Research Article

Received: 16 June 2020 Revised: 18 September 2020 Accepted article published: 29 September 2020 Published online in Wiley Online Library:

(wileyonlinelibrary.com) DOI 10.1002/ps.6109

Diversity of α-tubulin transcripts in *Lolium rigidum*

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Abstract

BACKGROUND: Tubulin, the target site of dinitroaniline herbicides, is encoded by small gene families in plants. To better characterize the mechanisms of target-site resistance to dinitroaniline herbicides in the globally important weedy species *Lolium rigidum*, attempts were made to amplify and sequence α-tubulin transcripts.

RESULTS: Four α-tubulin isoforms (TUA1, TUA2, TUA3 and TUA4) were identified in *L. rigidum*. Variations in the number and sequence of transcripts encoding these α-tubulin proteins were found in individuals from the two *L. rigidum* populations examined. Within and among populations, differences in the 5′- and 3′-untranslated regions of cDNA in TUA3 and TUA4 were identified. Furthermore, a novel double mutation, Arg-390-Cys+Asp-442-Glu, in the TUA3 transcript was identified and has the potential to confer dinitroaniline resistance.

CONCLUSION: This research reveals the complexity of the α-tubulin gene family in individuals/populations of the cross-pollinated weedy species *L. rigidum*, and highlights the need for better understanding of the molecular architecture of tubulin gene families for detecting resistance point mutations. Although TUA4 is a commonly expressed α-tubulin isoform containing most frequently reported resistance mutations, other mutant tubulin isoforms may also have a role in conferring dinitroaniline resistance.

Supporting information may be found in the online version of this article.

Keywords: Tubulin; mutation; microtubule inhibitors; *Lolium rigidum*; dinitroaniline resistance

1 INTRODUCTION

Microtubules, comprised principally of heterodimers of one α- and one β-tubulin polypeptide, are filamentous protein complexes with crucial roles in a number of diverse cellular processes.[1] Microtubules are intrinsically dynamic and can alternate between phases of polymerization and spontaneous depolymerization, in a process of dynamic instability, to perform various functions.[2, 3] Principally, microtubules constitute the major structural components of mitotic and meiotic spindles in eukaryotic organisms during cell division.[4] They also participate in several aspects of intracellular transport, in concert with actin filaments and intermediate filaments for cytoskeleton architecture.[5]

Several α- and β-tubulins are utilized to form functionally distinct microtubules. Incorporation of specific tubulin variants (known as tubulin isoforms) can control microtubule protofilament number and stability.[6, 7] The multi-tubulin family has been investigated at the molecular level in a variety of organisms, in particular for α-tubulin. For instance, in *Arabidopsis*, at least six α-tubulin isoforms are expressed.[8] At least eight α-tubulin genes have been identified in maize, and two in rice.[9, 10] In animals, there are nine α-tubulin genes in humans[11] and four in fruit flies.[12, 13]

Given their essential roles and ubiquitous distribution in cells, microtubules are excellent targets for disruptive chemicals, such as medical drugs and pesticides. Anti-microtubule chemicals include anti-tumour medicines (e.g. paclitaxel, colchicine, vinblastine and taxol), fungicides (e.g. benzimidazoles, griseofulvin) and herbicides (e.g. dinitroanilines, phosphoric amides and *N*-phenyl carbamates), depending on the target organism or cell type. These anti-microtubule agents interrupt the dynamic balance of microtubules, either as microtubule-stabilizers that maintain microtubule polymerization, or as destabilizers that promote microtubule depolymerization.[14-18]

With extensive application of anti-microtubule chemicals, evolution of resistance is inevitable. Here, we focus on resistance to the microtubule-disrupting dinitroaniline herbicides (trifluralin, pendimethalin, etc.) in crop weeds. In resistant weedy plants,

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target-site mutations in α-tubulin proteins confirmed to confer dinitroaniline resistance include Leu-136-Phe,[19] Val-202-Phe,[20] Thr-239-Ile,[21] Arg-243-Lys/Met[22] and Met-268-Thr.[23] In resistant weedy species studied to date, despite the presence of numerous tubulin isoforms, the target-site mutations identified have been largely located in a specific α-tubulin gene. For instance, in the weedy species *Lolium rigidum*, mutations at sites 202, 239 and 243 were all mapped in one isoform α-tubulin 4 (TUA4).[24] Considering the multiple isoforms of tubulin, the redundancy in isoform function, and the level of dinitroaniline resistance in *L. rigidum*, we hypothesize that in addition to TUA4, mutations in other α-tubulin isoforms may also contribute to resistance. In this study, we attempt to: (i) characterize the α-tubulin gene family in *L. rigidum*, (ii) look for potential dinitroaniline resistance tubulin mutations in the isoforms identified, and (iii) validate the functions of newly found α-tubulin mutations using rice genetic transformation.

