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Transgenic corn plants with modified ribosomal protein L3 show decreased ear rot disease after inoculation with *Fusarium graminearum*

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Abstract

Cereal crops are susceptible to various *Fusarium* species worldwide. *Fusarium graminearum* is a major pathogen of corn that causes ear rot disease and contaminates kernels with mycotoxins such as deoxynivalenol (DON). DON binds with the 60S ribosomal protein L3 (RPL3), blocking the translational machinery in the eukaryotic cells. Previously, the modified rice *Rpl3* gene (resulting in a change in amino acid residue at 258 from tryptophan to cysteine) was transformed into tobacco and resulted in increased resistance to DON. In the present study, the same modified *Rpl3* gene was used to develop two types of transgenic corn plants in which the modified *Rpl3* gene was controlled by a constitutive 35S CaMV promoter or a silk-specific *ZmGRP5* promoter. The transgenic lines were evaluated for the ear rot disease in the field by inoculating *F. graminearum* spores in silk and kernel tissues. The overall disease symptoms in the transgenic lines were significantly lower than wild type plants. Transgenic plants having the *Rpl3* gene with 35S CaMV promoter showed a 23 to 58% decrease in disease scores whereas the transgenic plants expressing the modified *Rpl3* gene under silk specific promoter showed a 27 to 62% decrease in disease scores compared to wild type plants. The kernel inoculation gave lesser disease symptoms in both types of transgenic plants compared to silk inoculation. The maximum decrease in disease scores was observed (up to 62%) in transgenic plants expressing modified *Rpl3* gene with silk specific promoter when inoculations were done through kernels. The present report is the first field study of transgenic corn plants developed to reduce *F. graminearum* infection. Taken together, the results suggest that the modified *Rpl3* gene is effective in reducing ear rot disease in corn.

Keywords: Corn, ear rot, *Fusarium*, mycotoxins, *Rpl3* gene, trichothecene
Abbreviations: 2,4 D- 2,4-Dichlorophenoxyacetic acid, BAP- 6-Benzylaminopurine; DON- Deoxynivalenol, MSI- multiple shoot initials, *Rpl3*- modified ribosomal Protein L3 with substitution of amino acid tryptophan to cysteine at position 258 of rice cDNA, WT- wild type.

Introduction

Cereal production throughout the world is adversely affected by various *Fusarium* species. *Fusarium graminearum* (teleomorph *Gibberella zeae*) is one of the most economically important pathogens that causes head blight in cereals like wheat and barley and ear rot and stalk rot in corn. *Fusarium* contaminated grains produce mycotoxins that cause serious health risks following consumption by humans and animals. The infected grains can not be used for food or feed, causing significant economic loss to farmers (Munkvold, 2003). Mycotoxin, such as DON produced by *F. graminearum* is a potent inhibitor of eukaryotic protein synthesis and is believed to play a role in fungal pathogenesis on cereal crops worldwide (Harris et al., 1999; Desjardins et al., 1996). Previously, research has been done to develop resistance in cereal crops against *F. graminearum* through molecular breeding and transgenic resistance. However, complete resistance is still a challenge due to the complex genetics of resistance controlled by multiple genes (Ali et al., 2005; Buerstmayr et al., 2009). The genetic transformation methods of cereal crops have improved in recent years and are performed routinely in several laboratories worldwide. Transgenic resistance in cereal crops have been deployed against *Fusarium* species. Genes that have resulted in increased resistance in plants include those encoding antifungal proteins (Mackintosh et al., 2007), pathogenesis-related proteins or defense response proteins (Anand et al., 2003; Makandar et al., 2006), trichothecene detoxifying enzymes (Higa et al., 2003; Igawa et al., 2007), ribosomal inactivating proteins (Logemann et al., 1992) or DON-resistant form of RPL3 protein (Harris and Gleddie, 2001). Different mutations in the ribosomal protein L3 domain have been studied in yeast (*Saccharomyces cerevisiae*) that confers resistance against trichothecenes produced by *Fusarium* species (Fried and Warner 1981; Mitterbauer et al., 2004). A single base pair substitution in cDNA which results in the change of tryptophan to cysteine at amino acid residue 255 in the RPL3 protein has shown resistance against trichothecene in yeast (Mitterbauer et al., 2004). Similarly, a mutation in the *Rpl*3 gene of rice which resulted in the change of tryptophan to cysteine at amino acid residue 258 (modified *Rpl3*) conferred tolerance to trichothecene in tobacco protoplasts (Harris and Gleddie, 2001). This approach of transforming genes for modified targets of DON could potentially be used in cereal crops. The aim of this study was to develop transgenic corn plants expressing a modified *Rpl3* gene from rice and their evaluation under field conditions against ear rot disease by *F. graminearum*. Two types of transgenic plants were developed, *Rpl3* gene with a constitutive promoter, 35S CaMV (Ishida et al., 1996) and the second having *Rpl3* gene with silk specific promoter, *ZmGRP5 (*Tao et al., 2006; Johnson et al., 2007*). F. graminearum* was inoculated via kernel or silk in both types of plants. The results showed that the transgenic corn plants displayed significantly lower disease symptoms compared to

