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Title: Expression of a truncated form of yeast ribosomal protein L3 in transgenic wheat improves resistance to Fusarium head blight

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1 Expression of a truncated form of yeast ribosomal protein L3 in transgenic wheat improves
2 resistance to Fusarium head blight
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26 **ABSTRACT**

27 Fusarium head blight (FHB) is a disease that causes major economic losses in wheat
28 and barley production worldwide. Contamination of food with the trichothecene mycotoxin
29 deoxynivalenol (DON) produced by *Fusarium* is a major health concern for humans and
30 animals because trichothecenes are potent cytotoxins of eukaryotic cells. Trichothecene
31 mycotoxins inhibit translation by targeting ribosomal protein L3 at the peptidyltransferase
32 center. We previously showed that expression of an N-terminal fragment of yeast L3 (L3Δ)
33 in transgenic tobacco plants reduced the toxicity of DON. Here, we produced transgenic
34 wheat plants that express the same yeast L3 (L3Δ) fragment and evaluated their susceptibility
35 to *F. graminearum* infection and their ability to accumulate DON. Following *F.*
36 *graminearum* infection in greenhouse tests, two transgenic wheat lines expressing the highest
37 levels of L3Δ showed reductions in disease severity and kernel DON levels, compared to
38 non-transformed plants. In a field test, a transgenic wheat line with the highest L3Δ
39 expression controlled by the maize *Ubi1* promoter had significant reductions in visually
40 scabby kernels and kernel DON levels. These results demonstrate that expression of a
41 modified form of the ribosomal protein that is the target of DON can improve FHB resistance
42 in wheat.

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45 ***Keywords*** Fusarium head blight, wheat and barley scab, trichothecene mycotoxin,
46 deoxynivalenol, transgenic wheat plants, ribosomal protein L3

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47
48 **1. Introduction**

49

50 Fusarium head blight (FHB) caused by several *Fusarium* species, primarily *F. graminearum*
51 Schwabe in the U. S., is an economically important disease of wheat and barley worldwide
52 [1]. It was estimated that several severe FHB outbreaks in U.S. wheat and barley from 1991
53 to 1997 resulted in $4.8 billion of total direct losses [2]. The contamination of wheat, barley
54 and maize related products with the trichothecene mycotoxin deoxynivalenol (DON) due to
55 the infection with *F. graminearum* poses a health threat to humans and animals [3, 4].
56 Disruption of the *F. graminearum* gene *Tri5*, which encodes the trichodiene synthase that
57 catalyzes the first step in the DON biosynthetic pathway, reduces FHB severity on
58 greenhouse- and field-grown wheat [5, 6]. The *Tri5* revertant strains of *F. graminearum*
59 regain their aggressiveness on field grown wheat [6]. These results have provided evidence
60 that DON acts as a virulence factor in FHB.

61 Trichothecene mycotoxins interact with the peptidyl transferase center of eukaryotic
62 ribosomes and inhibit protein synthesis [7]. Trichothecenes have been reported to have
63 diverse roles in the cell that are not limited to the inhibition of protein synthesis. A recent
64 genome-wide screen in *S. cerevisiae* revealed a critical role for the mitochondria in the
65 toxicity of a trichothecene mycotoxin [8]. The *tcm1* mutation that conferred resistance to the
66 trichothecene, trichodermin was identified in the yeast *RPL3* gene, which encodes the
67 ribosomal protein L3 [7, 9, 10]. L3 participates in the formation of the peptidyltransferase
68 center [11, 12]. There have been attempts to over-express the *RPL3* gene carrying the *tcm1*
69 mutation (W255C) in transgenic plants to achieve resistance to DON. A modified rice *RPL3*
70 cDNA containing the W255C mutation was transformed into tobacco [13]. The transgenic
71 tobacco calli and protoplasts displayed greater regeneration efficiency and viability in the
72 presence of DON. The expression of a tomato *RPL3* cDNA with the *tcm1* mutation in

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73 transgenic tobacco plants improved the ability of these plants to adapt to DON, but did not
74 result in constitutive resistance, possibly because the mutant protein did not accumulate in the
75 transgenic plants [14].

76 We cloned the two different L3 genes (*RPL3A* and *RPL3B*) from tobacco and showed
77 that their expression is coordinately regulated [15]. Increasing L3 levels in transgenic
78 tobacco resulted in leaf overgrowth and mottling, and led to an increase in cell number and a
79 decrease in cell size. The rRNA precursor and the mature rRNAs accumulated in these
80 plants, suggesting that ribosome biogenesis was upregulated. In contrast, L3 deficiency led
81 to a reduction in cell number and an increase in cell size [15]. Since altering endogenous L3
82 expression led to an abnormal phenotype in tobacco, we expressed the full length and a
83 truncated form of the yeast L3 gene corresponding to the first 99 amino acids of L3 in
84 tobacco to determine if expression of the yeast L3 gene confers resistance to DON.
85 Transgenic tobacco plants expressing yeast L3Δ were phenotypically normal and, in a
86 germination test, showed resistance to DON and to pokeweed antiviral protein (PAP), which
87 also targets L3 [16]. PAP is a 29-kDa ribosome inactivating protein (RIP) isolated from
88 pokeweed plants that inhibits translation by binding to L3 and catalytically removing a
89 specific purine residue from the highly conserved α-sarcin/ricin loop (SRL) of the large
90 rRNA [17]. Ribosomes from tobacco plants expressing PAP and yeast L3Δ were not
91 depurinated, even though PAP was associated with ribosomes. These results demonstrated
92 that yeast L3Δ conferred resistance possibly by protecting ribosomes from the translation
93 inhibitory effects of DON and PAP [16].

