seedlings as determined in our previous study [17]. Forty-eight hours after treatment, susceptible plants with obvious symptoms of swollen roots, and normally-growing resistant plants, were removed from the agar, washed and transplanted into pots filled with potting soil (50% peatmoss, 25% sand and 25% pine bark). The phenotypes (i.e. susceptible or resistant) of the rescued seedlings (16 from F2S1 and 24 from F2R2) were recorded and the plants were kept in the glasshouse for one month with regular watering and fertilization, before leaf tissue was taken for genomic DNA isolation. The rescued seedlings were then individually genotyped by Sanger DNA sequencing for the presence or absence of the Val-202-Phe substitution, using the primer pair LrTubulinF1/5-R-seq (LrTubulinF1: GGCCTTGGTTCTCTTCTCCTT, 5-R-seq: CAGGCCATGTACTTGCCGTG), specifically for Val-202-Phe detection in *L. rigidum*. The PCR cycling was as follows: 94°C 5 min, 35 cycles of 94°C 30 s, 60 °C 30 s, and 72°C 60 s, followed by a final extension step of 10 min at 72°C. The chromatogram files of all sequences were visually checked using the Chromas software (version 2.5.1; Technelysium Pty Ltd, Australia).

2.5 Investigation of non-target-site resistance mechanisms

Seeds from S, WT and 202FT were imbibed on moistened filter paper at 24 °C for 5 days with a 12 h photoperiod until the coleoptile reached 2-3 cm. Seedlings from each population were randomly

selected and transferred into a 10 ml glass beaker (10 plants per beaker per replicate) containing 700 µl ^{14}C-trifluralin (ring-^{14}C [U]; 16 mCi/mmol stock dissolved in ethanol; American Radiolabeled Chemicals, St Louis, Missouri) solution at a concentration of 1.35 Bq/µl (equivalent to 2 µM trifluralin), ensuring all roots were immersed in the solution. The beakers were sealed with Parafilm to minimize volatilization, and kept in an incubator at 20/15 °C day/night in the dark for 48 h.

To examine for trifluralin uptake and translocation pattern, S and 202FT plants were removed from the beaker, and each was washed with 5 ml 20% methanol containing 0.2% Triton X-100, and blotted dry. Then, plants were pressed and oven-dried at 70 °C for 48 h, prior to exposure to a storage phosphor screen (BAS-IP MS2040; GE Healthcare, Little Chalfont, UK) for 24 h. Trifluralin translocation was visualised using a phosphor imager (Typhoon 5, GE Healthcare Life Sciences).

To examine for trifluralin metabolism, fresh ^{14}C-trifluralin-treated, methanol-washed S, WT and 202FT seedlings were extracted in 80% methanol and partitioned against hexane as described in Chen et al. [25], with ten seedlings per replicate and four replicates per population. Aliquots of the methanol and hexane phases were taken for scintillation counting, and the distribution of ^{14}C between the two phases was determined and compared between populations.

2.6 Statistical analysis

Dose-response curves were generated with SigmaPlot® (version 13.0; Systat Software, Inc., San Jose, CA, USA) to determine LD_{50} (the herbicide rate causing 50% seedling mortality) by non-linear regression. The data were fitted to the four-parameter logistic model:

$$y = C + (D\text{-}C)/[1+(x/x_0)^b]$$

Or the three-parameter logistic model:

$$y = \frac{D}{1 + (x/x_0)^b}$$

where *C* is the lower limit representing plant survival at infinitely large herbicide rates, *D* is the upper limit representing plant survival at low herbicide rates close to untreated controls, x_0 is the LD_{50} and *b* is the slope around x_0. Significant differences in estimated LD_{50} values between WT and 202FT, and the radioactivity distribution after partitioning among S, WT and 202FT was determined by one-way analysis of variance with post-hoc Tukey HSD Test, using the software Prism® (version 5.0, GraphPad Software, Inc., San Diego, CA). The resistance index was calculated through dividing the LD_{50} of 202FT by the LD_{50} of WT.

3 Results

3.1 Plants containing the α-tubulin mutation Val-202-Phe are resistant to dinitroaniline herbicides

From dinitroaniline dose-response curves, the LD_{50} values were obtained and compared between WT and the resistant 202FT populations. WT plants with the amino acid valine at 202 were nearly 100% controlled at the field rate of each herbicide (Table 1), while 202FT plants with the phenylalanine at 202 survived (Fig. 1). The 202FT plants displayed low levels of resistance (3- to 6-fold) to trifluralin, pendimethalin and ethalfluralin; and high level (68-fold) resistance to oryzalin (Table 2).