2 MATERIALS AND METHODS

2.1 Origin of the plant material

Two *L. rigidum* populations were used in this research. The susceptible control population, SVLR1 (hereafter referred to as S), has no herbicide application history and is normally susceptible to all herbicides active on this species. The resistant population was purified from M4/16 collected originally from a cropping field in Western Australia.[25] This resistant population (referred to originally as 202FT and hereafter as R) was reported to contain the Val-202-Phe α-tubulin mutation.[20] Leaf tissue (~ 500 mg) was collected from four untreated S plants, and eight R plants that survived 960 g trifluralin ha^{-1}. The trifluralin treatment method and plant growth conditions were as described previously.[24] RNA was extracted with the ISOLATE II RNA Plant Kit (Bioline, Alexandria, Australia), and reverse-transcribed into cDNA using SuperScript® III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). The cDNA was used as the template in subsequent molecular experiments.

2.2 Amplification and cloning of α-tubulin transcripts in *L. rigidum*

Three new pairs of primers were designed for different isoforms according to published *L. rigidum* transcriptome sequences[26, 27] and α-tubulin sequences from *Eleusine indica* (AJ005599.1), *Setaria viridis* (AJ586805.1) and *Alopecurus aequalis* (AB514115.1–AB514118.1). Information on all α-tubulin primers for TUA1–TUA4 is listed in Table 1, including the pair A4F/R for TUA4 published previously.[24] These primers are located in the untranslated region (UTR), except A1F/A1R. With cDNA as the template, a polymerase chain reaction (PCR) was performed with the following cycles: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, X °C for 30 s, and 72 °C for 90 s, followed by a final extension step of 7 min at 72 °C (here, X is the corresponding annealing temperature of each pair of primers) (Table 1). All successfully amplified fragments were purified from agarose gel with the Wizard SV gel and PCR clean-up system (Promega, Sydney, Australia), and initially sequenced with the PCR primers in Table 1. Sequencing results were checked visually using the software Chromas (version 2.5.1; Technelysium Pty Ltd, South Brisbane, Australia).

For TUA3 and TUA4, sequencing with amplification PCR primers failed repeatedly due to multiple peaks in nucleotide chromatography. The presence of insertion/deletion sequences in the UTR post the sequencing primer was hypothesized. Thus, one S and one R plant (referred as S1 and R1 respectively hereafter) were used for sequence cloning to detect differences. Purified PCR products of TUA3 and TUA4 from S1 and R1 were ligated to the pGEM-T Easy Vector (Promega). After successful transformation into *Escherichia coli* competent cells (Promega), eight colonies were selected for PCR re-amplification. Recombinant plasmids were isolated using the Wizard Plus SV Minipreps DNA Purification Systems and sequenced with M13F (5′-GTTTTCCCAGTCACGAC-3′)/M13R (5′-CAGGAAACAGCTATGAC-3′) primers. Plasmid inserts were sequenced using the primers listed in Table 2.

All nucleotide and predicted-protein alignments were conducted online with the Clustal Omega tool run by EMBL-EBI (https://www.ebi.ac.uk/Tools/msa/clustalo/). Single nucleotide polymorphisms (SNPs) and amino acid substitutions of the four transcripts (TUA1–TUA4) were checked between S and R plants. Protein secondary structure prediction was deduced using an online secondary structure prediction server.[28] An optimal

Table 2. Sequencing primers for different α-tubulin isoforms in *Lolium rigidum*

α-Tubulin isoform	Primer name	Primer sequence (5′–3′)
TUA2	TUA2seqF1	GGATTCTTGGTGTTCAATGCTGTC
	TUA2seqF2	CCGAGAAGGCTTACCATGAG
	TUA2seqR1	TCTTTCCATAATCAACTGAGAGGC
TUA3	TUA3seqF1	TACACAGTTGGAAGAGAAGTAGTG
	TUA3seqF2	CCGTTCCAGAGATCACCAAC
	TUA3seqR1	CAGATGTCGTAGATGGCCTC
TUA4	TUA4seqF1	GGCCTTGGTTCTCTTCTCCTT
	TUA4seqF2	GCGCACTATCCAGTTTGTGG
	TUA4seqR1	GGGTGGACAGCACACTGTTGTATG

Table 1. PCR primer pairs and annealing temperatures for amplification of the four α-tubulin isoforms from *Lolium rigidum*

Target transcript	Primer	Sequence 5′–3′	Annealing temperature (°C)	Length of target transcript (bp)
α-Tubulin1	A1F	TCGGCAACGCGTGCTGGGAACTTTA	55	1520
	A1R	TTAATGAAGCAGGACAAGACCGAAG		
α-Tubulin2	A2F	CTTCTCCTCCGCCCCGCCCCCTTTT	57	1530
	A2R	ATACAGAACATAGAGCCAATCATTA		
α-Tubulin3	A3F	TCTGGCTGCTTCCTCTTGTTCGTGC	57	1457
	A3R	TTTCCGGCCAAGCTCAACTCAGTAC		
α-Tubulin4	A4F	ACCGCAGGGACAGGCGTCTTCGTAC	56	1633
	A4R	CGAAGCAGGTTCAAACATAGCACAG		

phylogenetic tree for α-tubulin protein sequences was generated using the tool MEGA (v. 10.1.8) with the sum of branch length = 0.52. The tree included α-tubulin protein sequences from model plants: *A. thaliana* and *Oryza sativa*, and from other weedy species: *E. indica*, *S. viridis* and *A. aequalis*. TUA6 from humans was used as an outgroup.[29]