94 In this study, we aimed to test whether expression of the yeast L3Δ gene could protect
95 wheat ribosomes from DON and thus reduce FHB severity in wheat plants carrying L3Δ
96 transgenes. Bread wheat (*Triticum aestivum* = *Ta*) is hexaploid and thus contains six
97 different *RPL3* genes: *TaRPL3A1*, *TaRPL3A2*, *TaRPL3A3*, *TaRPL3B1*, *TaRPL3B2* and

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98 *TaRPL3B3* [18]. We transformed hexaploid wheat cultivar Bobwhite with yeast L3Δ under
99 the control of two different promoters and assayed the resultant transformants for *RPL3* and
100 L3Δ expression levels and for FHB resistance and DON accumulation in the greenhouse and
101 field.

102 1
103 2

2. Materials and methods

104

105 3

2.1. Production of transgenic wheat plants

106

107 The coding sequence of the N-terminal 99 amino acids of yeast ribosomal protein L3
108 was cloned in place of the *bar* coding region in the wheat expression vector pUBK [19]. The
109 resulting construct, called pLem-L3d , contained the L3Δ gene between the barley tissue-
110 specific *Lem1* promoter [20] and the nopaline synthase (NOS) 3' transcription terminator
111 (Fig. 1). The L3Δ gene was also cloned into a similar wheat expression vector containing the
112 maize *Ubi1* promoter/first intron [21], resulting in construct pUbi-L3d (Fig. 1).

113 Embryogenic calli of a hexaploid FHB-susceptible spring wheat (*Triticum aestivum*
114 L. em. Thell. cv Bobwhite) were co-bombarded with a 2:1 (pLem-L3d) or 4:1 (pUbi-L3d)
115 molar ratio of the L3Δ constructs and pUBK containing the *bar* selectable marker gene under
116 the control of *Ubi1*. Regenerants were selected with bialaphos as described previously [19,
117 22], except that the callus recovery media after bombardment contained 5 μM $CuSO_4$ and the
118 regeneration media contained 5 μM $CuSO_4$, 0.1 mg l^{-1} 6-benzylamino purine, and 3 mg l^{-1}
119 bialaphos. T_0 plants containing pLem-L3d or pUbi-L3d were identified using PCR of wheat
120 genomic DNA, as described previously [19], with forward primer UbiA2 [19] or Lem752 [5'-
121 GACAGTGGGAGTGGGGTTTG-3'] in combination with reverse primer L3-L3d [5'-
122 CGACGTAACCGACAACACC-3'] and an annealing temperature of 62°C.

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124 *2.2. Real-time PCR analysis to determine the gene expression levels in transgenic wheat*
125 *plants*
126
127 Total RNA was isolated from the florets and immature seeds or leaves of transgenic
128 wheat plants using TRIzol® Reagent (Invitrogen) according to the manufacturer's protocol.
129 The RNA samples were treated with RQ1 RNase-Free DNase (Promega) following the
130 manufacturer's instructions and further purified with phenol/chloroform extraction and
131 ethanol precipitation. SuperScript® reverse transcriptase (Invitrogen) and oligo dT were used
132 to produce the first strand cDNA from 5 μg of total RNA. ABI PRISM 7000 Sequence
133 Detection System (Applied Biosystems) was used to perform real-time PCR analysis using
134 gene-specific primers designed by ABI Primer Express software, following the
135 manufacturer's protocols. The primers for yeast L3Δ were L3d67F, 5'-
136 GCCTCCATCAGAGCTAGAGTTAAGG-3' and L3d145R, 5'-
137 AACCCAAGAAGGAAGTTAGAGCAA-3'. The primers used to measure the levels of
138 wheat *TaRPL3A* were WL3A829F, 5'-CACCGCACAGAGATGAACAAA-3' and
139 WL3A900R, 5'-AGTGCAGGCCTCGTGAGACTC-3'. The primers for the wheat *TaRPL3B*
140 were WL3B829F, 5'-CACCGAACTGAGATGAACAAG-3' and WL3B900R, 5'-
141 GGTAGAGGCATCATGAGTTTC-3'. The relative gene expression levels of *TaRPL3* were
142 measured using wheat α-tubulin (GenBank Access #U76558) as an internal control with
143 primers WTub942F, 5'-CAGCTGAGAAGGCTTACCATGA-3' and WTub1000R, 5'-
144 AAAGGCGCTGTTGGTGATCT-3'. The $2^{-\Delta\Delta C_T}$ method [23] was used to determine the
145 relative gene expression levels.
146
147 *2.3. Greenhouse screening of transgenic wheat lines for resistance to F. graminearum*
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149 Plants were grown in pots (15 cm x 15 cm x 16.5 cm; Belden Plastics, Roseville MN)
150 containing commercial potting medium (Metromix 200, Scotts Co.) in a greenhouse
151 maintained at 20±2°C with a 16-h light and 8-h dark cycle. Six to seven seeds were planted
152 in each pot. Five pots per line were thinned to six plants at Zadoks growth stage (GS) 13-14.
153 In addition to the transgenic lines, two check lines were included in the greenhouse screening.
154 The FHB checks used were Wheaton, a hard red spring wheat, released cooperatively by the
155 Minnesota Agricultural Experiment Station and USDA-ARS in 1984, which is highly
156 susceptible to FHB, and Alsen, a hard red spring wheat released from North Dakota State
157 University and moderately resistant to FHB. The FHB resistance in Alsen is derived from the
158 Chinese wheat Sumai 3. Bobwhite was included as an untransformed control. The planting
159 was replicated. Each pot was fertilized with 5.8 g of slow release 14-14-14 (N-P-K) fertilizer
160 (Osmocote Classic, Scotts Co.) at GS 13-14. Insect pests were controlled with an application
161 of imidacloprid (Marathon 60WP, Olympic Hort. Products). Powdery mildew was controlled
162 with a single application of tridimefon (Bayleton 50% DF, Bayer CropScience).
163 Plants were inoculated when the main spike from each plant reached anthesis (GS 60-
164 65). A single spikelet at the central node of the main spike of each plant was inoculated with
165 10 μl of a macroconidial spore suspension (100,000 conidia ml^{-1}) of *F. graminearum* using a
166 repeating 500-μl Hamilton 700 syringe (model 80830), fitted with a PB600 dispenser (model
167 83700) (Hamilton Co.). Twelve isolates of *F. graminearum* collected from commercial fields
168 of wheat and barley in 2002 and 2003, which were naturally infected with *F. graminearum,*
169 were used to produce the inoculum according to the previously described procedure [24].
170 Immediately after inoculation, the plants were placed in a dew chamber for 72 h. Following
171 the dew period, the plants were returned to the greenhouse bench. Disease development was
172 assessed visually 21 days after the inoculation. FHB incidence is defined as the percentage of
173 spikes with visually symptomatic spikelets and FHB severity as the percentage of