3.2 Rice calli transformed with the α-tubulin mutant (Val-202-Phe) gene are resistant to dinitroaniline herbicides

To establish definitively if the Val-202-Phe substitution endows dinitroaniline herbicide resistance, rice calli were transformed with the *L. rigidum* WT and 202FT α-tubulin genes. This was confirmed by PCR amplification of the 562 bp hygromycin tag gene in the expression vector (Fig. 3). Transformed

rice calli grew vigorously on the control NB medium without herbicide. However, in the presence of herbicide, the transformed WT calli with valine at 202 stopped growing and became brown on growth medium containing at least 50 μg L^{-1} trifluralin, 7.5 μg L^{-1} pendimethalin and 50 μg L^{-1} ethalfluralin, respectively (Fig. 2). In contrast, the calli transformed with the mutant α-tubulin gene Val-202-Phe proliferated normally at discriminating rates of each dinitroaniline herbicide, and higher herbicide rates (e.g. 250 μg L^{-1} trifluralin, 20 μg L^{-1} pendimethalin and over 200 μg L^{-1} ethalfluralin) were needed to inhibit their growth (Fig. 2).

3.3 Phenotype-genotype correlation analysis

For the *ψ*-F2 family F2S1, 10 seedlings were identified as resistant and six as susceptible to trifluralin. For F2R2, 10 seedlings were identified as the resistant phenotype, and 14 as the susceptible (Table 3). Sequencing results showed that susceptible phenotype was either homozygous for WT (Val-202), or heterozygous (Val-202/202-Phe) genotype. In contrast, 17 of the 20 resistant phenotypes were identified as homozygous for 202-Phe, establishing the co-segregation of the Val-202-Phe mutation with trifluralin resistance. We note that three resistant individuals from F2R2 had a wild type homozygous susceptible Val-202 genotype, suggesting that a potential non-target-site resistance mechanism is also present in the population.

3.4 Enhanced trifluralin metabolism in *L. rigidum* mutants

Root-absorbed [^{14}C]-trifluralin translocated readily throughout seedlings from both the S and 202FT plants (Fig. 4), although not examined in WT plants. After phase partitioning of tissue extracts from ^{14}C-trifluralin-treated seedlings, there was a significant difference between the S and 202FT (p<0.05), and between S and WT (p<0.01) in the relative distribution of radioactivity in the polar (methanol) and non-polar (hexane) phases (Fig. 5). No significant difference was identified between WT and

202FT plants (Fig. 5). In the methanol phase containing the polar trifluralin metabolites, more than 42% of the total extractable radioactivity was detected in the resistant 202FT and WT samples whilst less than 36% was recovered in the S samples (Fig. 5).

4 Discussion

4.1 The α-tubulin substitution Val-202-Phe endows dinitroaniline resistance in *L. rigidum*

Our previous work showed that trifluralin resistance in *L. rigidum* involves both target-site resistance (TSR) and non-target-site resistance (NTSR) [25]. This work identified the Val-202-Phe substitution as likely endowing resistance. In order to prove dinitroaniline resistance conferred by the Val-202-Phe mutation and to offset the possible contribution of NTSR, the WT sub-population, rather than an S population as published before [28], was compared to the 202FT sub-population in whole plant dose response experiments. This clearly demonstrated that the Val-202-Phe substitution endows resistance to dinitroaniline herbicides (Table 2, Fig. 1). In contrast to other confirmed α-tubulin mutations in higher plants, which occur at highly conserved amino acid residues (e.g. Thr-239 or Arg-243), the Val-202 residue is not conserved across kingdoms, as the amino acid at this site can be Phe in animals and fungi, or Val/Ile in plants and protists (Fig. 6). It is interesting that dinitroanilines are not effective inhibitors of animal or fungus microtubules, and they do not bind to these microtubules *in vitro* [29-31]. Moreover, both Val and Ile are present in different α-tubulin isoforms of Arabidopsis (Fig. 7). Therefore, to confirm the results of whole-plant studies and to provide direct evidence if the Val-202-Phe mutation is responsible for the observed resistance, rice genetic transformation was employed. This transgenic approach established that the Val-202-Phe mutation does confer resistance to dinitroaniline herbicides in *L. rigidum* (Fig. 2).

