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1 **Generation of a *ToxA* knockout strain of the wheat tan spot pathogen *Pyrenophora tritici-*
2 ***repentis***

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17 **SUMMARY**

18 The necrotrophic fungal pathogen *Pyrenophora tritici-repentis* causes tan spot, a major disease of
19 wheat throughout the world. The proteinaceous effector ToxA is responsible for foliar necrosis on
20 ToxA sensitive wheat genotypes. The single copy *ToxA* gene was deleted from a wild-type race 1 *P.*
21 *tritici-repentis* isolate via homologous recombination of a knockout construct. Expression of the *ToxA*
22 transcript was found to be absent in transformants (*toxa)*, as was ToxA protein production in fungal
23 culture filtrates. Plant bioassays were conducted to test transformant pathogenicity. The *toxa* strains
24 were unable to induce necrosis on ToxA sensitivity wheat genotypes. To our knowledge, this is the
25 first demonstration of a targeted gene knockout in *P. tritici-repentis*. The ability to undertake gene
26 deletions will facilitate the characterisation of other pathogenicity effectors of this economically
27 significant necrotroph.

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29 **INTRODUCTION**

30 The necrotrophic fungus *Pyrenophora tritici-repentis* (Died.) Drechs. [anamorph: *Drechslera tritici-*
31 *repentis* (Died.) Shoem.] is an economically significant pathogen and is the causal agent of tan (or
32 yellow) spot, a devastating foliar disease of wheat. This leaf spotting disease affects the major wheat
33 (*Triticum aestivum* L.) and durum (*T. turgidum* L.) growing areas of the world, and causes severe yield
34 losses by reducing the leaf photosynthetic area (De Wolf *et al.*, 1998). It is economically the most
35 damaging disease of wheat in Australia (Murray & Brennan, 2009).

36 *P. tritici-repentis* produces at least three effectors (host-selective toxins), referred to as ToxA, ToxB
37 and ToxC (Lamari *et al.*, 2003). These effectors interact in a highly-specific manner with the host plant
38 (Tan *et al.*, 2010), leading to development of two distinct foliar symptoms: tan necrosis and/or
39 extensive chlorosis. The type of symptom depends both on the effectors produced by a particular
40 isolate, and the susceptibility genes present in the infected wheat host. So far, eight races of *P. tritici-*
41 *repentis* have been defined, based on their ability to produce the three effectors alone or in
42 combination (Lamari et al., 2003). ToxA causes the most severe damage by inducing necrosis on the
43 leaves of ToxA sensitive wheat genotypes (possessing the *Tsn1* susceptibility gene) (Faris *et al.*,
44 2010), while ToxB and ToxC both induce chlorosis, although on different wheat genotypes harbouring
45 the *Tsc2* and *Tsc1* loci respectively (Friesen & Faris, 2004, Effertz *et al.*, 2002). ToxA is the
46 predominant effector in the tan spot-wheat pathosystem, and is present in the majority of isolates
47 worldwide (Friesen *et al.*, 2006). A single copy gene (*ToxA*) encodes the small (13.2 kDa) secreted
48 ToxA protein (Ciuffetti *et al.*, 1997, Tuori *et al.*, 1995). The *ToxA* gene is sufficient for the
49 pathogenicity of *P. tritici-repentis*, since transformation of a non-pathogenic isolate with *ToxA* was
50 sufficient to render that isolate pathogenic on ToxA-sensitive wheat lines (Ciuffetti et al., 1997).

51 ToxA was first identified over twenty years ago as a necrosis toxin (Ballance *et al.*, 1989) and isolation
52 of the *ToxA* gene followed eight years later (Ciuffetti et al., 1997). However, there have been no
53 reports of *ToxA* gene knockout mutants, and moreover, to our knowledge, there have been no reports
54 of any gene deletions of *P. tritici-repentis*. Recent work has proved successful in the generation of
55 partial knockdown mutants of *P. tritici-repentis* using a sense- and antisense-mediated RNA-silencing
56 mechanism to reduce the expression of *ToxB* and an exo-1,3-β-glucanase gene (Aboukhaddour *et*
57 *al.*, 2012, Fu *et al.*, 2013). Here, we report the successful generation of a *ToxA* knockout strain, and

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58 investigate mutant pathogenicity on a set of differential wheat genotypes. To our knowledge, this is
59 the first study in which a gene was successfully deleted in *P. tritici-repentis*.

60

61

62 RESULTS

63 *ToxA* gene disruption

64 Using a PCR fusion strategy, a phleomycin resistance cassette (*Phleo*^{R}) was integrated into the *ToxA*
65 target site, by means of flanking sequences homologous to the targeted *ToxA* locus. Protoplasts of an
66 Australian *P. tritici-repentis* isolate (M4) were transformed with the knockout construct, and putative
67 transformants were screened via PCR for absence of the *ToxA* gene. Four transformants found to
68 have undergone homologous recombination were selected for further study, and were designated as
69 *toxa-1*, *toxa-2*, *toxa-3* and *toxa-4*. Absence of *ToxA* gene expression was confirmed in all four
70 transformants based on RT-PCR analysis (Figure 1a).

71 To exclude the possibility that the *ToxA* gene deletion construct had integrated ectopically elsewhere
72 in the genome, mutants were screened via PCR to ensure that the construct had integrated at the
73 intended homologous site (Figure 1b). Correct targeted integration was confirmed for all four
74 transformants.

75 The copy number of the phleomycin cassette was determined for each of the four mutants via qPCR
76 (Figure 1c). As expected, the phleomycin resistance gene was not detected in the wild-type isolate.
77 The *Phleo*^{R} copy numbers revealed for each of the transformants closely correlated to the known
78 single copy of the *Act1* actin gene in the *P. tritici-repentis* genome sequence, thus confirming single
79 integration of the *ToxA* knockout construct.