2.3 Rice calli transformation and growth response to trifluralin

Transgenic studies were conducted in rice calli transformed with vectors containing wild-type or mutant α-tubulin cDNA. Vector construction was similar to that published in Chu *et al.*,[22] except that the insertion α-tubulin fragment was replaced, and no hemagglutinin or c-myc epitope tags were attached. Vectors containing the three mutant α-tubulin cDNAs (i.e. the single mutation Arg-390-Cys, Asp-442-Glu, and the double mutation Arg-390-Cys +Asp-442-Glu) were constructed. Transcripts carrying the Asp-442-Glu or double mutation were sequences cloned originally from *L. rigidum* R plants, whereas the Arg-390-Cys transcript was generated by site-directed mutagenesis on the wild-type transcript using the primer pairs: α-Tub3-KpnI-F (5′-AAAAAGGTACCATGA GGGAGATCATCAGCATC-3′)/R390C-R (5′-GAACTTGCGGTCGATGC ACGAGAACACCTCAG-3′) and R390C-F (5′- CTGAGGTGTTCTCG TGCATCGACCGCAAGTTC-3′)/α-Tub3-SpeI-R (5′-AAAACACTAGTT-CAGTACTCCTCGC CTTC-3′) (see Appendix S1).

Successfully transformed rice calli are able to grow normally on media containing hygromycin. These calli were then tested for growth in the presence of trifluralin. The conditions for calli

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TUA3      MREIISIHIGQAGIQVGNSCWELYCLEHGIHPDGLMPSDTSVGVAKDAFNTFFSETGSGK      60
TUA2      MREIISIHIGQAGIQVGNACWELYCLEHGIQQDGTMPSDTTVGVAHDAFNTFFSETGAGK      60
TUA1      ----------------------------------TSGDKTIGGGDDAFNTFFSETGAGK      25
TUA4      MRECISIHIGQAGIQVGNACWELYCLEHGIQPDGQMPGDKTVGGGDDAFNTFFSETGAGK      60
                                              *   *   *********** **

TUA3      HVPRALFVDLEPTVIDEVRTGAYRQLFHPEQLISHNEDAANNFARGHYTVGREVVDLCLD      120
TUA2      HVPRAIFVDLEPTVIDEVRTGAYRQLFHPEQLISGKEDAANNFARGHYTVGKEIVDLCLD      120
TUA1      YVPRAVFVDLEPTVIDEVRTSAYRQLFHPEQLISGKEDAANNFARGHYTIGKEIVDLCLD      85
TUA4      HVPRAVFVDLEPTVIDEVRTGTYRQLFHPEQLISGKEDAANNFARGHYTIGKEIVDLCLD      120
           **** **************  ************  ************* * * ******

TUA3      RIRKLADNCTGLQGFLVFNAVGGGTGSGLGSLLLERLSVDYGRKSKLGFTIYPSPQISTA      180
TUA2      RVRKLADNCTGLQGFLVFNAVGGGTGSGLGSLLLERLSVDYGKKSKLGFTIYPSPQVSTA      180
TUA1      RIRKLADNCTGLQGFLVFNAVGGGTGSGLGSLLLERLSVDYGKKSKLGFTVYPSPQVSTS      145
TUA4      RIRKLADNCTGLQGFLVFNAVGGGTGSGLGSLLLERLSVDYGKKSKLGFTVYPSPQVSTS      180
          * ************************************** ******* ***** **

TUA3      VVEPYNSVLSTHSLIEHTDVVVMLDNEAIYDICKRSLCIERPSYTNLNRLISQVISSLTT      240
TUA2      VVEPYNSVLSTHSLLEHTDVAVLLDNEAIYDICRRSLDIERPTYTNLNRLISQIISSLTT      240
TUA1      VVEPYNSVLSTHSLLEHTDVSILLDNEAIYDICRRSLDIERPTYTNLNRLVSQVISSLTT      205
TUA4      VVEPYNSVLSTHSLLEHTDVAVLLDNEAIYDICRRSLDIERPTYTNLNRLVSQVISSLTA      240
          ************** *****   ********** *** **** ******* ** *****

TUA3      SLRFDGAINVDITEFQTNLVPYPRIHFMLSSYAPIISAEKAFHEQHSVPEITNSVFEPAS      300
TUA2      SLRFDGAINVDVTEFQTNLVPYPRIHFMLSSYAPVISAEKAYHEQLSVPEITNAVFEPSS      300
TUA1      SLRFDGALNVDVTEFQTNLVPYPRIHFMLSSYAPVISAEKAYHEQLSVSEITNSAFEPSS      265
TUA4      SLRFDGALNVDVNEFQTNLVPYPRIHFMLSSYAPVISAEKAYHEQLSVAEITNSAFEPSS      300
          ******* ***  ********************* ****** *** ** ****  *** *