In cases documented so far, α-tubulin mutations identified in resistant weeds were either in a homozygous state, or co-occurred as two different resistance-endowing tubulin mutations. For example, homozygous Thr-239-Ile mutations were reported in *S. viridis* [11], *E. indica* [32] and *L. rigidum* [16], while the Val-202-Phe mutation together with the Leu-125-Met or Leu-136-Phe were reported in *A. aequalis* [15], and Val-202-Phe with Thr-239-Ile in *L. rigidum* [16]. It seems that more than one mutant tubulin allele or gene copies are required for dinitroaniline resistance, and this is also true in the current study, where only the homozygous Val-202-Phe mutant plants were resistant to dinitroaniline herbicides. Indeed, our recent genetic inheritance study on this resistant population revealed a recessive nature of the Val-202-Phe mutation for resistance genetic control [33]. Nevertheless, this recessive trait may not be reflected by herbicide screening using transgenic rice calli in petri dishes. This is because (1) in transgenic rice calli the mutant tubulin gene is over-expressed, and (2) the recessiveness is herbicide rate dependent and the herbicide rates used are not comparable across the two scenarios.

4.2 Enhanced trifluralin metabolism also contributes to dinitroaniline resistance in sub-population 202FT

Agar-based phenotype-genotype test on the segregating *ψ*-F2 family F2R2 identified three plants without the Val-202-Phe mutation (WT genotype) that displayed the resistant phenotype, indicating possible involvement of non-target-site resistance in these WT plants derived from the 202FT parental population. This result is consistent with our genetic study, showing more than expected numbers of plants from pseudo-F2 families surviving 480 g ha^{-1} trifluralin [33]. Indeed, using the phase partitioning method reported in our previous study [25] for diagnosis of trifluralin metabolism, more

polar trifluralin metabolites were detected in 202FT and WT plants than in S plants (Fig. 5), suggesting involvement of both TSR and NTSR in 202FT and NTSR in WT plants.

Herbicide resistance conferred by TSR and NTSR mechanism is common in resistant *L. rigidum*. For example, both target-site mutations and enhanced herbicide metabolism were found to endow resistance to ALS- and ACCase-inhibitors in many *L. rigidum* populations [27, 34-36]. In particular, in a recent study, 70% of the diclofop-resistant *L. rigidum* populations were revealed to contain both target-site and non-target-site-based resistance mechanisms [37]. Similarly, TSR and NTSR to trifluralin was demonstrated in one *L. rigidum* population [25] and is now shown in another population in this current study. Based on previous studies on enhanced herbicide metabolism in *L. rigidum* [38] and *Echinochloa phyllopogon* [39], it is likely that cytochrome P450s (P450) play a role in endowing metabolism-based trifluralin resistance. Although not examined in this current study, our previous study showed that the P450-inhibitor phorate can partially reverse trifluralin resistance in the *L. rigidum* population SLR31 [40]. P450 genes involved in trifluralin metabolism remain to be identified.

In summary, *L. rigidum* plants homozygous for the α-tubulin mutation Val-202-Phe exhibited target site resistance to dinitroaniline herbicides. Genetic transformation of rice calli confirmed that the Val-202-Phe mutation endows dinitroaniline resistance. Genotype-phenotype correlation analysis and quantification of trifluralin metabolism revealed both TSR and NTSR to dinitroaniline in this *L. rigidum* population.

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Table 1 Dinitroaniline herbicide rates applied to WT and 202FT populations in a whole-plant dose-response study.

Dinitroaniline Herbicides	Field rate (g ha^{-1})	Population	Rates applied (g ha^{-1})
Trifluralin (TriflurX® 480g	960	WT	0, 120, 180, 240, 720, 960, 1440
a.i.L^{-1}, Nufarm)		202FT	0, 240, 720, 960, 1440, 1920
Pendimethalin (Rifle®440,	594	WT	0, 31, 62, 124, 248, 495, 990
440 g a.i. L^{-1}, Nufarm)		202FT	0, 62, 124, 248, 495, 990
Ethalfluralin (Sonalan 35.4%,	360	WT	0, 22.5, 90, 180, 360, 540, 1080

Dow®)		202FT	0, 90, 180, 360, 540, 1080
Oryzalin (Surflan® 480 g a.i.	500	WT	0, 15, 31, 62, 125, 250, 500,
L^{-1}, Villa Crop Protection)			2000
		202FT	0, 125, 250, 500, 2000, 4000

Table 2 Parameter estimates of the nonlinear logistic analysis of the whole-plant dose-response to dinitroaniline herbicides for the purified WT and 202FT *Lolium rigidum* populations. Standard errors are in parentheses.