80 ToxA protein production was evaluated by SDS-PAGE of crude culture filtrates of wild-type, mutants
81 and the race 5 isolate DW5, which does not produce ToxA (but instead produces ToxB) (Figure 1d).
82 The presence of notable bands with a mass of approximately 13.2 kDa, corresponding to the
83 expected size of the ToxA protein (Tuori et al., 1995) were detected in the wild-type isolate, but not in
84 the four transformants or DW5, although faint bands of a similar size were visualised in the knockouts

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85 and race 5 isolate. Therefore, to confirm absence of ToxA, these gel bands were excised from wild-
86 type, *toxa-1* and DW5, and subjected to peptide analysis by electrospray ionisation mass
87 spectrometry. As expected, ToxA was only identified within the wild-type isolate, and was absent from
88 knockout and DW5 culture filtrates, which both contained other low molecular weight proteins (Figure
89 S1).

90

91 **Functional analysis of *ToxA* knockout mutants**

92 In order to assess mutants for pathogenicity and to functionally confirm deletion of *ToxA*, a set of five
93 tan spot differential wheat genotypes, which differ in their effector sensitivities, were inoculated (Table
94 1) (Lamari *et al.*, 1998, Lamari et al., 2003, Ciuffetti et al., 1997). Also included was the ToxA
95 sensitive line BG261 (a *Parastagonospora nodorum* ToxA differential line) (Friesen et al., 2006).
96 Plants were inoculated with conidial suspensions of the wild-type isolate and two of the independent
97 knockout mutants (*toxa-1* and *toxa-2*). As expected, the spreading necrosis typical of ToxA was
98 observed on the ToxA sensitive lines (cv. Glenlea, BG261 and cv. Katepwa) following infection with
99 the wild-type isolate (Figure 2). However, this spreading necrosis was absent on these lines following
100 inoculation with the two mutants, thus functionally confirming *toxa-1* and *toxa-2* as lacking *ToxA*. The
101 average disease scores of the wild-type isolate was determined to be significantly higher than the
102 mutants on these wheat lines (Figure 3).

103 All three strains were unable to induce chlorosis on the ToxB sensitive wheat line 6B662, although the
104 wild-type isolate and both mutants induced chlorosis on the ToxC sensitive wheat line 6B365 (Figures
105 2 and 3). No strain was able to successfully infect cv. Auburn, which is resistant to all races and
106 insensitive to all known effectors produced by *P. tritici-repentis* (Ciuffetti et al., 1997). Katepwa has
107 been reported as resistant to race 3 (ToxC producing) isolates (Lamari et al., 1998), so the
108 observation of mild chlorosis following inoculation with the *ToxA* knockouts is purportedly due to an as
109 yet unknown effector.

110 To further functionally validate the *ToxA* deletion, the differential wheat lines were infiltrated with
111 crude culture filtrates of wild-type, *toxa-1* and *toxa-2* mutant strains and purified ToxA protein (Figure

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112 4). The knockout mutants were unable to cause necrosis on the three ToxA sensitive lines (cv.
113 Glenlea, BG261 and cv. Katepwa).

114 The wild-type isolate (M4) used in this study was collected from Australia, where, so far, all isolates
115 tested (including M4) have been found to possess *ToxA* and lack *ToxB* (Antoni *et al.*, 2010).
116 Inoculation with M4 conidia and culture filtrate infiltration induced chlorosis on the ToxC differential
117 wheat line 6B365, thus confirming that this isolate also produces ToxC, and hence belongs to race 1
118 (ToxA$^+$, ToxB$^-$, ToxC$^+$). Both of the *toxa* mutants tested also retained this chlorosis-inducing ability
119 (Figures 2 and 4).

120 No observable effect on colony morphology, growth rate, sporulation or spore germination was
121 detected in the knockouts compared to the wild-type (data not shown).

122

123

124 **DISCUSSION**

125 Deletion of *ToxA* from *P. tritici-repentis* significantly reduced the extent of disease caused by this
126 pathogen on ToxA sensitive (*Tsn1*) wheat genotypes tested, and thus emphasises the role of ToxA as
127 a major necrotrophic effector. Although non-ToxA containing isolates are found in certain parts of the
128 world (e.g. North America, the Middle East and North Africa) (Strelkov *et al.*, 2002, Lamari *et al.*,
129 2005, Friesen *et al.*, 2005), the majority of *P. tritici-repentis* isolates worldwide produce ToxA (Friesen
130 et al., 2006, Lamari & Strelkov, 2010). In recent years, an increase in the prevalence of tan spot
131 disease, particularly in Europe, has been reported (Crop Monitor, 2008/2009, Jorgensen & Olsen,
132 2007), however, the proportion of isolates that produce ToxA has yet to be determined, and the
133 distribution of ToxA within *P. tritici-repentis* isolates worldwide requires further research.

134 The ToxA-*Tsn1* interaction is the best characterised host-effector interaction identified in wheat. The
135 dominant *Tsn1* gene was identified on the long arm of chromosome 5B (Faris *et al.*, 1996), and has
136 been demonstrated to confer sensitivity to purified ToxA using *Tsn1*-disrupted mutants (Liu *et al.*,
137 2006). Cloning of *Tsn1* revealed it to have disease resistance gene features such as S/TPK and NBS-
138 LRR domains. All three domains are required for ToxA sensitivity, as had been demonstrated via

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139 induced mutagenesis (Faris et al., 2010). Although *Tsn1* is required to mediate ToxA recognition, it is
140 unlikely to be the actual ToxA receptor. The Tsn1 protein does not contain any apparent
141 transmembrane domains, thus is likely located within the cell cytoplasm, and yeast two-hybrid assays
142 suggest the protein does not interact directly with ToxA (Faris et al., 2010). However, ToxA has been
143 reported to interact with a chloroplast-localised protein known as ToxABP1 (Manning *et al.*, 2007).
144 Viral-induced gene silencing of *ToxABP1* in wheat was reported to reduce the extent of ToxA-induced
145 necrosis, although it is likely that other ToxA-host protein interactions are required for full necrosis
146 (Manning *et al.*, 2010).