TUA3      VMAKCDPRHGKYMACCLMYRGDVVPKDVNAAVHSIKTKRTVQFVDWCPTGFKCGINYQPP      360
TUA2      MMAKCDPRHGKYMACCLMYRGDVVPKDVNAAVATIKTKRTVQFVDWCPTGFKCGINYQPP      360
TUA1      MMAKCDPRHGKYMACCLMYRGDVVPKDVNAAVATIKTKRTIQFVDWCPTGFKCGINYQPP      325
TUA4      MMAKCDPRHGKYMACCLMYRGDVVPKDVNAAVATIKTKRTIQFVDWCPTGFKCGINYQPP      360
           ******************************  ****** ******************

TUA3      TVVPGGDLAKVRRAVCMISNNTAVAEVFSRIDRKFDLMYAKRAFVHWYVGEGMEEGEFSE      420
TUA2      SVVPGGDLAKVQRAVCMISNNTAVAEVFARIDHKFDLMYAKRAFVHWYVGEGMEEGEFSE      420
TUA1      TVVPGGDLAKVQRAVCMISNSTSVVEVFSRIDHKFDLMYAKRAFVHWYVGEGMEEGEFSE      385
TUA4      SVVPGGDLAKVQRAVCMISNSTSVVEVFSRIDHKFDLMYAKRAFVHWYVGEGMEEGEFSE      420
           ********** ******** * * *** *** ***************************

TUA3      AREDLAALEKDYEEVGGEGEED--EDEGEEY  449
TUA2      AREDLAALEKDYEEVGAEGAD-DDGDDGDDY  450
TUA1      AREDLAALEKDYEEVGAEGGDDEEGEDEDDY  416
TUA4      AREDLAALEKDYEEVGAEFDEGEDGDEGDEY  451
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Figure 1. Alignment of deduced amino acid sequences of the four α-tubulins from S *Lolium rigidum*. '–' denotes gaps for alignment; '*' indicates a conserved residue.

culture were as described previously,[22] except that the trifluralin rates used in this study were 0, 400, 800, 1600 and 3200 μg L^{-1}. Photographs were taken 21 days after treatment and calli growth was evaluated. The whole transformation experiment was repeated.

3 RESULTS

3.1 Multiple α-tubulin transcripts in *L. rigidum*

Overall, four α-tubulin isoforms (TUA1, TUA2, TUA3 and TUA4) were deduced from the transcripts in four S and eight R *L. rigidum* plants examined. In S plants, TUA1 and TUA2 were each encoded by one transcript, and TUA3 and TUA4 were each encoded by two transcripts. The only difference between the two transcripts coding TUA3 or TUA4 occurs in the UTR. In R plants, TUA1, TUA2 and TUA4 were each encoded by one transcript, and TUA3 by two transcripts. Again, the difference between the two TUA3 transcripts exists in the UTR only. Full coding sequences (CDS) of TUA2, TUA3 and TUA4 were obtained, and a major part of the TUA1 sequences was amplified in both S and R. The CDS of TUA1–TUA4 were submitted to NCBI database with Accession numbers MT514930 to MT514933 for R and MT514934 to MT514937 for S. Transcripts of TUA2, TUA3 and TUA4 contain a CDS length of 1353, 1350 and 1356 bp, respectively, and share 71.2% similarity in nucleotide identity. The corresponding α-tubulin gene product is predicted to be 450, 449 and 451 amino acids, with a molecular weight of 49.57, 49.88 and 49.74 kDa, respectively. Alignment of deduced tubulin proteins TUA1–TUA4 with S is shown in Fig. 1. Together, the four tubulin proteins share 72% similarity in identity. Very high amino acid similarities (94.5%) were observed among TUA2–TUA4 (Fig. 1). However, when short 5′- and 3′-UTRs of TUA3 and TUA4 were obtained by cloning, and the alignment showed significant sequence differences, both within and between S and R (Fig. 2).

Diversity of α-tubulin nucleotide sequences was identified among individuals and between populations. In four S plants analysed, all four α-tubulin isoforms were successfully amplified as indicated by the targeted length of PCR fragments in an agarose gel (Fig. S1). In R plants, the same primer worked for targeted

TUA3 5′ UTR

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R_T1          TCTGGCTGCTTCCTCTTGTTCGTGCAGGTACTAATTCTGAAGATCGGAGGCTGCAGAAGA
R_T2          TCTGGCTGCTTCCTCTTGTTCGTGCAGGTACTAATTCTGAAGATCGGAGGCTGCAGAAGA
S_T1          TCTGGCTGCTTCCTCTTGTTCGTGCAGGTACTAATTCTGAAGATCGGAGGCTGCAGAAGA
S_T2          TCTGGCTGCTTCCTCTTGTTCGTGCTGCTACTAATTCTGAAGATCGGAGGCGACAGA---
              ************************* * ********************** ****

R_T1          GAAGAGCAGAGCAGAGCAGAGCAGAAGAACAGAGAGAGGAGATGAGGGAGATCATCAGCA
R_T2          GAAGAGCAGAGCAGAG--------CGGAACAGAGAGAGGAGATGAGGGAGATCATCAGCA
S_T1          GAAGAGCAGAGCAGA----------AGATCAGAGGGAGGACATGAGGGAGATCATAAGCA
S_T2          -----GCAGAGAAGA----------AGAACAGAGAGAGGAGATGAGGGAGATCATAAGCA
                   ****** ***           ** ***** ***** ************** ****
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TUA4 5′ UTR