Herbicide	Population	*D*	*C*	*b*	LD_{50} (g ha^{-1})	*P* [a]	Resistance index [b]
Trifluralin	WT	101 (7)	3.7 (6)	2.3 (1)	204 (27)	<0.01	6
	202FT	99.6 (1)	-15.2 (9)	2.9 (0.3)	1230 (81)		
Pendimethalin	WT	99.2 (4)	3.7 (4)	2.4 (0.4)	106 (9)	<0.01	4
	202FT	98.5 (1)	23.8 (2)	4.2 (0.6)	457 (12)		
Ethalfluralin	WT	99.8 (1)	-0.7 (2)	2.8 (0.2)	174 (5)	<0.01	3
	202FT	99.0 (1)	14.5 (2)	6.3 (0.5)	465 (8)		
Oryzalin	WT	100 (3)	10.4 (2)	3.5 (0.6)	57 (3)	<0.01	68
	202FT	97 (1)	-	5.5 (1)	3889 (71)		

[a] Significance of a t-test comparison of LD_{50} values between the WT and 202FT.

[b] Resistance index was calculated as the ratio of 202FT: WT LD_{50} values.

Table 3 Correlation analysis of trifluralin resistance phenotype and the α-tubulin genotype at the Val-202 site in the two ψ-F2 (F2S1 and F2R2) segregating families.

F2 families	Phenotype	Plants number	Plants number of different genotypes		
			Phe/Phe	Phe/Val	Val/Val
F2S1	Resistant	10	10	0	0
	Susceptible	6	0	2	4
F2R2	Resistant	10	7	0	3
	Susceptible	14	0	5	9

Fig. 1 Dose response curves of *Lolium rigidum* population WT (filled circles) and 202FT (open circles) treated with dinitroaniline herbicides (a. trifluralin, b. pendimethalin, c. ethalfluralin, d. oryzalin), 21 days after treatment. Each data point represents the mean ± standard error of three replicates in a single dose-response experiment.

Fig. 2 Growth of rice calli transformed with the WT or mutant Phe-202 α-tubulin cDNA in media containing the indicated concentrations of trifluralin, pendimethalin or ethalfluralin. The control medium contained no herbicides but the same amount of DMSO as was used as a solvent for the herbicides. Photos were taken 21 days after treatment.

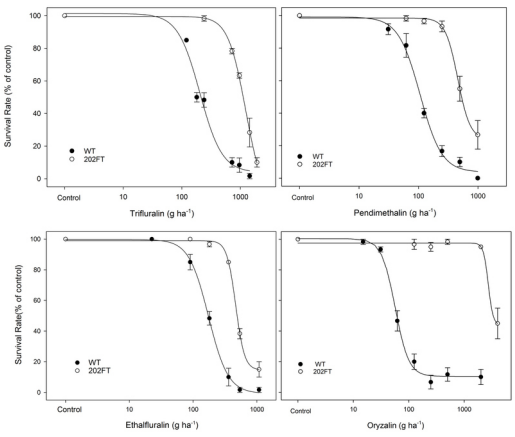
Fig. 3 Confirmation of rice callus transformation using PCR amplification of the hygromycin tag. Lanes 1-5 show the PCR products using DNA from calli transformed with the wild type tubulin transcript, and lane 6-10 from calli transformed with the 202-Phe tubulin transcript. The length of the hygromycin tag is 562 bp.

Fig. 4 Phosphor image of [^{14}C]-trifluralin-treated S (upper panel) and 202FT (lower panel) seedlings showing no visual differences in trifluralin uptake and translocation, 48 h after treatment. Roots of seedlings were immersed in [^{14}C]-trifluralin solution for 48 h prior to washing, oven-drying and imaging.

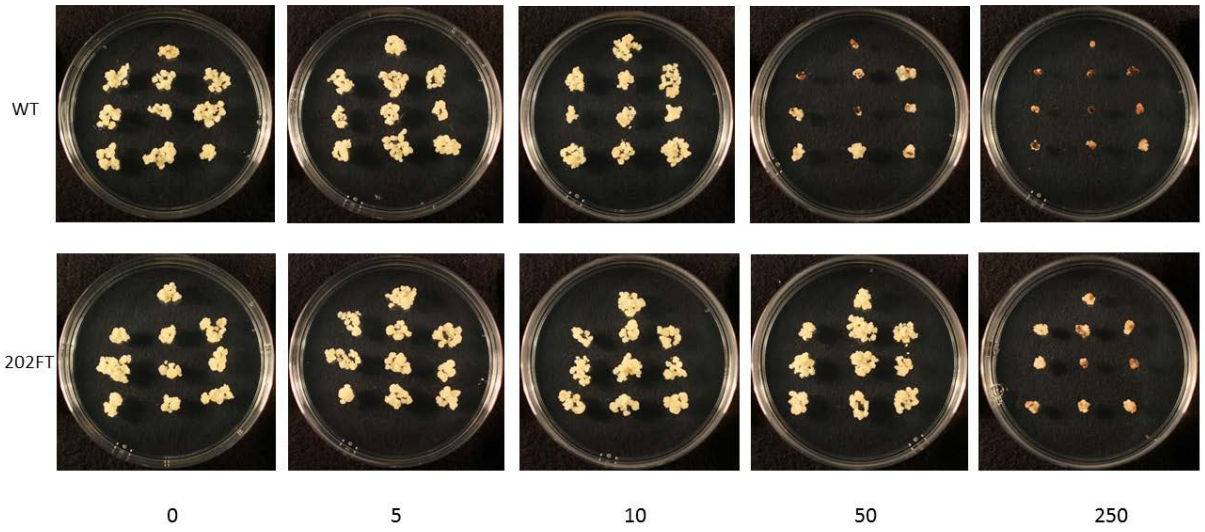
Fig. 5 Distribution of recovered radioactivity in the nonpolar (100% hexane) and polar (80% methanol) phases of extracts from the S, WT and 202FT populations treated with [^{14}C]-trifluralin. Values are means ± standard errors (n = 4), and asterisks above the bar denote differences between populations in the proportion of ^{14}C partitioning into the methanol phase (**: p<0.01, *: p<0.05, ns: no significant difference).