147 In order to fully understand the *P. tritici-repentis*-wheat pathosystem, the role of ToxA in disease
148 needs to be determined for all ToxA-producing races (races 1, 2, 7 and 8). An early report found that
149 ToxA insensitivity was associated with resistance to race 2 ($ToxA^+$ $ToxB^-$ $ToxC^-$) (Lamari & Bernier,
150 1991). However, in another study, inoculation of this same race 2 isolate on different ToxA insensitive
151 wheat genotypes resulted in necrotic lesions, though they developed more slowly compared to those
152 on *Tsn1* genotypes (Friesen *et al.*, 2003). Therefore, although production of ToxA is sufficient for
153 pathogenicity (Ciuffetti et al., 1997), it does not appear to be necessary for disease on all host
154 genotypes. Previous work to determine if ToxA insensitivity equates to race 1 ($ToxA^+$ $ToxB^-$ $ToxC^+$)
155 resistance, showed that wheat lines with mutations for ToxA insensitivity were susceptible following
156 inoculation with two race 1 isolates (Friesen *et al.*, 2002). This earlier work demonstrated that host
157 sensitivity to ToxA is not necessarily equivalent to resistance to race 1, and one or more other
158 effectors are in play, including ToxC. Indeed, our results agree with this, since we observed
159 symptoms on BG261. Determining the precise role that ToxA plays in tan spot disease will require
160 further characterisation of the *ToxA* knockout described herein, against a range of wheat genotypes,
161 as well as the generation of *toxa* mutant strains of other races.

162 The ToxA-Tsn1 interaction has also been evaluated using segregating wheat lines. For example, a
163 population of recombinant inbred lines (derived from Salamouni and Katepwa) have been evaluated
164 for reaction to race 1 and race 2 isolates (Faris *et al.*, 2012). Unsurprisingly, the *Tsn1* locus was
165 significantly associated with disease for all isolates tested. However, the amount of variation
166 explained by *Tsn1* varied considerably (ranging between 5 and 30 %), which is suggestive of possible
167 *ToxA* gene regulation variation among *P. tritici-repentis* isolates. This has been demonstrated for

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168 ToxA-producing isolates of another necrotrophic fungal pathogen of wheat, *Parastagonospora*
169 *nodorum* (syn. *Stagonospora nodorum*; teleomorph: *Phaeosphaeria nodorum*), which express *ToxA*
170 at different levels, such that expression of *ToxA* at higher levels caused more disease on *Tsn1* wheat
171 (Faris *et al.*, 2011). It is also conceivable that broad spectrum or race-non-specific resistance
172 mechanisms may impede ToxA-Tsn1 interactions (Faris & Friesen, 2005) or that the consequences of
173 host-effector interactions are reduced or masked due to epistatic effects with other host-effector
174 interactions (Friesen *et al.*, 2008).

175 The *ToxA* gene has been successfully deleted in *P. nodorum*, and similar to the approach presented
176 herein, culture filtrates of these mutants were infiltrated on the *P. nodorum* differential line BG261 and
177 were unable to induce necrosis, demonstrating that *ToxA* is necessary for complete virulence on *Tsn1*
178 wheat lines. (Friesen et al., 2006). The authors also performed infection assays on a wheat population
179 segregating for *Tsn1* (BR34 x Grandin) and found that the *Tsn1* QTL associated with the disease
180 phenotype was eliminated following inoculation with *ToxA*-disrupted *P. nodorum* mutants. In the same
181 study, expression of either *ToxA* from *P. tritici-repentis* or from *P. nodorum* in an avirulent (*ToxA*-
182 lacking) *P. nodorum* strain was sufficient to confer virulence that co-segregated with *Tsn1*. Despite
183 predicted amino acid differences, this demonstrates that *P. tritici-repentis ToxA* and *P. nodorum ToxA*
184 are functionally identical in their interaction with *Tsn1* and there is evidence that the *ToxA* gene was
185 horizontally transferred from *P. nodorum* to *P. tritici-repentis* (Friesen et al., 2006). The full suite of
186 native effectors will differ between the two pathogens, so it is important to separately dissect the role
187 of ToxA within each pathosystem.

188 This work presented herein now opens several new vistas for future research. Firstly, removal of
189 ToxA, the predominant effector of *P. tritici-repentis*, will facilitate identification of other, as yet
190 uncharacterised effectors, whose effects have so far been masked by extensive ToxA-induced
191 necrosis. This is in agreement with previous work whereby inoculation of *Tsn1*-disrupted mutants still
192 resulted in disease (Friesen et al., 2003) and sensitivity to ToxA was a non-significant factor in
193 disease development following inoculation of the BR34 x Grandin population with race 1 and 2
194 isolates (Faris & Friesen, 2005).

195 Secondly, the generation of *toxa* mutants will enable screening of *Tsn1* wheat cultivars for sensitivity
196 to other novel effectors. In particular, this will permit the screening of ToxA sensitive wheat mapping

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197 populations for novel effector sensitivity and disease resistance QTL, and thus facilitate the
198 identification of molecular markers for wheat breeders and potential targets for crossing. Thus far the
199 identification of novel QTL has been limited to just a handful of mapping populations with ToxA
200 insensitive parents.

201 Thirdly, the development of a targeted gene knockout method for such an economically significant
202 and global wheat pathogen is noteworthy. Over the last two years, an RNA-silencing method has
203 been successfully developed to reduce gene expression in *P. tritici-repentis* (Aboukhaddour et al.,
204 2012, Fu et al., 2013). This technique was utilised to create *ToxB*-silenced transformants, with ToxB
205 production ranging from 15 to 81% of western blot band intensity relative to wild-type (Aboukhaddour
206 et al., 2012). In the case of ToxB, such an approach was well justified since the *ToxB* gene is found in
207 multiple copies, the number of which varies among isolates (Strelkov *et al.*, 2005, Martinez *et al.*,
208 2001). Thus, to knockout all individual copies of *ToxB* would be inherently more challenging than
209 RNA-silencing. However, the RNA-silencing strategy is less suitable for single copy effector gene
210 discovery, since it results in partial gene knockdowns with variable expression levels, and thus will not
211 eliminate gene expression entirely. Here we show, as proof-of-concept, that *P. tritici-repentis*
212 possesses the necessary machinery to bring about the precise integration of exogenous sequences
213 through homologous recombination, and paves the way for the creation of knockouts of other
214 potential genes of interest, and the introduction of specific mutations into a target gene.