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R             CTCCTCC---TCTTCGTCGGCAGCGAAGTAGCCACCAGCCACCATGAGGGAGTGCAT
S_T1          ---CTCCATTTCGTCGGCGGCGAAGTAGCC--AGCCAGCCGCCATGAGGGAGTGCAT
S_T2          ---CTCCATTTCGTCGGCGGCGAAGTAGC---CAGCCACCACCATGAGGGAGTGCAT
                 ****    * *** * **   * **       *  ** ****************
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TUA4 3′ UTR

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R             GCGATGAGTACTAGAGCCTGCATCCTGGTGCTTTCCCAAGGCTGG--ACTACTGCTATCC
S_T1          GCGATGAGTACTAGAGCCTGCATCCTGGTGCTTTCCCAAGGCCGACTTCTACTGCTATCC
S_T2          GCGATGAGTACTAGAGCCTGCATCCTGGTGCTTTCCCAAGGCCGACT---ACTGCTATCC
              ****************************************** *      **********

R             AGTGATCTGCCCTGAGTGGCTTTATCTGTTATCTGTTTGTTTCAATGTTTGCTTTGTTGT
S_T1          AGTGATCTGCCATGAGTGGCTTTATCTGTTATCAGTTTGTTTGAACGTTTGCTTTG---T
S_T2          AGTGATCTGCCATGAGTGGCTTTATCTGTTATCTGTTTGTTTGAACGTTTGCTTTG---T
              *********** ******************** ******** ** **********   *

R             GACGTTTGGTTTACAACCTGTTCCGTTGTAAGAACCTGTGCTATGTTTGAACCTG
S_T1          GATGTTTGGTTTACAACCTGTTCCGTTGTAAGAACCTGTGCTATGTTTGAACCTG
S_T2          GATGTTTGGTTTACAACCTGTTCCGTTGTAAGAACCTGTGCTATGTTTGAACCTG
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Figure 2. Differences in 5′/3′-UTR identified in two α-tubulin transcripts (TUA3 and TUA4) from S and R *Lolium rigidum*. The start codon ATG and stop codon TAG are in bold. T1 and T2 mean Type 1 and Type 2 found in the same individual. '–' denotes gaps for alignment; '*' indicates a conserved residue.

Table 3. Amplification and direct sequencing results of the four α-tubulin isoforms using PCR primers in Table 1, from susceptible (S) and resistant (R) *Lolium rigidum*

	TUA1		TUA2		TUA3		TUA4	
Sample	Amplification	Sequencing	Amplification	Sequencing	Amplification	Sequencing	Amplification	Sequencing
S1	√	√	√	√	√	x	√	x
S2	√	x	√	√	√	√	√	x
S3	√	√	√	√	√	x	√	x
S4	√	√	√	x	√	x	√	x
R1	√	x	√	x	√	x	√	√
R2	x	x	√	√	√	x	√	√
R3	√	√	√	√	√	x	√	x
R4	x	x	√	√	√	√	√	NA
R5	x	x	√	√	√	√	√	NA
R6	x	x	√	√	√	√	√	NA
R7	x	x	√	√	√	√	√	NA
R8	√	√	√	√	√	√	√	NA

√, PCR band can be seen in the agarose gel after electrophoresis.
x, either the concentration of the purified PCR product is too low for sequencing, or the sequences cannot be clearly identified due to multiple peaks in sequencing chromatograms.
NA, not available. Instead, these fragments were directly sequenced using the sequencing primers in Table 2.

Table 4. Mutations in different α-tubulin transcripts identified in resistant *Lolium rigidum*

α-Tubulin isoforms	Mutations detected in resistant *L. rigidum*
TUA1	No known mutations
TUA2	No known mutations
TUA3	Arg-390-Cys + Asp-442-Glu, Asp-442-Glu
TUA4	Val-202-Phe, Thr-239-Ile, Arg-243-Met/Lys

fragment amplification of TUA2, TUA3 and TUA4, and TUA1 was successfully sequenced in only two of eight R plants (Table 3, Fig. S1).

3.2 Novel α-tubulin mutations in TUA3 in R

Within each isoform of α-tubulin protein, the amino acid sequences from S and R plants are identical, except at specific mutation sites (Table 4). In TUA1 and TUA2, no differences in clearly identified amino acids were observed between S and R. In TUA3, alignment of amino acid sequences derived from cloned S1 and R1 transcripts revealed two types of mutations in R1: a single mutation of Asp-442-Glu, due to codon mutation of GAC to GAG; and a double mutation of Arg-390-Cys (due to codon alteration from CGC to TGC) plus Asp-442-Glu in a single transcript. Moreover, direct sequencing showed that three in eight R plants contained at least an Arg-390-Cys mutation, demonstrating that the Arg-390-Cys mutation identified in the cloning sequences is not an artefact. In TUA4, valine (Val) at 202 substituted by phenylalanine (Phe) was found in R, which is consistent with our previous report.[20]