Fig. 6 Alignment of partial α-tubulin protein sequences from different species across the animal, fungus, plant and protist kingdoms. The 202 site is highlighted in red bold font. Sequences were aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo). All protein sequence numbers are from GenBank except the annual ryegrass sequence. The corresponding GenBank numbers are: human (P68363.1), crab-eating macaque (Q4R538.1), Chinese hamster (P68365.1), *Pneumocystis carinii* (P53372.1), *Zymoseptoria tritici* (O94128.1), *Neurospora crassa* OR74A (P38669.2), *Toxoplasma gondii* (P10873.1), *Chlamydomonas reinhardtii* (P09204.1), *Chlorella vulgaris* (Q9ZRJ4.1), rice (P28752.1), goosegrass (O22347.1), maize (P22275.1), Arabidopsis (P29511.1), wheat (Q9ZRB7.1), barley (Q96460.1).

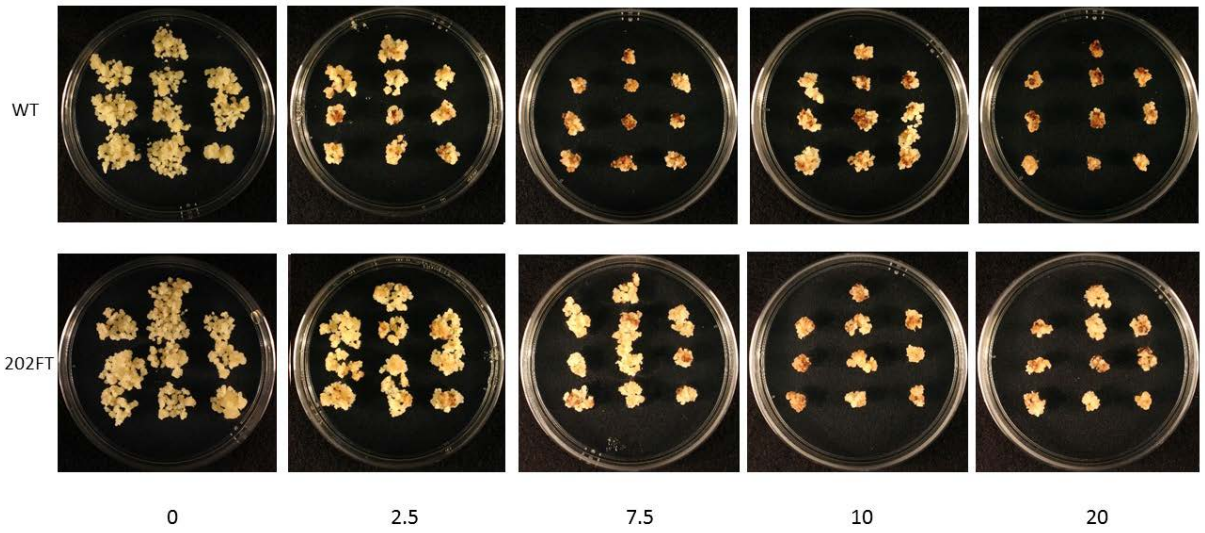
Fig. 7 Alignment of partial α-tubulin protein sequences of *Arabidopsis thaliana* showing variations in the 202 residue between α-tubulin isoforms TBA1-TBA6. Data are from https://www.arabidopsis.org. The 202 site is highlighted in red bold font.