215 The results of this study reiterate the need to increase the area sown to ToxA insensitive wheat
216 varieties, and ultimately phase out *Tsn1* cultivars, particularly in wheat growing regions with a high
217 proportion of *ToxA*-expressing isolates. A recent wheat cultivar trial found that there was no yield
218 penalty associated with growing ToxA insensitive varieties, and moreover, in the presence of disease
219 ToxA insensitive lines substantially out-performed the sensitive lines (Oliver *et al.*, in press).

220 In Australia, effector-assisted breeding has been adopted in response to the significant combined
221 losses caused by ToxA-producing pathogens ($212 million due to tan spot and $108 million from
222 septoria nodorum blotch) (Murray & Brennan, 2009). Semi-purified ToxA has been delivered to wheat
223 breeders since 2009 as a selection tool towards the development of disease resistant germplasm,
224 with current delivery of 30,000 doses per annum (Vleeshouwers & Oliver, 2014). As a result, there
225 has been a considerable decrease in the area sown to ToxA sensitive wheat varieties, a major step to

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226 reduce the huge scale of losses due to tan spot. However, in accordance with this study and earlier
227 findings, screening germplasm with ToxA should not be used in place of fungal inoculation by
228 breeding programs, since ToxA sensitivity is not always required for susceptibility to race 1. This is
229 likely due to the presence of ToxC and other effectors not yet identified, and there are currently no
230 Australian commercial wheat varieties rated as resistant to tan spot (DAFWA, 2014). A thorough
231 understanding of the role of ToxA and other effectors in tan spot disease is required, and knockout
232 capability can be expected to expedite strategies targeting the release of resistant lines.

233

234

235 **EXPERIMENTAL PROCEDURES**

236 **Fungal material and growth conditions**

237 The pathogenic *P. tritici-repentis* race 1 isolate M4 was collected from Meckering, Western Australia
238 in 2009. PCR amplification of M4 gDNA confirmed presence of the *ToxA* gene and absence of *ToxB*
239 using primers ToxAscreeningF/R and TB10f/TB12r respectively (Antoni et al., 2010). Fungi were
240 grown on V8-PDA plates (Campbell's V8 juice 150 ml l^{-1},potato dextrose agar 10 g l^{-1}, $CaCO_3$ 3 g l^{-1},
241 agar 15 g l^{-1}) and incubated at 22 ^{o}C under 12 h cycles of light. Sporulation was induced by flooding
242 the plates with ultrapure water and flattening colonies using an L-shaped glass rod. Plates were
243 placed under near-UV and fluorescent lights for 24 h, followed by incubation at 15 ^{o}C in darkness for
244 24 h. Liquid cultures were started with crushed mycelia in Fries media (Liu *et al.*, 2004) and grown at
245 27 ^{o}C and 100 rpm in darkness. For culture filtrates, liquid cultures were shaken for 3 days followed
246 by 2.5 weeks of stationary growth. The filtrate was harvested by filtration through sterile gauze,
247 MiraCloth (CalBiocem) and passed through a 0.2 μm syringe filter unit (Pall Life Sciences). For spore
248 germination assays, 100 conidia suspended in water were germinated per strain on 1.5 % agarose at
249 4 ^{o}C. After 17 h, the number of germ tubes was counted per conidium. Three independent replicates
250 were performed per fungal strain.

251

252 **Development of the *ToxA* knockout construct**

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253 A fusion PCR approach was undertaken for the inactivation of *ToxA*, whereby two homologous
254 flanking regions and the phleomycin resistance cassette were amplified separately and then fused in
255 a single PCR reaction. Flanking regions of the *ToxA* gene (PTRG_04889) were amplified from
256 genomic M4 DNA. A 1639 bp upstream flanking region was amplified using PtrToxA5'f and PtrToxA5'r
257 primers, while PtrToxA3'f and PtrToxA3'r primers were used to amplify a 1561 bp downstream
258 sequence. A phleomycin cassette (*Phelo*^{R}) was amplified from pAN8-1 using the primers pAN8f and
259 pAN8r, as described previously (Solomon *et al.*, 2006). Incorporated into the 5' regions of the
260 PtrToxA3'f and PtrToxA5'r primers were 25 bp and 23 bp of sequence homologous to the 3' and 5'
261 ends of the phleomycin fragment. Fragments were gel-extracted using a QIAquick Gel Extraction Kit
262 (Qiagen), and equimolar amounts were combined as template for a single fusion PCR reaction using
263 primers PtrToxA5'f and PtrToxA3'r at 50 µM final concentration. The fusion PCR was performed using
264 iProof High-Fidelity Master Mix (Bio-Rad) with the following cycling conditions: 98°C/30 s; (98°C/5 s,
265 67°C/30 s, 72°C/5 min) x 35; 72°C/5 min. The fusion PCR product (5624 bp) was gel-extracted and
266 resuspended in sterile water. A further amplification with nested primers (PtrToxA5'Nf and
267 PtrToxA3'Nr) was performed to generate the final gene deletion construct. All primer sequences are
268 detailed in Table 2.

269

270 **Fungal transformation**

271 A PEG-mediated protoplast transformation method was utilised (Aboukhaddour et al., 2012).
272 Transformations were performed using 5 µg DNA per 1 x 10^{7} protoplasts. Protoplasts were overlaid
273 with RM agar amended to a final concentration of 10 µg ml^{-1} phleomycin. Resistant colonies were
274 transferred to V8-PDA agar containing 15 µg ml^{-1} phleomycin for a second round of screening.
275 Putative transformants were screened via PCR in order to verify absence of the *ToxA* gene (primers
276 PtrToxAF2 and PtrToxAR2) and correct genomic integration of the gene deletion construct (primer
277 combinations PtrToxA5'f/Phleo5 and Phleo3/PtrToxA3'r which amplify the 5' and 3' flanking regions
278 from positive transformants, respectively). Thermal cycling conditions were as follows: 94°C/3 min;
279 (94°C/30 s, 58°C/30 s, 72°C/2 min) x 35; 72°C/5 min. Single spore re-isolation was performed for all
280 true transformants to ensure mutant purity.