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174 symptomatic spikelets from the total spikelets observed. The DON content of grain was
175 determined on the kernels harvested from the four spikelets adjacent to the inoculated
176 spikelet of inoculated heads. Heads were harvested at maturity and threshed by hand.
177

178 *2.4. Field testing of transgenic wheat lines for resistance to F. graminearum*

179

180 The 2008 field screening nursery was located at UMore Park, Rosemount MN. In
181 addition to the transgenic wheat lines, untransformed Bobwhite, the moderately resistant
182 wheat Alsen and the FHB susceptible wheat Wheaton were included as checks. The
183 experimental design was a randomized block with four replicates. Plots were 2.4 m long
184 single rows. All plots, except a non-inoculated Wheaton check, were inoculated twice. The
185 first inoculation was applied at anthesis and the second 3 days after anthesis. Inoculum was a
186 composite of 41 *F. graminearum* isolates at a concentration of 100,000 macroconidia ml^{-1}.
187 Polysorbate 20 (Tween 20) was added at 2.5 ml l^{-1} to the inoculum as a wetting agent. The
188 inoculum was applied using a CO_2-powered backpack sprayer fitted with a SS8003 TeeJet
189 spray nozzle with an output of 10 ml sec^{-1} at a working pressure of 275 kPa. FHB incidence
190 and severity were assessed visually 20-25 days after inoculation on 20 arbitrarily selected
191 spikes per plot. FHB incidence and severity were defined as stated above. The harvested
192 seeds from each plot were split using a Borrner divider (Seedburo) to obtain a 50 g sub-
193 sample, which was then cleaned by hand. These samples were used to estimate the
194 percentage of visually scabby kernels (VSK %), according to method of Jones and Mirocha
195 [25], and then analyzed for deoxynivalenol (DON).

196

197 *4 2.5. Determination of DON level in kernels of the*
198 *inoculated wheat plants*
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200 Grain samples were analyzed for DON according to the published protocol [26]. Briefly, the
201 samples were ground for 2 min with a Stein Laboratories Mill (model M-2, Stein
202 Laboratories Inc.). DON was extracted from 4 g of the ground sample in 16 ml of
203 acetonitrile-water (84:16 vol/vol) placed on an Eberbach reciprocal shaker (model 6010,
204 Eberbach) for 1 h. A 1–ml sample eluted through a specially prepared cleanup column [27]
205 was evaporated to dryness with nitrogen. DON was derivatized and analyzed using gas
206 chromatography-mass spectrometry (GC/MS) (Model QP-2010, Shimadzu Ltd.) in selected
207 ion monitoring (CSIM) mode. DON concentration was determined based on retention times
208 and peak areas by comparing to standards.
209
210 **3. Results**
211
212 *3.1. Generation of transgenic wheat plants expressing yeast ribosomal protein L3Δ*
213
214 To determine if expression of L3Δ in transgenic wheat would confer resistance to
215 DON, embryogenic calli of susceptible spring wheat (*Triticum aestivum* L. em. Thell. cv
216 Bobwhite) were transformed *via* particle gun bombardment with genes encoding L3Δ under
217 the control of either the barley *Lem1* [20] (pLem-L3d) or the maize *Ubi1* [21] promoter
218 (pUbi-L3d) (Fig. 1). The *bar* gene, conferring resistance to the herbicides bialaphos and
219 BASTA, under control of *Ubi1*, was co-bombarded with the L3Δ constructs into the calli as a
220 selection marker to identify the stably transformed T_0 plants. Several bialaphos resistant T_0
221 wheat plants were regenerated from one bombardment experiment with each expression
222 vector. Some of these T_0 plants also contained *Lem1*::L3Δ or *Ubi1*::L3Δ, as determined by
223 PCR with oligonucleotide primers specific for the promoters and L3Δ (data not shown). The
224 integration of the L3Δ transgenes was confirmed by PCR analyses of genomic DNA from T_1