wild type (WT) plants. Transgenic plants having a modified *Rpl3* gene with silk-specific promoter had highest tolerance to *F. graminearum* and the lowest disease symptoms under field conditions.

Results

Transformation and characterization of transgenic corn plants

Agrobacterium mediated transformation was used to develop the transgenic corn plants expressing the modified *Rpl3* gene either constitutively using CaMV promoter (Ishida et al., 1996) or in silk tissues using a silk-specific promoter (Tao et al., 2006; Johnson et al., 2007). For this, two types of vectors were designed expressing, (i) modified *Rpl3* gene with constitutive 35S CaMV promoter (PML14 plasmid), and (ii) modified *Rpl3* gene with silk-specific promoter, *ZmGRP5* (PML16 plasmid). The structures for the two vectors are shown in Fig 1. The transgenic plants were analyzed by Polymerase Chain Reaction (PCR) to confirm the presence of different genes. The amplified bands of transformed genes are shown in Fig 2. Eight selected T0 plants were regenerated from transformations with plasmid PML14 that had the *Rpl3* gene with 35S CaMV promoter showed an amplified band of 977 bp corresponding to the presence of *Rpl3* (Fig 2A). Similarly, six T0 plants obtained after co-cultivation with *Agrobacterium* containing plasmid PML16 that has the silk promoter and *Rpl3* gene, had an amplified band of 650 bp (Fig 2B). All the transgenic plants that showed the presence of *Rpl3* gene also tested positive for the *BAR* gene, having a 455 bp band amplified using *Bar* gene specific primers (Fig 2C). Among the PCR positive transgenic plants, three lines of PML14 (PML14-102, PML14-110 and PML14-133) and two lines of PML16 (PML16-53 and PML16-61) produced seeds at maturity and these lines were further advanced to the next generation. The PML14-102 lines had tassels and were self-pollinated, while plants of PML14-110 and PML14-133 were fertilized with the WT pollen because sometimes tissue culture regenerated corn plants do not produce tassels (Li et al., 2002) that might have occurred in PML14-110 and PML14-133 transgenic lines. In the next generation, all T1 plants from both PML14 and PML16 transformations produced normal tassels and were self-pollinated to produce T2 seeds. All the T1 plants were PCR positive for the presence of *Rpl3* and *BAR* gene.