281

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282 **RNA extraction and transcript expression**

283 Total RNA was isolated from 1-week old fungal liquid cultures using TRIzol Reagent, as per the
284 manufacturer's instructions (Invitrogen). RNA was reversed transcribed using iScript reverse
285 transcriptase (Bio-Rad) according to the manufacturer's protocol. The resulting cDNA was quantified
286 using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Scientific). Primers PtrToxAF2 and
287 PtrToxAR2 were used to test for *ToxA* expression in wild-type and transformants. As a control,
288 expression of the housekeeping actin gene (*Act1*) was tested using Act1F4 and Act1R4 primers. PCR
289 products were visualised by agarose gel electrophoresis on a 1.5 % agarose gel using SYBR Safe
290 DNA Gel Stain (Life Technologies).

291

292 **Determination of copy number**

293 A quantitative PCR (qPCR) was performed to confirm single integration of the *ToxA* knockout
294 construct into the M4 genome, as has been described previously to determine the copy number of
295 introduced gene cassettes in fungal transformants (Solomon *et al*., 2008). Genomic DNA was
296 extracted from wild-type and transformants using the Biosprint 15 DNA kit (Qiagen) as per the
297 manufacturer's instructions. For detection of the *ToxA* knockout construct copy number, a 150 bp
298 region of the phleomycin resistance cassette ($Phleo^R$) was amplified using primers PhleoF4 and
299 PhleoR4. As an endogenous control, primers Act1F4 and Act1R4 were used to amplify a 150 bp
300 fragment from the single copy actin gene (*Act1*) (Ellwood *et al*., 2012). Thermal cycling conditions
301 were: 95°C/15 min; (94°C/15 s, 63°C/30 s, 72°C/30 s) x 35; 72°C/30 and were performed in a CFX96
302 Real-Time PCR Detection System (Bio-Rad). Each 20 µl qPCR reaction consisted of 50 ng DNA, 10
303 µl QuantiTect SYBR Green PCR mix (Qiagen) and 300 nM of the appropriate primers. PCR
304 efficiencies of the target ($Phleo^R$) and reference (*Act1*) qPCR amplifications were tested to be
305 approximately equal. The $Phleo^R$ copy number was normalised to *Act1* copy number using the ΔΔCt
306 method. Samples were analysed in triplicate with two technical replicates.

307

308 **ToxA protein production**

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309 Fungal culture filtrates were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis
310 (SDS-PAGE) performed on a Mini-PROTEAN 3 vertical gel apparatus (Bio-Rad). Culture filtrates were
311 passed through PD-10 desalting columns to remove salts and low-molecular weight impurities, as per
312 the manufacturer's instructions (GE Healthcare). Proteins were resolved via a 16.5 % polyacrylamide
313 separating gel using the Tris-tricine buffer separation system (Schagger & Vonjagow, 1987).
314 Approximately 40 µg of each total protein sample was loaded per lane and the Precision Plus Protein
315 Standard (Bio-Rad) was used as protein molecular weight standard. Gels were fixed and visualised
316 via Coomassie G250 colloidal staining (Neuhoff *et al.*, 1988). Bands of the expected ToxA size were
317 excised individually from the gel for M4, DW5 and *toxa-1*, trypsin-digested and peptides were
318 extracted according to standard techniques (Bringans *et al.*, 2008). Peptides were analysed by
319 electrospray ionisation mass spectrometry using the Shimadzu Prominence nano HPLC system
320 (Shimadzu) coupled to a 5600 TripleTOF mass spectrometer (AB Sciex). Tryptic peptides were
321 loaded onto an Agilent Zorbax 300SB-C18, 3.5 µm (Agilent Technologies) and separated with a linear
322 gradient of water/acetonitrile/0.1 % formic acid (v/v). Spectra were analysed to identify proteins of
323 interest using Mascot sequence matching software (Matrix Science) with Ludwig NR database.

324

325 **Plant material and pathogenicity assays**

326 Wheat seeds (*Triticum aestivum* L.) were obtained from the Australian Winter Cereals Collection
327 (AWCC). For culture filtrate assays, seeds were sown in Grade 2 vermiculite (The Perlite and
328 Vermiculite Factory, Australia) in seed trays and grown at 20 ^{o}C under a 12 h day/night cycle in a
329 controlled growth chamber. Fully extended leaves of 2-week old wheat plants were infiltrated with
330 crude culture filtrate from the wild-type or *toxa* mutants. A needleless 1-ml syringe was used to
331 infiltrate the adaxial surface of second leaves, and the infiltration boundaries were marked with a
332 permanent marker pen. Leaves were evaluated 10 days post-infiltration. All infiltration experiments
333 were repeated twice with consistent results, using a minimum of 4 plants per line each time. The ToxA
334 protein was purified as described previously (Tan *et al.*, 2012) and infiltrated at 50 µg ml^{-1}.

335 For the infections, pots (10 cm in diameter) containing P500 perlite (The Perlite and Vermiculite
336 Factory, Australia) and vermiculite were sown with 4 seeds and grown at 21 ^{o}C under a 12 h day/night
337 cycle. Inoculum was prepared consisting of approximately 2000 conidia per ml in 0.25 % gelatin.

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338 Infection assays were performed by evenly spraying 2-week old plants (at the 2-3 leaf stage) with the
339 inoculum using a spray bottle until run-off. The plants were incubated in a misting chamber for 2 days
340 (relative humidity ≥ 95 %) with continuous moisture supplied by a humidifier. Seven days post-
341 inoculation, the second leaves were harvested, photographed and visually assessed for disease
342 severity, based on the Australian wheat disease resistance ratings scale (DAFWA, 2014), whereby
343 varieties are rated between 1 and 9 (where 1 represents absence of infection and 9 denotes total
344 necrosis). All infection experiments were independently repeated twice with consistent results, using a
345 minimum of 4 replicates (pots) per treatment and run as blind experiments.