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225 plants, demonstrating that the transgene locus was inherited (data not shown). All (12/12) the
226 progeny of one T_0 plant containing *Lem1*::L3Δ inherited a *Lem1*::L3Δ transgene, suggesting
227 that the original transformant contained at least two independent transgenic loci. Three
228 homozygous T_2 sublines derived from this event were identified by PCR (data not shown)
229 and named 771, 772 and 774. The progeny of two independent T_0 plants containing
230 *Ubi1*::L3Δ segregated 3:1 (8/10 and 11/15) for the transgene, suggesting they each contained
231 a single transgene locus. Homozygous T_2 plants from these two transformants were identified
232 by PCR and were named 8133 and 8153. Eight to ten homozygous T_3 plants from each of the
233 five lines were grown in the greenhouse for expression and resistance analyses. No
234 developmental or seed set differences were observed between the transgenic plants and their
235 parent.
236
237 *3.2. Expression of yeast L3Δ in transgenic wheat plants*
238
239 For each of the five homozygous transgenic wheat lines, expression of the L3Δ
240 transgene was measured at anthesis, the stage at which they would be most susceptible to
241 *Fusarium* infection. The florets and immature seeds were sampled since most of the natural
242 infection occurs in these organs. Total RNA was extracted from these samples and used in
243 real-time polymerase chain reaction (PCR) assays to measure the expression level of the
244 yeast L3Δ gene, using a set of primers specifically designed for the 5' end of the yeast *RPL3*
245 gene, corresponding to the N-terminal amino acids +22 to +48 of L3. As shown in Fig. 2A,
246 these amino acids are significantly divergent among the yeast and TaRPL3 proteins.
247 Experimental results showed that these primers did not hybridize to the endogenous wheat
248 *TaRPL3* genes (data not shown). The relative L3Δ expression levels were determined using
249 the wheat α-tubulin mRNA as an internal control. Real-time PCR analysis was performed on

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250 RNA from four to five different plants of each homozygous line with two replicates per plant
251 and results were averaged. As shown in Fig. 3A, the L3Δ expression was detected at high
252 levels in the florets/immature seeds of transgenic wheat plants. Transgenic lines 772 and
253 8153 contained the highest levels of the yeast L3Δ transcripts.
254
255 *3.3. Relative steady state levels of the endogenous wheat TaRPL3 transcripts in transgenic*
256 *wheat plants*
257
258 We have shown previously that the endogenous *TaRPL3* genes were up-regulated in
259 the transgenic tobacco plants expressing the yeast L3Δ [16]. In order to determine if
260 expression of the yeast L3Δ affected expression of the endogenous *TaRPL3* genes in wheat,
261 we measured the *TaRPL3* expression levels relative to those of α-tubulin in the homozygous
262 lines of transgenic wheat plants. The *TaRPL3* gene family consists of three alleles of both
263 *TaRPL3A* and *TaRPL3B* [18]. The following six different wheat *TaRPL3* gene sequences
264 from the GenBank were used in this study: *TaRPL3A1* (Accession #AY343327), *TaRPL3A2*
265 (Accession #AY343328), *TaRPL3A3* (Accession #BK001237), *TaRPL3B1* (Accession
266 #BK001235), *TaRPL3B2* (Accession #BK001234) and *TaRPL3B3* (Accession #AY347532).
267 Comparison of the amino acid sequences among the three wheat *TaRPL3A* genes and three
268 wheat *TaRPL3B* genes showed that TaRPL3A and TaRPL3B protein sequences are very
269 similar within the A and B groups. However, the sequences of the L3 proteins encoded by
270 the *TaRPL3B1/B2/B3* genes are approximately 5% divergent from the sequences of the
271 proteins encoded by the *TaRPL3A1/A2/A3* genes. Most of the divergence occurs between
272 amino acids 277 to 300 as shown in Fig. 2B. Therefore, two sets of primers corresponding to
273 this most divergent region were designed to distinguish between the expression levels of
274 *TaRPL3A1/A2/A3* and *TaRPL3B1/B2/B3* by real-time PCR analysis. As shown in Fig. 3B,

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275 the relative expression levels of *TaRPL3A1/A2/A3* were higher in 772 and 8153 transgenic
276 wheat lines. Fig. 3C also shows that the relative *TaRPL3B1/B2/B3* expression levels were
277 higher in 772, 8133 and 8153 transgenic wheat lines. In line 772 the *TaRPL3A1/A2/A3*
278 expression levels were about 2.5-fold and the *TaRPL3B1/B2/B3* expression levels were 3.5-
279 fold higher than the wild type (wt) wheat plants, while there was an approximately 4-fold
280 increase in the *TaRPL3A1/A2/A3* expression levels and 2.5-fold increase in the
281 *TaRPL3B1/B2/B3* expression levels in 8153 compared to the wt plants. The increases
282 observed in the endogenous *TaRPL3* expression level in the transgenic wheat plants were of
283 similar magnitude to those previously documented in transgenic tobacco plants [16].
284

285 *3.4. Greenhouse test of transgenic wheat plants for FHB resistance*

286

287 To determine if the transgenic wheat plants expressing the yeast L3Δ were resistant to
288 infection by *F. graminearum*, T_3 generation homozygous lines and non-transformed
289 Bobwhite plants were evaluated for resistance in a greenhouse. Wheaton, a hard red spring
290 wheat, was used as the susceptible check and Alsen, another hard red spring wheat, was used
291 as the resistant check. Twenty-one days after the single spikelet at the central node of the
292 main spike was inoculated with a macroconidial spore suspension, whole spikes of wt plants
293 had turned brown (Fig. 4A). In the transgenic plants, except in line 774, browning was
294 mainly confined to the inoculated spikelets (Fig. 4A). Table 1 lists the averages and ranges
295 of incidence of *F. graminearum* infection in all the wheat lines including Alsen and Wheaton
296 from two separate experiments. All transgenic wheat lines except 774 exhibited a
297 significantly lower incidence of infection spread than the untransformed wt Bobwhite as
298 assessed by the Student *t* test (excluding Alsen and Wheaton). The average FHB incidences
299 for the five transgenic lines in these greenhouse tests were inversely correlated with their

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300 levels of the L3Δ mRNA (Fig. 3A). The FHB severity parameter measures the percentage of
301 spikelets with symptoms. Fig. 4B indicates that on average a 50% reduction in disease
302 severity was observed in line 772, a 49% reduction in line 8153, and a 48% reduction in line
303 8133, as compared to the wt Bobwhite. These results demonstrated that transgenic wheat
304 plants expressing the yeast L3Δ showed improved resistance to FHB over the untransformed
305 Bobwhite plants.