Southern hybridization analysis

Southern hybridization analysis was conducted to confirm the presence of the *Rpl3* and *BAR* genes in the T1 transgenic plants (Fig 3). The *Rpl3* probe was synthesized by PCR that was complementary to the first 200 bp of the *Rpl3* gene. (Fig 1) The Dig-labeled 200 bp *Rpl3* probe detected the transformed *Rpl3* gene in both PML14 and PML16 transformed plants (Fig 3A). Each of the PML14 and PML16 plasmids had two *Pst1* restriction enzyme sites with fragments of 2734 bp and 3148 bp, respectively (Fig 1) served as controls in Southern hybridization analysis. Genomic DNA of the transgenic plants was digested with the *Pst1* restriction enzyme to detect the presence *Rpl3* gene. The transgenic corn plants developed from PML14 transformation showed the expected hybridization band of 2734 bp and for PML16 transformed plants showed a band of 3148 bp (Fig 3A). Southern hybridization was also done with the probe developed from *Bar* gene specific sequences. The DNA of PML14 and PML16 transgenic plants was digested with *Dar1* restriction enzyme and probed with *Bar* gene sequence gave hybridization band of expected size 2500 bp (Fig 3B). The results from Southern hybridization has confirmed the integration of *Rpl3* and *Bar* gene in plant genomic DNA.

Field evaluation of transgenic plants for ear rot disease

The T2 progenies of the transgenic corn plants were tested under field conditions for *Fusarium* ear rot disease development after *F. graminearum* inoculations. Two types of transgenic corn plants were developed expressing the *Rpl3* gene either with a constitutive promoter or silk specific promoter. All transgenic corn plants showed normal growth and development. Two types of inoculation were conducted to inject spores either through kernels or onto the silks protruding outside the ears. The disease evaluation was performed at maturity by scoring the disease symptoms on the ears. The transgenic lines tested in the field were T2 segregating populations and two transgenic lines (PML14-110 and PML14-133) were produced after crossing with the WT plants. Therefore the field experiments consisted of mixtures of homozygous and heterozygous plants for the *Rpl3* gene. For this reason detailed results of disease scores from one representative replication is described in Table 1 that clearly shows the performance of individual transgenic plants compared to WT (non-transgenic) plants. The disease scoring was done on a scale of 1 to 7; where 1 means no disease and 7 as sever disease, the representative corn ears of different scores are shown in Fig 4. The results for disease scores in transgenic plants inoculated through kernel and silk are described as following:

Disease scores after kernel inoculation of transgenic corn plants

For kernel inoculation, the transgenic plants developed from plasmid PML14 (35SCaMV promoter) showed responses from highly tolerant to susceptible with disease scores ranging from 1 to 7. The transgenic line PML14-102-1 had the highest number of plants with low disease scores followed by transgenic lines PML14-110-3, PML14-110-4, and PML14-133-1. The plants of transgenic line PML14-102-1 were self-pollinated to produce T1 and T2 seeds, therefore their progeny might have higher numbers of tolerant plants due to homozygosity. The transgenic lines PML14-110 and PML14-133 were crossed with the WT plants in first generation (T1) resulting in heterozygosity in the successive T2 generation. Out of the 60 transgenic plants inoculated, 29 transgenic plants (48%) produced disease scores lower than 4. None of the WT plants had disease scores of ≤ 4 and their disease severity was higher, in the range of 5 to 7 (Table 1). The PML16 transgenic plants had lower disease symptoms upon kernel inoculation. The majority of these transgenic plants had disease scores between 1 and 4. Out of a total of 105 transgenic plants inoculated, 76 transgenic plants (72%) had disease scores in the range of 1 to 4. All of the WT plants showed higher disease scores of 6 and 7. Amongst the different progenies tested, the PML16-53-2, PML16-53-7, PML16-61-5 and PML16-61-15 lines had the highest numbers of plants (5 each) that did not show any disease symptoms i.e. a disease score of 1 (Table 1). The result shows that PML16 transgenic plants which were developed using *Rpl3* gene with a silk-specific promoter had better disease resistance compared to PML14 plants which had the *Rpl3* gene with constitutive promoter.

Table 1. Disease scores in corn plants. Number of plants obtained with different disease scores in a representative replication after *F. graminearum* inoculation under field conditions. Transgenic corn lines expressing modified *Rpl3* gene with 35S promoter (PML14 plants) and silk promoter (PML16 plants) were inoculated separately through kernel and silk tissues. The disease scoring was on a scale of 1 to 7; where 1 means no disease and 7 as sever disease. The hyphen means there was no plant in that corresponding disease score.