346 Disease scores were analysed by ANOVA to determine any significant differences between the
347 isolate virulence ($p \leq 0.05$), followed by Fisher's LSD post-hoc analysis to identify which mean values
348 were significantly different ($p \leq 0.01$). Prior to ANOVA, raw data were checked to ensure homogeneity
349 of variance.

350

351

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355 of isolate DW5 and wheat lines 6B662 and 6B365. We also thank the Australian Winter Cereals
356 Collection (AWCC) for the provision of seeds.

357

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482 **FIGURE LEGENDS**

483 **Figure 1.** Confirmation of four independent *ToxA* knockout strains. (**a**) RT-PCR detection of *ToxA*
484 transcript (PCR product size of 393 bp) in the four knockout strains and wild-type (WT), as visualised
485 by agarose gel electrophoresis. *Act1* was included as a positive control (PCR product size of 150 bp).
486 (**b**) Correct integration of the *ToxA* gene deletion cassette at 5' and 3' ends in four replicates of the
487 knockout strains. PCR product sizes are for the 5' and 3' amplicons are 1.7 kb and 1.6 kb
488 respectively. (**c**) Phleomycin resistance cassette ($Phleo^R$) copy number as detected by qPCR. Error
489 bars depict standard deviation. (**d**) SDS-PAGE of culture filtrate proteins. Open arrows indicate ToxA
490 (13.2 kDa). Two independent wild-type (WT) culture filtrate samples were analysed.

491

492 **Figure 2.** Reactions of a differential set of wheat lines to inoculation with wild-type (WT), *toxa* mutants
493 and DW5. Inoculation with 0.25 % gelatin (Ctrl) is included as a negative control. Images were taken 7
494 days post-inoculation and show representative second leaf symptoms.

495

496 **Figure 3.** Pathogenicity assays of the *ToxA* knockout strains. Average disease scores of individual
497 plants from one round of infection. Leaf symptoms were assessed for disease severity 7 days post-
498 inoculation using a nine point scale, where 1 represents absence of infection and 9 denotes total
499 necrosis. ANOVA revealed a significant effect of fungal strain on disease score ($p \leq 0.05$). Average
500 disease scores with asterisks are significantly different as determined by Fisher LSD post-hoc
501 analysis ($p \leq 0.01$).

502

503 **Figure 4.** Sensitivity of differential wheat lines infiltrated with culture filtrates of wild-type (WT) and
504 *toxa* mutants. Infiltrations with purified ToxA protein (ToxA) and Fries media (Ctrl) are included as
505 positive and negative controls respectively. Images were taken 10 days post-infiltration and show
506 representative first leaf symptoms.

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508 **SUPPORTING INFORMATION**

509 **Figure S1.** *P. tritici-repentis* proteins identified from SDS-PAGE excised bands of culture filtrates. (**a**)
510 SDS-PAGE of culture filtrate proteins (as shown in Figure 1a) with arrows indicating excised bands.
511 Two bands (upper and lower) were excised from 3 strains: wild-type (WT), DW5 and *toxa-1*. (**b**) The
512 peptide fragmentation data from tandem mass spectrometry (LC/MS/MS) were searched against the
513 non-redundant Ludwig NR database using the MASCOT sequence matching software. Only
514 significant hits (score >35) with a significance threshold of $p < 0.01$ and a minimum of 2 peptide
515 matches are shown.

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Effector	*Differential Wheat Line*				
	Glenlea	6B662	Katepwa	6B365	Auburn
ToxA	N	-	N	-	-
ToxB	-	C	C	-	-
ToxC	-	-	-	C	-

533 **Table 1.** Differential wheat genotypes and their corresponding effector sensitivities (adapted from
534 (Lamari et al., 1998)). Where N and C denote necrosis and chlorosis respectively, while a dash
535 indicates insensitivity.

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Primer name	Sequence
PtrToxA5’f	TCCGCTCTCGATTACCGGCTCA
PtrToxA5’r	**TGTGACTTTTGGTTACGCCGTCT**TGTCAATGTCGACTTGGCCGATG
PtrToxA3’f	**TCTCCTATGAGTCGTTTACCCAGAA**TCAATGGGAATAAGTCTCCCCACCA
PtrToxA3’r	GCGCTCTCGGTACGCTCCTC
pAN8f	AGACGGCGTAACCAAAAGTCACA
pAN8r	TTCTGGGTAAACGACTCATAGGAGA
PtrToxA5’Nf	TGTTCGAGCCTGGTTCAGAT
PtrToxA3’Nr	CCTATCTTAAGGGCGGCTTC
PtrToxAF2	ACCGGCAGGACTAATCGCCTCA
PtrToxAR2	CCAACACGTGCCGTTCCGGT
Phleo5	CTCCGTCTTCCGTAGCCGTG
Phleo3	CCAATACGCCGGCCGAAAC
Act1F4	CGAGACCTTCAACGCTCCCGC
Act1R4	GCGTGGGGAAGAGCGAAACCC
PhleoF4	GACCGAGATCGGCGAGCAGC
PhleoR4	TCAAGCTCCTGGGACCCGTGG

539 Table 2. Primers used throughout this study. Bold text refers to sequence complementary to

540 the phleomycin resistance cassette primers (pAN8f and pAN8r).

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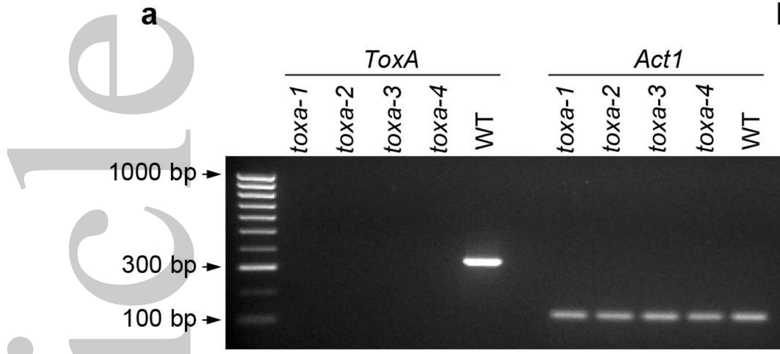