306

307 *3.5. Reduction of DON toxin levels in some transgenic wheat plants in greenhouse test*

308

309 To determine if the FHB resistance of L3Δ transgenic wheat plants would result in
310 reduction of the fungal toxin DON, the mature kernels above and below 20 to 25 inoculated
311 spikelets were extracted, and their DON levels were measured by GC/MS. Since the
312 susceptible Wheaton was heavily infested (Table 1 and Fig. 4B), we were not able to harvest
313 sufficient seeds to be able to determine the DON levels in this line. However, compared to
314 the untransformed wt Bobwhite, there was a 58% reduction in DON levels in the transgenic
315 line 772, a 46% reduction in DON levels in line 8153, and a 36% reduction in line 771 (Fig.
316 4C). These were also the lines with the highest L3Δ mRNA levels. Surprisingly, lines 774
317 and 8133 had higher DON levels than their non-transformed parent in the greenhouse tests.

318

319 *3.6. Field testing of transgenic wheat plants for FHB resistance*

320

321 In order to assess resistance to FHB in the field, the transgenic wheat lines were
322 planted in the field during spring of 2008 along with untransformed Bobwhite and resistant
323 and susceptible check cultivars (Materials and Methods). The disease pressure in this trial
324 was very high, as indicated by FHB incidence of 91% for moderately resistant check Alsen.

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325 The FHB incidence was 100% for all the transgenic wheat lines, the non-transformed wt
326 Bobwhite plants, and the susceptible check Wheaton. However, plants from line 8153 had a
327 significantly lower percentage of visually scabby kernels (VSK%) and DON levels,
328 compared to the untransformed wt Bobwhite plants (Table 2).
329

330 **4. Discussion**

331

332 Outbreaks of FHB over the last 15 years have caused significant yield losses in wheat
333 and barley in the United States [2] and the resultant DON contamination in small grain crops
334 is a major health concern for human food and animal feed. Control of FHB disease is a
335 proactive way to prevent DON from getting into the food supply. Although wheat cultivars
336 with partial resistance are available, development of completely resistant cultivars by
337 conventional breeding methods has proven to be difficult. The fungicides that have been
338 approved to control FHB are also only partially effective. Thus, additional sources of host
339 plant resistance, from breeding or genetic engineering, are needed to protect small grains
340 from FHB in the field.

341 In the present study, we report the production of transgenic Bobwhite wheat plants
342 expressing the N-terminal 99 amino acids of the yeast L3 protein under the control of either
343 the barley *Lem1* or the maize *Ubi1* promoter. Endogenous wheat *TaRPL3* genes were up-
344 regulated in most of the transgenic wheat lines. Most noticeably, 772 expressing pLem-L3d
345 and 8153 expressing pUbi-L3d had higher gene expression levels of L3Δ compared to the
346 other transgenic wheat lines. These two lines demonstrated the highest resistance to the
347 spread of FHB (Type II resistance) and the lowest DON levels when adult plants were
348 challenged with *F. graminearum* in greenhouse tests. These transgenic lines also showed
349 higher levels of accumulation of the endogenous *TaRPL3As*. Even though the FHB incidence

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350 in the field test reported here was very high, plants from line 8153 demonstrated the lowest
351 VSK percentage and lowest level of DON, indicating that high levels of L3Δ and/or
352 endogenous RPL3 can protect plants from FHB and reduce DON accumulation in wheat
353 seeds.

354 The difference in the behavior of 8153 and 772 in the field may be due to the different
355 activities of their promoters. The *Ubi1* promoter is expressed throughout the plant, so any
356 protective protein encoded by *Ubi1*::L3Δ would be present during all phases of plant
357 development [28]. Thus 8153 could be protected both from the spread of FHB (Type II
358 resistance), as measured by the greenhouse tests, and the recurring infection by FHB that
359 occurs in the field (Type I resistance). The *Lem1* promoter has much more restricted
360 expression [20] and it may be that the L3Δ does not accumulate in 772 in all the tissues in
361 which it is needed for Type I protection.

362 Our resistance test results are comparable to those reported by others using the
363 transgenic approach to engineer FHB resistance by expressing genes with different modes of
364 action. The *Fusarium TRI*101 gene, which encodes a trichothecene 3-*O*-acetyltransferase,
365 was expressed in transgenic wheat plants, where it conferred partial protection against the
366 spread of *F. graminearum* in greenhouse tests [19]. Several groups have expressed
367 individual plant defense genes under control of constitutive promoters in transgenic wheat
368 [29-31]. Greenhouse tests of plants carrying such constructs showed that Type II resistance
369 was improved 20 to 50% compared with their non-transformed parents. However, when
370 several of these transgenic plants were challenged with *Fusarium* infection in field
371 experiments [19, 29, 31], only those over-expressing a wheat alpha-thionin, a barley beta-1,3-
372 glucanase, or a barley thaumatin-like-protein showed any improvement in resistance [31].
373 Among these, only a line carrying the glucanase transgene showed reduction of multiple
374 disease indices in the field, including DON accumulation, percentage visually scabby kernels,

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375 and disease severity, compared with the non-transformed parent [31]. Improved Type II
376 resistance in greenhouse tests was also reported by Makandar et al. [32], who expressed the
377 *Arabidopsis thaliana NPR1* (At*NPR1*) gene in Bobwhite wheat. Since At*NPR1* regulates the
378 activation of systemic acquired resistance, the heightened resistance in their wheat plants was
379 attributed to the transgenic plants being more responsive to an endogenous activator of plant
380 defense. Recently, an antibody fusion protein consisting of a *Fusarium* specific recombinant
381 antibody derived from chicken and an antifungal peptide from *Aspergillus giganteus*
382 conferred Type I and Type II resistance to FHB [33].