Number of plants with corresponding disease scores													
Disease scores	WT	PML14 plants				WT	PML16 plants						
		102-1	110-3	110-4	133-1		53-1	53-2	53-4	53-6	53-7	61-5	61-15
Kernel Inoculation													
1	-	4	3	3	2	-	4	5	4	3	5	5	5
2	-	2	1	1	1	-	1	2	2	2	2	2	2
3	-	4	2	1	1	-	1	2	2	-	2	2	3
4	-	2	-	3	1	-	3	4	3	3	3	3	-
5	2	1	2	2	3	1	5	1	3	3	2	2	3
6	6	2	3	4	3	6	-	-	1	2	-	1	1
7	7	-	4	1	4	7	1	1	-	-	1	-	1
Silk Inoculation													
1	-	2	3	3	-	-	1	1	-	-	1	1	-
2	-	2	1	1	1	-	-	1	1	2	-	1	1
3	-	2	2	-	4	-	2	2	2	4	1	2	1
4	-	3	-	3	2	-	3	2	4	4	2	1	3
5	-	4	2	1	2	-	2	3	3	3	5	6	4
6	5	2	3	4	3	3	4	2	4	2	3	3	5
7	10	-	4	3	3	12	3	4	-	1	3	1	1

Table 2. Average disease score of transgenic plants after inoculation with *F. graminearum* spores. Transgenic plants were developed by expressing *Rpl3* gene with a constitutive promoter (PML14 plants) or silk promoter (PML16 plants). The disease score was on a scale 1 to 7, where 1 = no visual signs of disease, 2 = 1-3%, 3 = 4-10%, 4 = 11-25%, 5 = 26-50%, 6 = 51-75% and 7 = 76-100% disease. Data shown is mean ± SD (*n*=3). Disease scores within each treatment of inoculation with different letters (a-d) are significantly different at $P<0.05$ (Fisher's protected LSD test). The WT and transgenic plants with disease score <4 were considered as resistant, disease score 4 to 5 as moderately resistant and disease scores >5 as susceptible.

Genotype	Average Disease Score		Disease Severity	
	Kernel Inoculation	Silk Inoculation	Kernel Inoculation	Silk Inoculation
PML14 plants				
WT	6.9 ± 0.74a	7.0 ± 0.78a	Susceptible	Susceptible
102-1	2.9 ± 0.28d	3.8 ± 0.43d	Resistant	Resistant
110-3	3.6 ± 0.37c	4.4 ± 0.47cd	Resistant	Moderate
110-4	4.4 ± 0.36b	4.9 ± 0.54bc	Moderate	Moderate
133-1	5.1 ± 0.48b	5.4 ± 0.41b	Susceptible	Susceptible
PML16 plants				
WT	6.9 ± 0.77a	7.0 ± 0.81a	Susceptible	Susceptible
53-1	3.4 ± 0.41b	4.0 ± 0.52cd	Resistant	Resistant
53-2	2.6 ± 0.31c	3.6 ± 0.41d	Resistant	Resistant
53-4	3.3 ± 0.38bc	4.6 ± 0.49bc	Resistant	Moderate
53-6	3.4 ± 0.38b	3.6 ± 0.39d	Resistant	Resistant
53-7	2.9 ± 0.32bc	4.4 ± 0.38bc	Resistant	Moderate
61-5	3.2 ± 0.35bc	5.1 ± 0.54b	Resistant	Susceptible
61-15	3.1 ± 0.36bc	5.0 ± 0.56bc	Resistant	Moderate

Disease scores after silk inoculation of transgenic corn plants

For silk inoculation, the PML14 transgenic lines showed a range of disease scores from 1-7 (Table 1). Among 60 transgenic plants inoculated, 24 plants (40%) had disease scores of ≤4. The maximum number of transgenic plants (9 plants) were observed with lower disease scores (≤4) in the PML14-102-1 transgenic line followed by PML14-110-4, PML14-110-3 and PML14-133-1. The PML16 transgenic plants inoculated through silk had disease scores 1 to 4 in 43 of 105 transgenic plants (42%), while all the WT plants scored 6 to 7 (Table 1). The transgenic plants PML16-53-2 and PML16-53-6 produced lower disease scores among all PML16 progenies. The silk inoculation resulted in more severe disease symptoms compared to the kernel inoculation in transgenic and WT plants.