PS_5561_Figure 1.JPG



Trifluralin (µg L^{-1})



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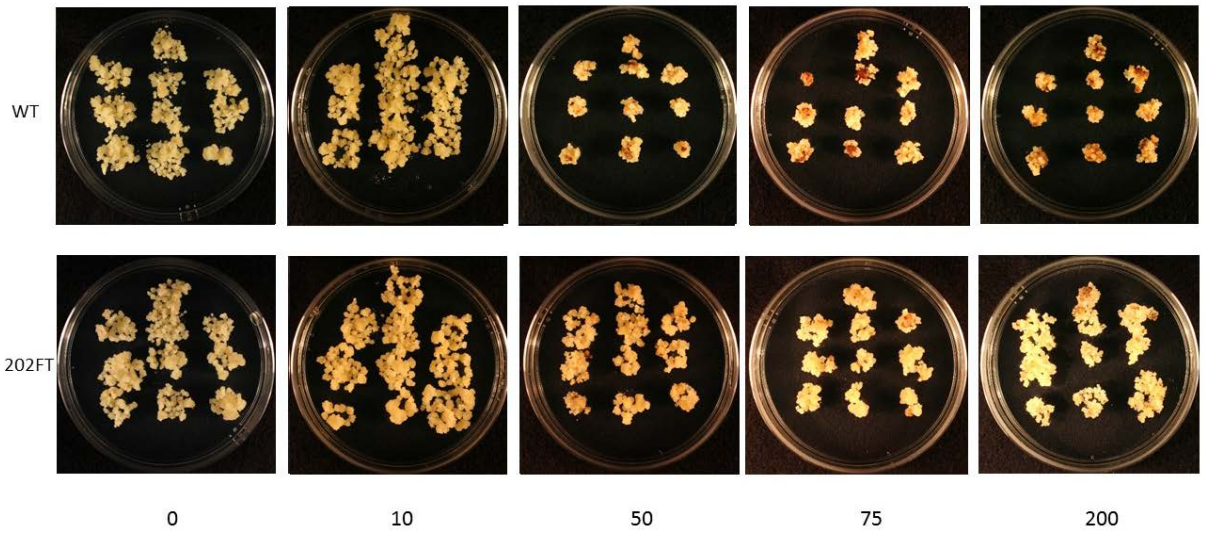
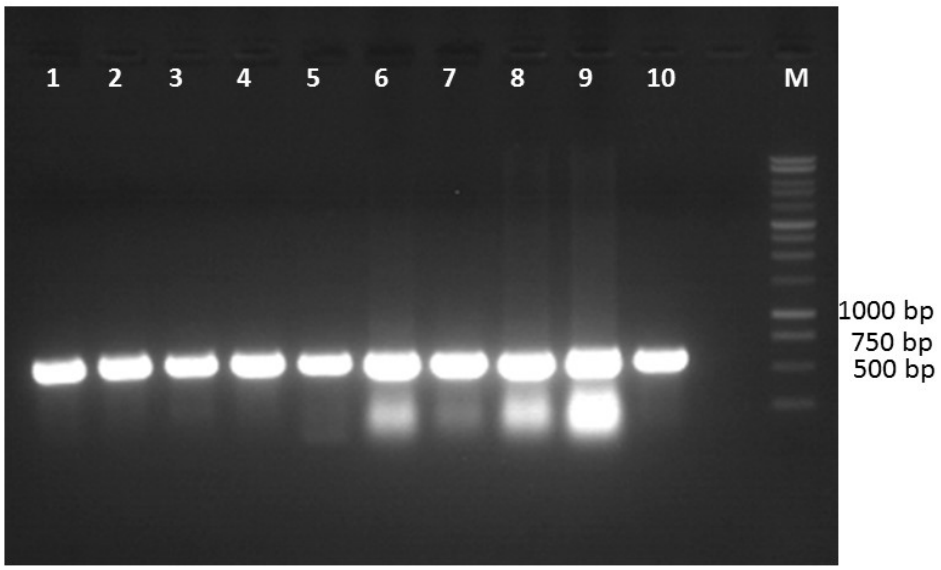


Ethalfluralin (µg L^{-1})