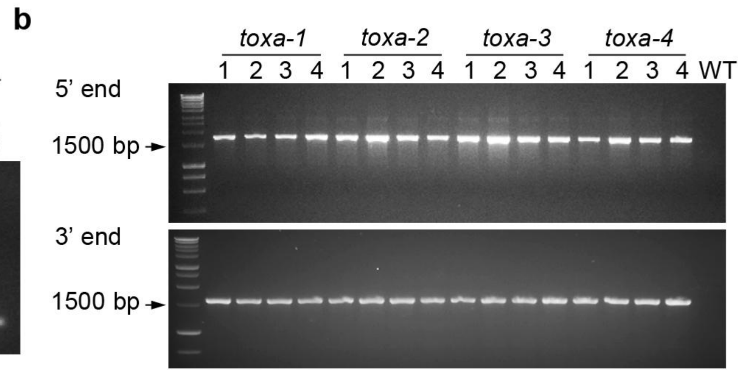

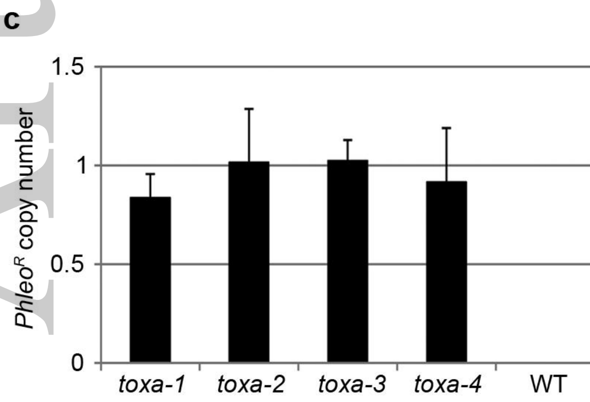

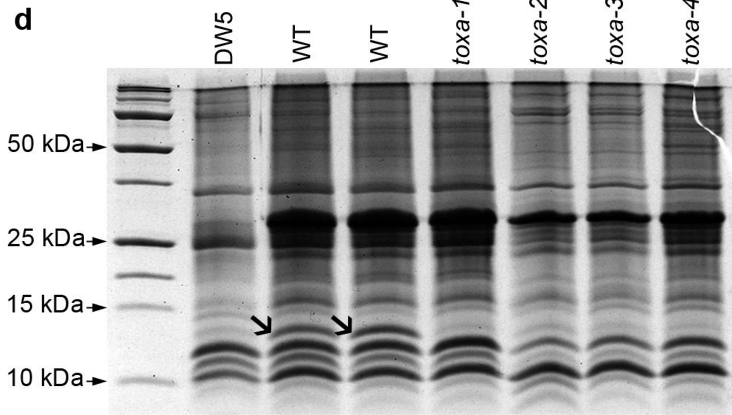

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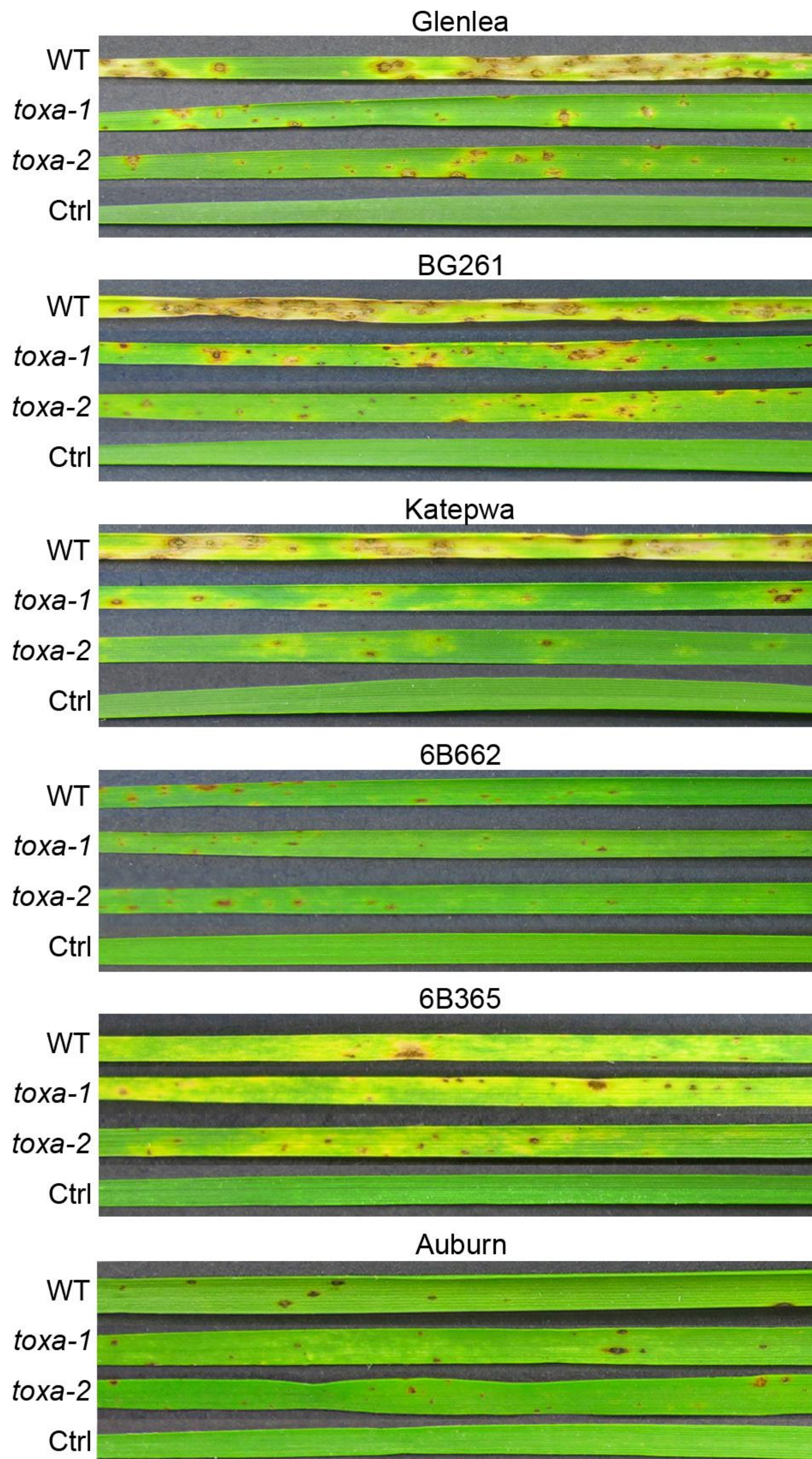