3.3 A double mutation in TUA3 contributing to dinitroaniline resistance

Four *L. rigidum* TUA3 α-tubulin cDNA variants (S, 390-Cys, 442-Glu and 390-Cys+442-Glu) were successfully introduced into rice calli, all of which survived on media containing hygromycin. Proliferation of transformed rice calli was compared on growth medium containing increasing rates of trifluralin. The S calli were susceptible to trifluralin, and were necrotic at trifluralin concentrations ≥ 800 μg L^{-1} (Fig. 3). Similarly, calli transformed with the single mutation 390-Cys or 442-Glu stopped growing at 800 μg L^{-1} trifluralin. However, calli with the double mutation (390-Cys+442-Glu) survived and continued to grow at 800 μg L^{-1} trifluralin (Fig. 3). This result demonstrated that neither Arg-390-Cys nor Asp-442-Glu single mutation on their own is sufficient for resistance; however, the double mutation, 390-Cys+442-Glu, imparts trifluralin resistance.

4 DISCUSSION

4.1 Diverse α-tubulin transcripts and isoforms reveals complexity of tubulin family

Tubulin is a small gene family involving several members that share highly conservative protein sequences. Here, the complexity of the α-tubulin family in *L. rigidum* was investigated using PCR and cloning of *α-tubulin* family members. Because of sequence variances in UTR, and SNPs, even the same α-tubulin protein isoform is encoded by different transcripts in S and R plants. In this research, a total of 11 α-tubulin transcripts were identified in *L. rigidum*, coding for α-tubulin isoforms TUA1 to TUA4. In S plants, six transcript sequences were obtained; one for TUA1, one for TUA2, two for TUA3 and two for TUA4. In R plants, five transcripts sequences were obtained; one for TUA1, one for TUA2, two for TUA3 and one for TUA4. The number of TUA4 transcript in R plants is likely to be more than one because TUA4 from plant R3 was amplified but not successfully sequenced with PCR primers due to multiple peaks (Table 3), indicating multiple amplicons of similar length. For one specific tubulin variant, taking TUA1 as an example here, with the same primer and under the same PCR conditions, failure of the amplification suggests that