383 Here we use a novel approach for FHB resistance by expressing a modified form of
384 the trichothecene target in transgenic plants. Our results demonstrate that expression of yeast
385 L3Δ in transgenic wheat plants can result in improved FHB resistance and a reduction in
386 DON accumulation in wheat kernels. We postulate that introduction of a modified form of L3
387 into transgenic plants may prevent DON from targeting ribosomal protein L3 at the
388 peptidyltransferase center. Consequently, ribosome-bound L3 may be protected from the
389 inhibitory effects of DON. The tryptophan residue at position 255 in the yeast L3 that is
390 mutated in *tcm1* makes the closest approach of any amino acid to the peptidyltransferase
391 center of the large subunit [34]. Previous studies showed that substitution of W255 with
392 cysteine (W255C) conferred resistance to trichodermin, anisomycin, and affected
393 translational fidelity by decreasing the peptidyltransferase activity [34]. Since expression of
394 L3Δ in yeast has similar effects on resistance to anisomycin and on translational fidelity as
395 the *tcm1* mutation [35], an alternative model is that L3Δ may be making the ribosomes less
396 sensitive to DON by decreasing their peptidyltransferase activity. Further studies are needed
397 to investigate the observed resistance.

398 Lucyshyn et al. (2007) reported that the amino acids encoded by the *TaRPL3* genes
399 were the same in wheat cultivars differing in *Fusarium* resistance [18], ruling out qualitative

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400 mutations in *TaRPL3* alleles that could account for resistance in varieties Frontana and CM-
401 82036. In the latter cultivar, the locus for *TaRPL3-A3* was mapped in a low recombination
402 region that included an FHB resistance QTL [27]. In light of our observations that *TaRPL3*
403 levels were increased in some of our L3Δ transgenic plants, it would be interesting to
404 measure the levels of *TaRPL3-A3* transcripts in CM-82036 to determine whether quantitative
405 differences in *TaRPL3* levels could account for this variety's resistance.

406 Regardless of the mechanism, protection of wheat ribosomes from the inhibitory
407 effects of DON would rob *Fusarium* fungi of one of their most potent virulence factors, thus
408 decreasing the severity of FHB. Further field testing is needed to confirm the potential of this
409 strategy for increasing both Type I and Type II resistance and decreasing mycotoxin
410 accumulation in cereals.

411

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413

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422 **4.1**
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533

534 **Table 1** Averages and ranges of incidence of *F. graminearium* infection from two separate

535 experiments

536

537

Line	Number of plants tested	Percentage of infected spikes[1]	Range of percentage
771	24	22.3±18.3*	11.7 - 54
772	25	18.7±5.9*	10.1 - 25.9
774	24	43.3±19.8	20.3 - 60.5
8133	24	31.4±16.9*	17.4 -51.5
8153	25	20.5±16.9*	10.8 - 35.1
wt Bobwhite	23	42.0±17.2	22.2 - 55
Alsen (resistant check)	25	10.8±5.7*	6.5 - 20.8
Wheaton (susceptible check)	16	65.7±29.4	27.7 - 95.7

549

550 [1] average ± standard deviation

551 *The percentage of infected spikelets in these transgenic plants was significantly different

552 from the wt Bobwhite as assessed by the Student *t* test ($p < 0.05$).

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553
554 **Table 2** Field testing of transgenic wheat lines in 2008. The mean percentage of visually
555 scabby kernels (VSK%) was assessed with 4 replicates after the seeds were harvested. The
556 DON levels in the kernels were analyzed as described in Materials and methods.
557
558

Line	VSK%	Range of VSK%	DON[1] (ppm)	Range of DON level
771	26.3±7.8	17.5-35.0	39.2±8.5	29.3-47.5
772	24.4±5.5	17.5-30.0	36.5±2.5	33.9-39.2
774	25.6±9.4	17.5-35.0	35.8±9.8	28.1-49.2
8133	26.3±13.6	12.5-45.0	34.5±5.9	29.7-42.8
8153	16.9±3.2*	12.5-20.0	28.0±4.9*	23.5-34.9
wt Bobwhite	28.8±1.4	27.5-30.0	35.3±8.2	27.4-46.6
Alsen (resistant check)	5.8±2.4*	4.0-9.0	13.0±6.4*	5.7-21.1
Wheaton (susceptible check)	58.1±19.5	40.0-75.0	53.3±14.5	42.6-74.7

572
573 [1] average ± standard deviation
574
575 * The values were significantly different from those of wt Bobwhite plants as assessed by the
576 Student *t* tests ($p < 0.05$).
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578

579 **Fig. 1.** Constructs used to generate the transgenic wheat plants. A gene fragment from yeast
580 that encodes the N-terminal 99 amino acids (L3Δ) of the ribosomal protein L3 was cloned
581 into wheat expression vectors under the control of either the barley *Lem1* (pLem-L3d) or the
582 maize *Ubi1* promoter/first intron (pUbi-L3d). The nopaline synthase (NOS) 3' transcription
583 termination sequence was used as the transcription terminator in both constructs.

584

585 **Fig. 2.** Alignment of the amino acids of yeast L3Δ with the wheat (*Triticum aestivum*) L3
586 proteins. Amino acids that differ among the aligned sequences are boxed. **A.** Comparison of
587 the N-terminal 99 amino acids of the yeast with the wheat L3 encoded by *TaRPL3A1,*
588 *TaRPL3A2, TaRPL3A3, TaRPL3B1, TaRPL3B2* and *TaRPL3B3*. The region used to design
589 primers specific for the yeast L3Δ transgene transcripts is over-lined. **B.** Diversity of amino
590 acids 277 to 300 among the wheat L3 proteins.