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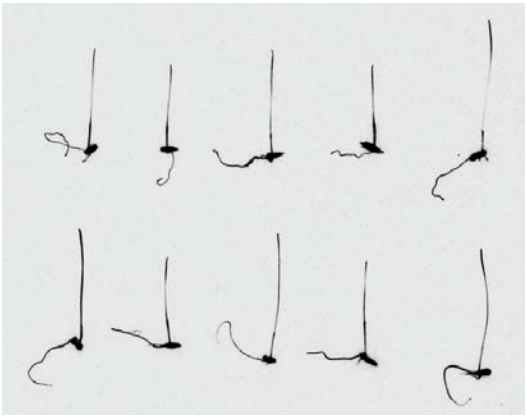
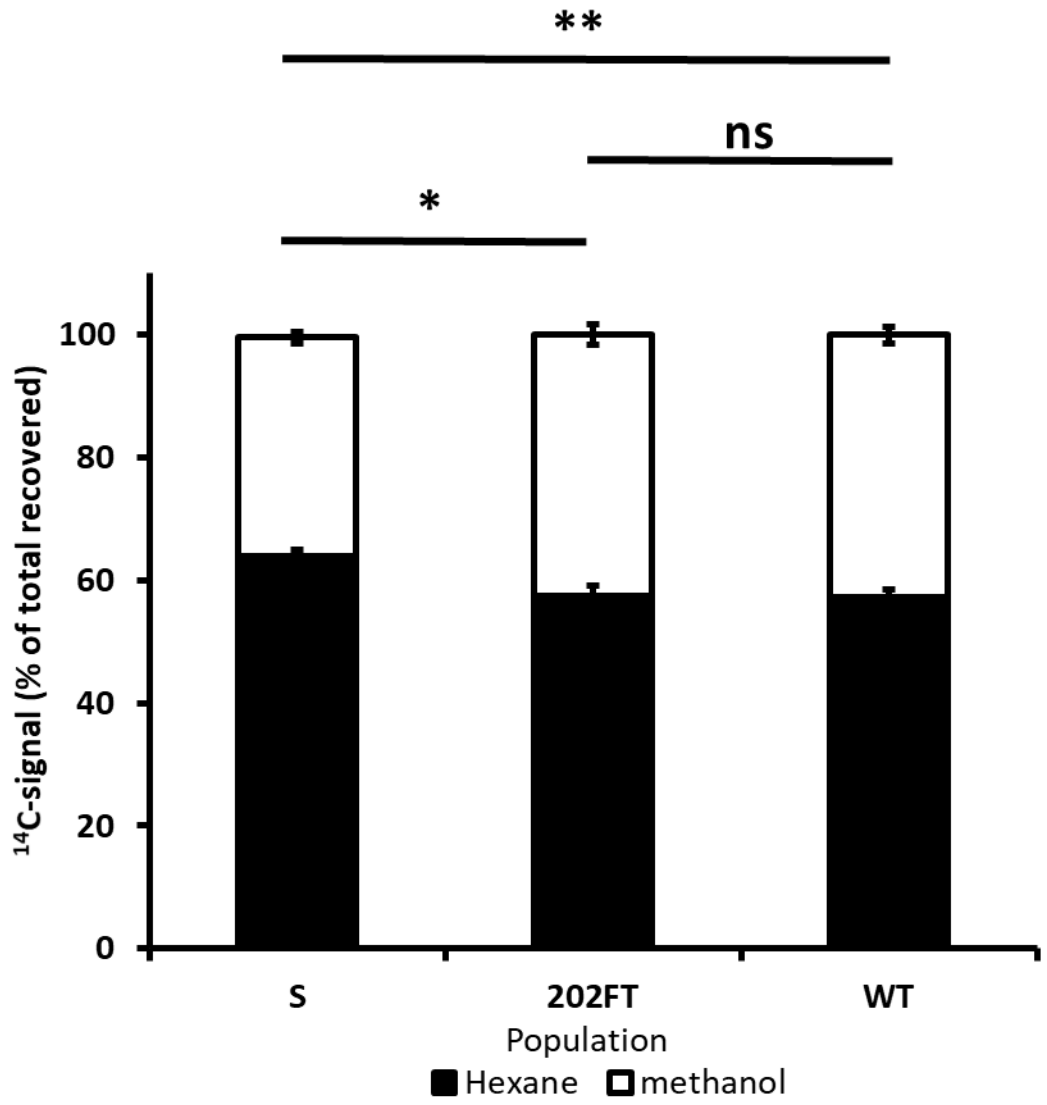
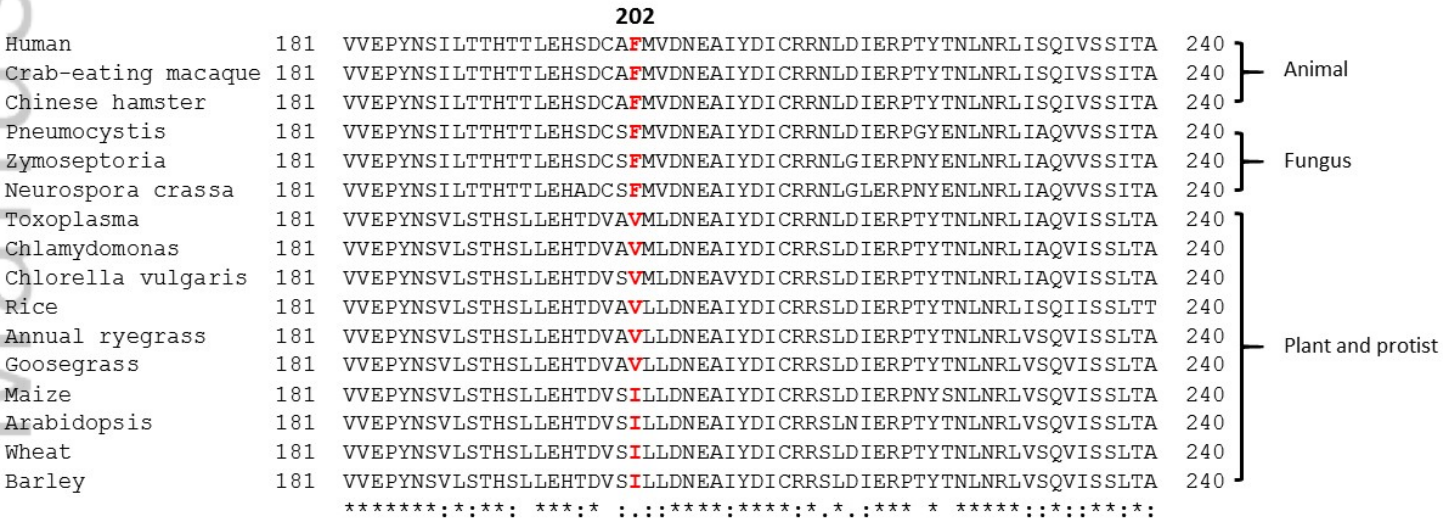