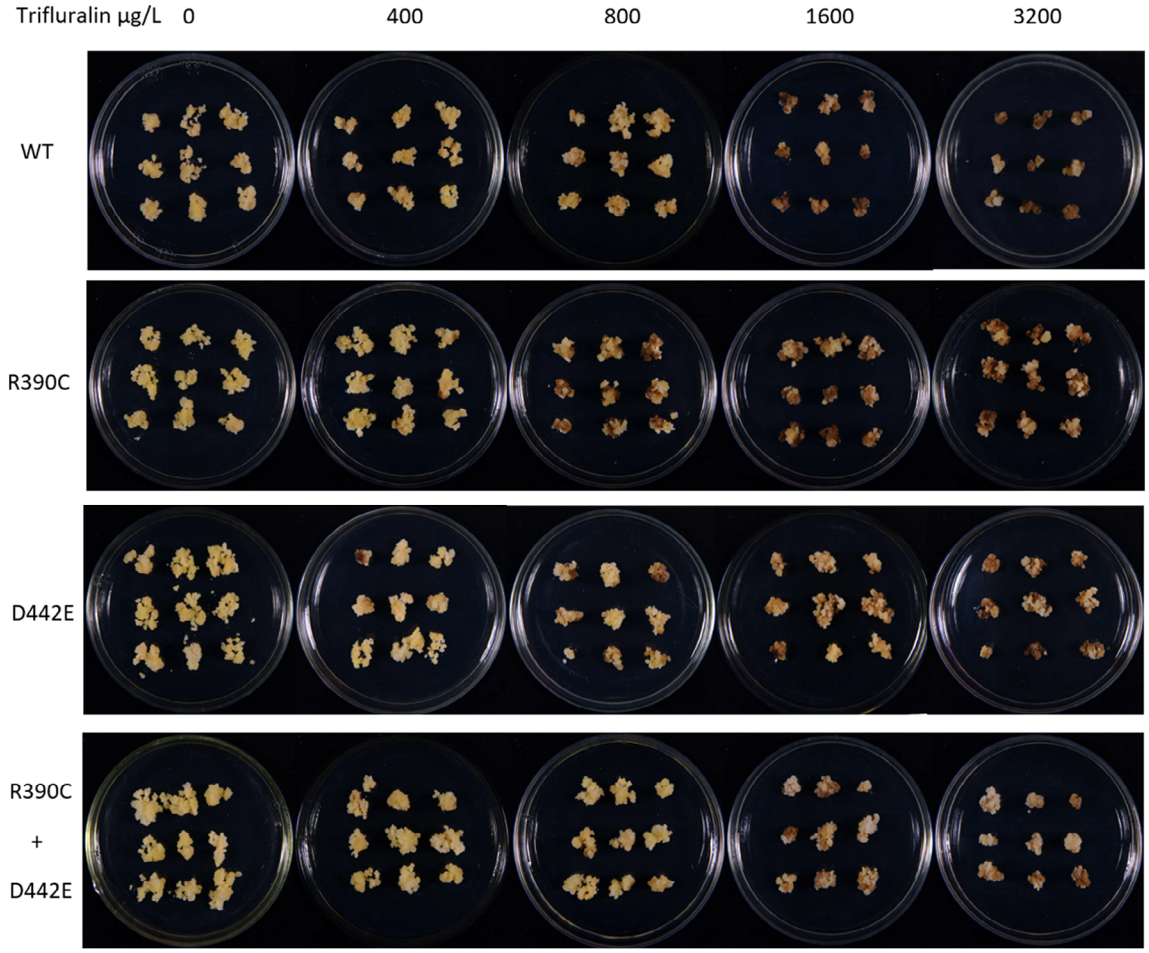

Figure 3. Growth response of rice calli transformed with wild-type (WT) or mutant α-tubulin cDNA in media containing the indicated rates of trifluralin. The control medium contained no herbicide but the same amount of DMSO that was used as a solvent for trifluralin. Photographs were taken 21 days after treatment.

not all tubulin variants are conserved in the primer-targeted regions (Table 3, Fig. S1). Meanwhile, for the amplified fragments, for example TUA3 or TUA4, the presence of multiple peaks in direct sequencing results indicates nucleotide variances (SNPs, indels) in the UTR in a single plant (Table 3, e.g. successful amplification of TUA3 in S1, S2, S3 and S4, but only S2 was able to be sequenced). The occurrence of various UTRs in a single isoform (e.g. TUA3, TUA4) further hampers and complicates tubulin direct sequencing. It remains to be determined whether the observed UTR differences are due to alternative splicing in mRNA, or to different genomic tubulin copies. Moreover, it is known that 5′- and 3′-UTRs are crucially involved in post-transcriptional regulation, which has been shown to be the primary regulator controlling tubulin gene expression.[4, 30] Yet the specific role of these variant transcripts with subtle differences in UTR from TUA3 or TUA4 is unclear. Revealing the source, function and roles of different tubulin isoforms awaits more upstream sequence of tubulin (e.g. promoter sequences) or genome information for *L. rigidum* becoming available.

Both traditional Sanger sequencing from PCR and next generation sequencing are important for understanding the plant gene family. Previously, researchers have found that PCR-based amplification may not be sufficient to reveal all target-site resistance mutations.[31, 32] Here, we demonstrated that it is equally important to validate sequencing data from RNA-seq by standard PCR, so that: (i) computational errors could be checked and corrected; and (ii) the nucleotide diversity of different individuals/populations, not limited to the few plants with transcriptome data, can be revealed.

4.2 Multiple mutant tubulin isoforms possibly co-work to confer trifluralin resistance

In our previous research, four resistance-conferring mutations were reported in the TUA4 transcript of *L. rigidum*, including Val-202-Phe, Thr-239-Ile, Arg-243-Lys/Met.[20, 22, 24] The relatively easy PCR amplification of TUA4 transcripts suggests that TUA4 is commonly present and relatively conserved in both S and R plants/populations (Fig. S1). Indeed, phylogenetic analysis indicates that the amino acid sequence of TUA4 in *L. rigidum* is closer to specific isoforms in other model plants or weedy species containing dinitroaniline resistance mutations (Fig. 4). These isoforms include: TUA1 in *A. aequalis* with the Leu-136-Phe or Val-202-Phe mutation,[33] TUA2 in *S. viridis* with the Leu-136-Phe or Thr-239-Ile mutation,[19] and TUA1 in *E. indica* with the Thr-239-Ile or Met-268-Thr mutation.[23] This evidence suggests that mutations in TUA4 mainly contribute to dinitroaniline resistance in *L. rigidum*.

Identification of resistance mutations in TUA4, however, does not exclude the possibility that mutations from other tubulin isoforms also impact on resistance. The double mutation in TUA3, Arg-390-Cys+Asp-442-Glu, revealed in this study has the potential to endow resistance. The Arg-390 residue is highly conserved in tubulin from