591

592 **Fig. 3.** Real time PCR analysis of the L3Δ and *TaRPL3* mRNA levels in the transgenic wheat
593 lines. Yeast L3Δ mRNA (**A.**) endogenous *TaRPL3A* (**B.**) and *TaRPL3B* (**C.**) mRNA levels
594 were quantified by real-time PCR analysis using gene-specific primers. Total RNA was
595 isolated from the florets and immature seeds of the wheat plants at the stage just prior to
596 when they would be inoculated with *F. graminearum* for the greenhouse resistance tests. The
597 relative gene expression levels were determined using the $2^{-\Delta\Delta C_T}$ method and wheat α-tubulin
598 mRNA as an internal control. The relative gene expression levels were measured from four
599 to five plants per line, averaged and presented as bar graphs with standard errors.

600

601 **Fig. 4.** Susceptibility of transgenic wheat plants to FHB in the greenhouse and accumulation
602 of DON in the kernels. **A.** The central spikelets of the main spike of transgenic and wt wheat

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603 plants, 20 to 25 plants per line, were inoculated with 10 μl of a macroconidial spore
604 suspension (100,000 conidia ml^{-1}) of *F. graminearum* where indicated by the arrows.
605 Disease spread was recorded 21 days after the inoculation. Representative plants are shown.
606 **B.** FHB severity was assessed by counting the number of symptomatic spikelets for each
607 spike and expressing the infection level as the percentage of the total number of spikelets for
608 each spike. FHB severity was averaged from two separate experiments for each line and
609 presented with standard errors. **C.** The mycotoxin DON was extracted from kernels
610 harvested from the four spikelets adjacent to the inoculated spikelet of inoculated heads.
611 DON was analyzed using GC/MS. DON levels from each line were averaged from two
612 separate experiments and shown in the bar graph with standard errors.
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Figure(s)

Fig. 1

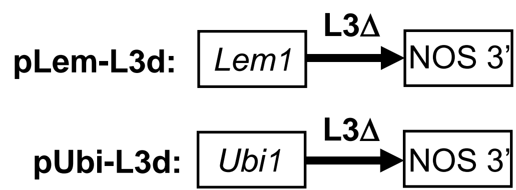


Fig. 2

A.

```
1 MSHRKYEAPRHGHLGFLPRKRAASIRARVKAFPKDDRSKPVALTSFLGYK   Yeast L3Δ
  MSHRKFEHPRHGSLGFLPRKRCSRHRGKVKSFPKDDQQKPCHLTAFLGYK   Wheat RPL3A1
  MSHRKFEHPRHGSLGFLPRKRCSRHRGKVKSFPKDDQQKPCHLTAFLGYK   Wheat RPL3A2
  MSHRKFEHPRHGSLGFLPRKRCSRHRGKVKAFPKDDQQKPCHLTAFLGYK   Wheat RPL3A3
  MSHRKFEHPRHGSLGFLPRKRCSRHRGKVKAFPRDDQSKKCHLTAFLGYK   Wheat RPL3B1
  MSHRKFEHPRHGSLGFLPRKRCSRHRGKVKAFPRDDQSKKCHLTAFLGYK   Wheat RPL3B2
  MSHRKFEHPRHGSLGFLPRKRCSRHRGKVKAFPRDDQSKKCHLTAFLGYK   Wheat RPL3B3

  AGMTTIVRDLDRPGSKFHKREVVEAVTVVDTPPVVVVGVVGYVETPRGL
  AGMTHIVREVEKPGSKLHKKETCEAVTIIETPPLVIVGLVAYVKTPRGL
  AGMTHIVREVEKPGSKLHKKETCEAVTIIETPPLVIVGLVAYVKTPCGL
  AGMTHIVREVEKPGSKLHKKETCEAVTIIETPPLVIVGLVAYVKTPRGL
  AGMTHIVREVEKPGSKLHKKETCEAVTIVETPPIVIVGLVAYVKTPRGL
  SGMTHIVREVEKPGSKLHKKETCEAVTIVETPPIVIVGLVAYVKTPRGL
  AGMTHIVREVEKPGSKLHKKETCEAVTIVETPPIVIVGLVAYVKTPRGL 99
```

B.

```
277 HRTEMNKKIYKMGKSGQESHEACT       Wheat RPL3A1
    HRTEMNKKIYKMGKSGQESHEACT       Wheat RPL3A2
    HRTEMNKKIYKMGKSGQESHEACT       Wheat RPL3A3
    HRTEMNKKVYKIGKVGQETHDAST       Wheat RPL3B1
    HRTEMNKKVYKIGKVGQETHDAST       Wheat RPL3B2
    HRTEMNKKVYKIGKVGQETHDAST 300   Wheat RPL3B3
```