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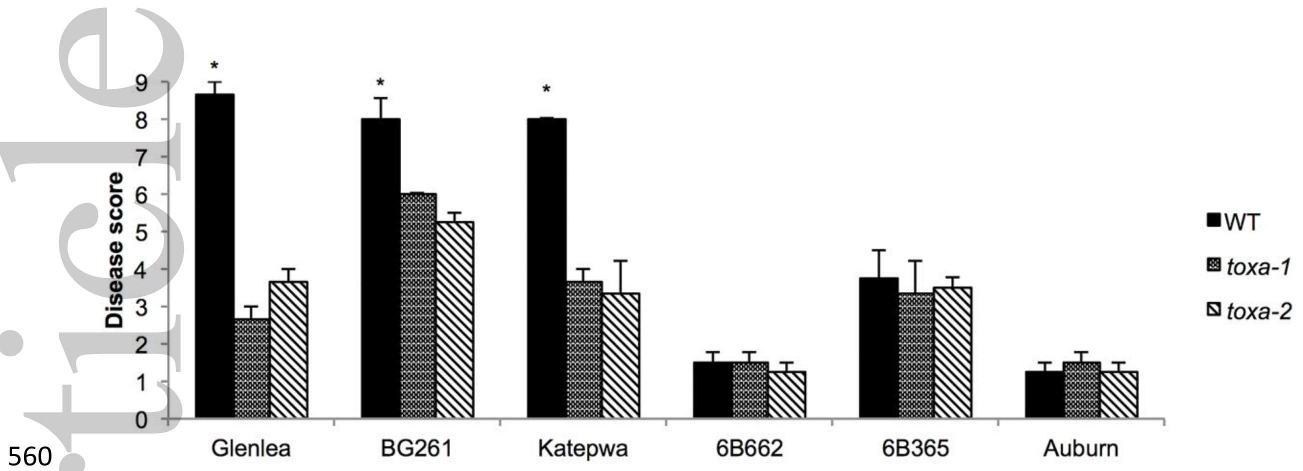

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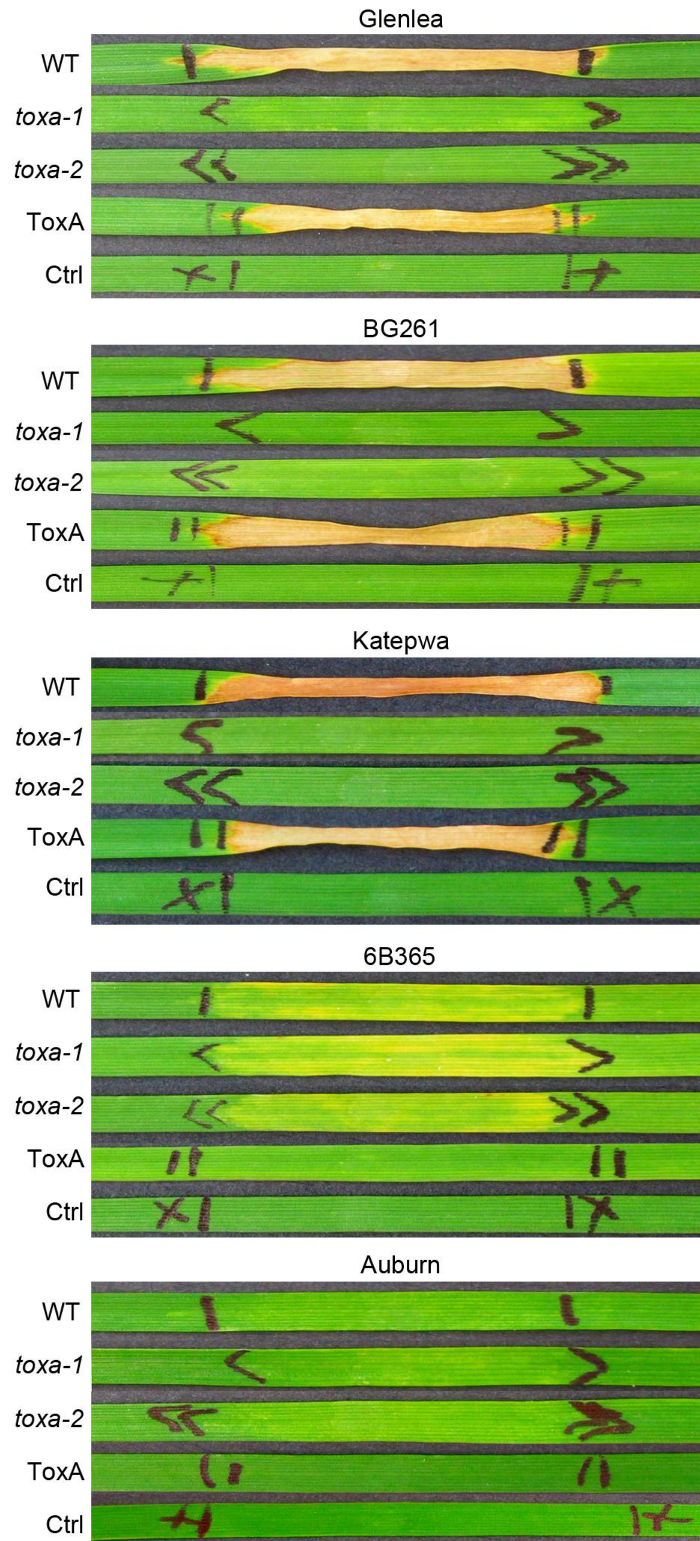

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