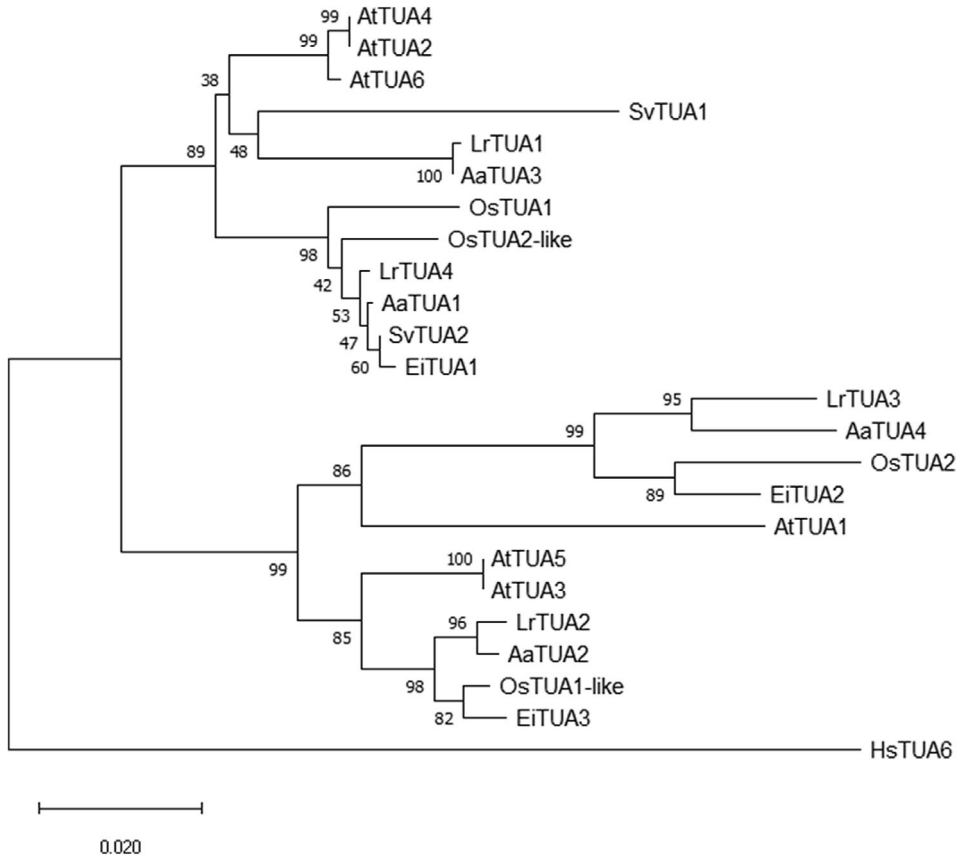

Figure 4. Phylogenetic relationship of α-tubulins in *Lolium rigidum* (Lr), *Arabidopsis thaliana* (At), *Eleusine indica* (Ei), *Setaria viridis* (Sv), *Alopecurus aequalis* (Aa) and *Oryza sativa* (Os). *Homo sapiens* (Hs) α-tubulin 6 (NP_116093) was used as an outgroup. The optimal tree with a sum of branch length of 0.52647296 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

a variety of different species (Fig. S2). According to published tubulin modelling work, helix 11 of α-tubulin (where 390 is located in Fig. S3) is positioned on the outer surface of the microtubule and is part of a cluster of basic residues responsible for interaction with β-tubulin.[34] This suggests that amino acid 390 may be directly involved in α/β-tubulin dimer interaction. In animals, α-tubulin with mutations at Arg-390 either causes resistance to microtubule inhibitors or results in severe disease.[35, 36] In addition, in protozoan *Toxoplasma gondii*, mutation at the neighbouring position Met-391 gave weak resistance to dinitroaniline inhibitors.[37] This indicates that Arg-390 is involved in the normal function of tubulin and α/β tubulin interaction.

The Asp-442 residue is located at the C-terminus of tubulin protein, which is quite distinct from the highly conserved 390 residue. The C-terminus of tubulin is notably variable in both the number and character of its amino acids.[34, 38] Also, Asp and Glu are two acidic amino acids of similar structure and chemical properties. Hence, the Asp-442-Glu mutation alone is not predicted to have a large effect on tubulin function or to provide resistance, which is consistent with our transgenic validation results (Fig. 3). How Arg-390-Cys+Asp-442-Glu confers dinitroaniline resistance is unclear.

Accumulation of multiple target-site mutations from isoforms/copies of target molecules occurs in herbicide-resistant polyploid species (e.g. wild oat[39]), and is also expected to occur with resistance to herbicides targeting multigene families in diploid species. The simultaneous existence of multiple mutations from various tubulin family members in one biotype or individual was previously found in trifluralin-resistant *A. aequalis*.[33] In this species, mutation of Val-202-Phe in TUA1 co-occurs with Leu-136-Phe in TUA3. Additionally, researchers artificially created other allelic tubulin mutations that can work synergistically to confer resistance,[40] or can offer trade-offs on resistance cost.[41] Therefore, the co-existence of multiple mutant tubulin variants could conceivably enhance resistance to microtubule inhibitors. We propose that this synergism between TUA isoforms is the way in which Arg-390-Cys+Asp-442-Glu in TUA3 and Val-202-Phe in TUA4 likely interact to confer resistance in R. Considering *L. rigidum* is an obligate cross-pollinated species and tubulin is a nuclear gene family, a mixture of different target-site resistance mutations simultaneously in a single individual is expected due to continuous crossing and selection by herbicides that occurs in fields. This study further reveals the complexity of target-site resistance mechanisms in multi-gene families like tubulin. These herbicide targets provide a greater challenge to researchers, especially in the development of molecular resistance test markers. Meticulous care should be taken when drawing conclusions about the presence or absence of target-site mutations from gene families involved in herbicide resistance, and this is also applicable to our own published work.[20, 42]

ACKNOWLEDGEMENTS

This work was supported by a Scholarship for International Research Fee (SIRF), UWA-CSC (China Scholarship Council) Scholarship to Jinyi Chen. AHRI is supported by the Australian Grains

Research and Development Corporation and Australian Research Council (LP170100903). The authors declare no conflict of interest.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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