Fig. 3

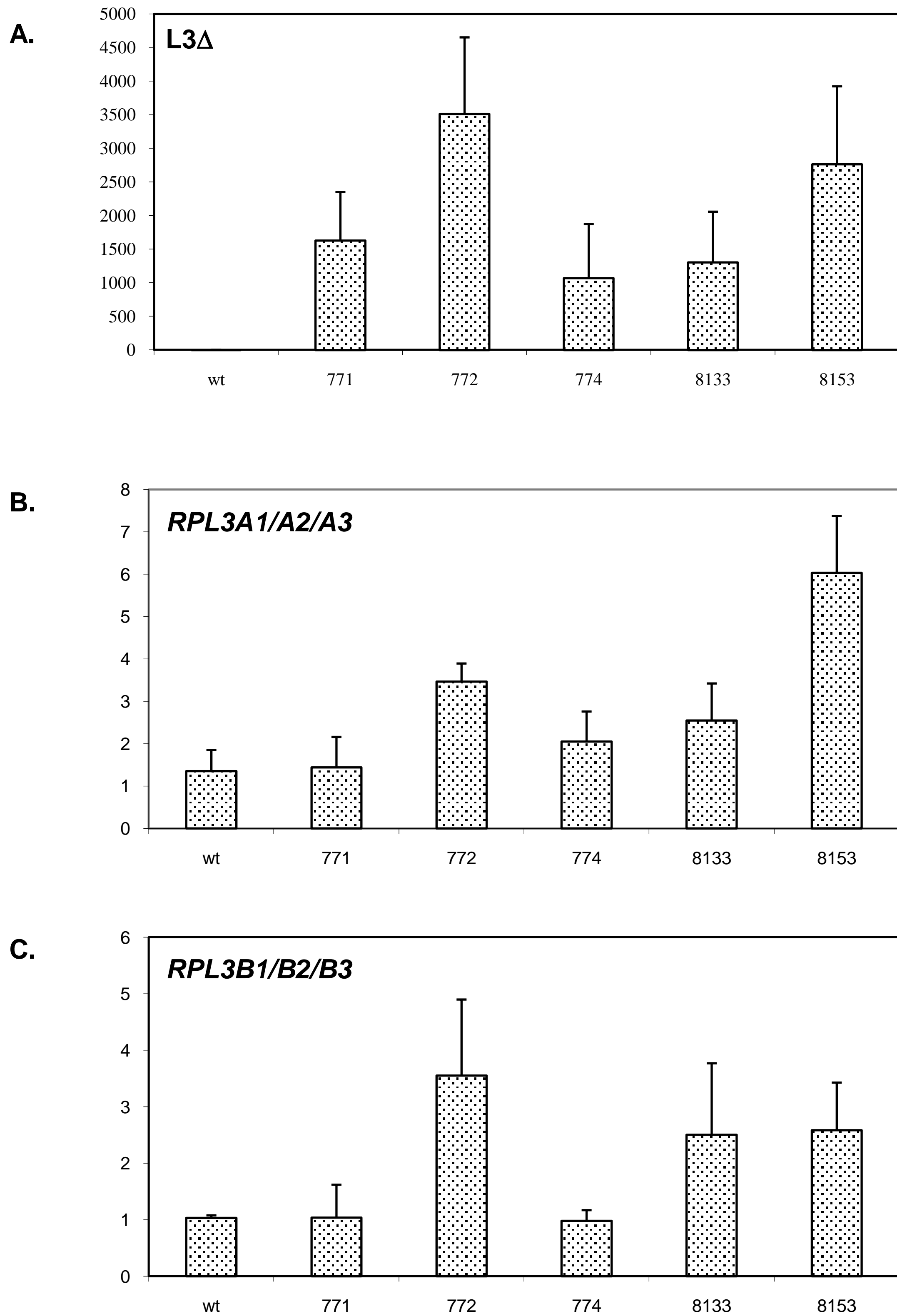
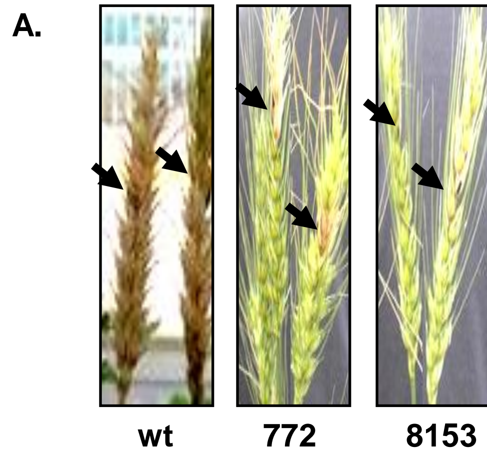
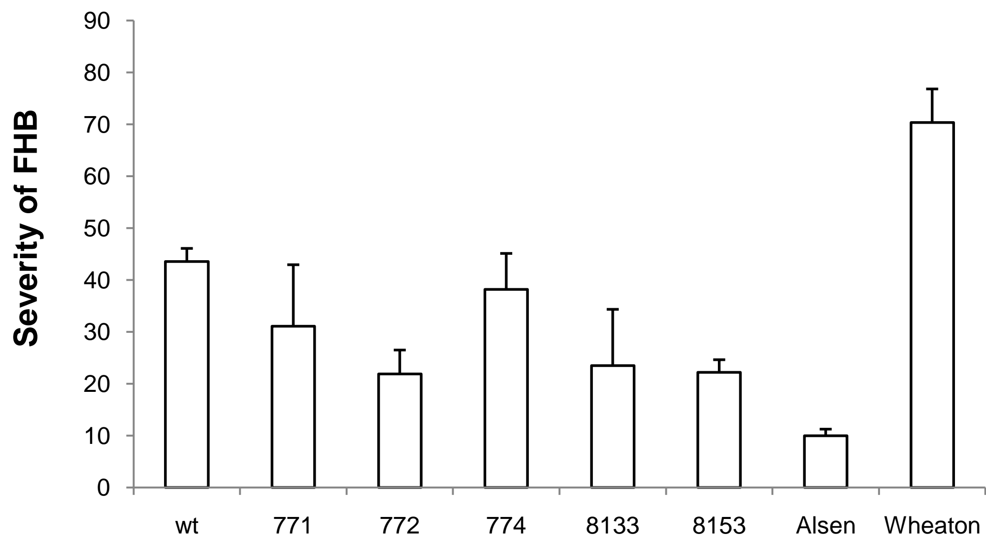


Fig. 4

A.



B.



C.