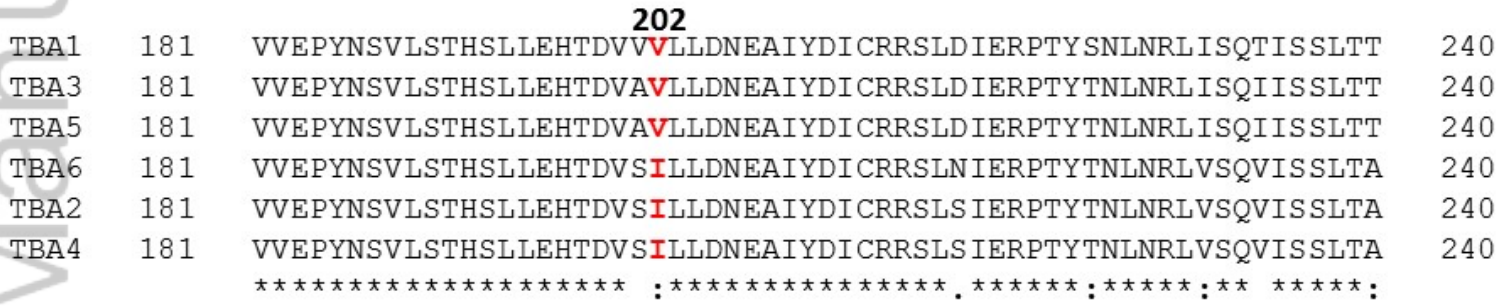
S

202FT





PS_5561_Figure 6.tif

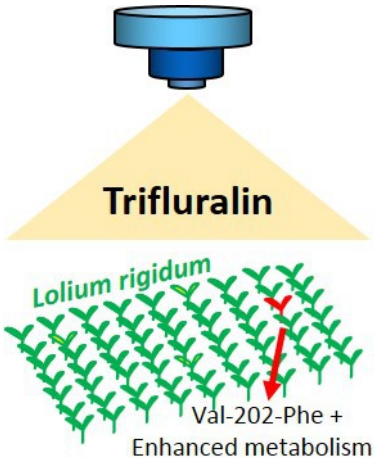


PS_5561_Figure 7.tif

Target-site and non-target-site resistance to dinitroaniline herbicides in *Lolium rigidum*

Jinyi Chen, Zhizhan Chu, Heping Han, Danica Goggin, Qin Yu*, Chad Sayer, Stephen Powles

Dinitroaniline resistance in *Lolium rigidum* has reached a concerned level in Australian agriculture. This article demonstrated that α-tubulin target-site mutation Val-202-Phe and enhanced trifluralin metabolism conferred resistance in *L. rigidum*.



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