Mean analysis of disease severity in transgenic corn plants

Table 2 shows the average disease scores recorded in transgenic and WT plants. Despite the mixed population of homozygous and heterozygous transgenic plants tested under field conditions, the disease symptoms in the transgenic plants was significantly lower compared to the WT plants. Transgenic plants with the *Rpl3* gene with a constitutive promoter resulted in a 23 to 58% decrease in disease scores. The transgenic plants expressing a modified *Rpl3* gene with a silk-specific promoter showed 27 to 62% decrease in disease scores, compared to WT plants. Among the inoculation methods used, the kernel inoculated transgenic plants had significantly lower disease scores compared to silk inoculated transgenic plants. The maximum decrease in disease scores was observed (up to 62%) in transgenic plants expressing modified *Rpl3* with silk specific promoter when inoculated

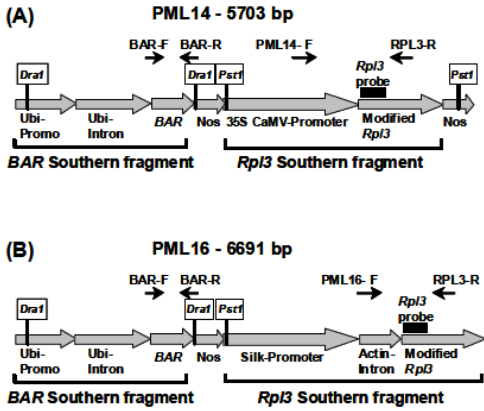

Fig 1. Schematic diagram of vectors used for corn transformation. A- Plasmid PML14 having the modified *Rpl3* gene fused with 35S CaMV promoter. B- Plasmid PML16 having the *Rpl3* gene with a silk-promoter, *ZmGRP5* and rice actin intron. The plasmids had herbicide resistant *BAR* gene with *Ubiqutin* promoter. The restriction sites, probes and positions of PCR primers are also shown.

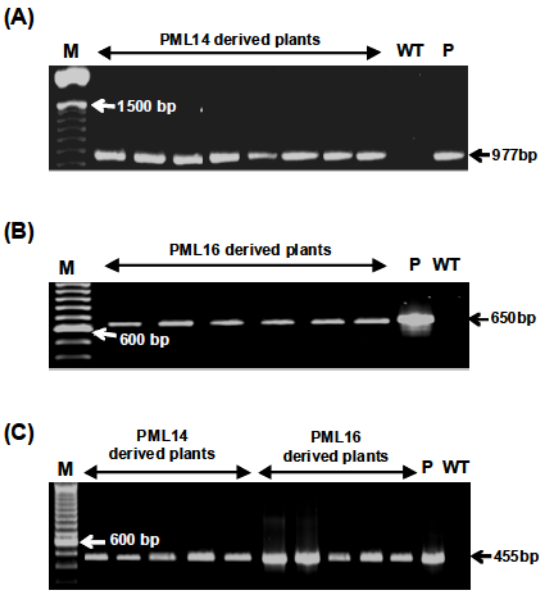

Fig 2. PCR analysis of *Rpl3* and *BAR* genes in transgenic corn plants. A- PML14 transgenic plants expressing *Rpl3* gene with 35S CaMV promoter had PCR amplified band of 977 bp. B- PML16 transformed plants expressing *Rpl3* gene with silk promoter had PCR amplified band of 650 bp. C- Presence of *BAR* gene was confirmed in PML14 and PMl16 transgenic plants had PCR amplified band of 455 bp. WT- non-transgenic wild type plant, P- plasmid, M- marker ladder.

through kernels. The lower disease symptoms in transgenic plants compared to WT plants suggest that transformation with *Rpl3* gene resulted in tolerance against ear rot disease in transgenic plants.

Discussion

This is the first report of field evaluation of transgenic corn plants engineered to tolerate ear infections caused by *F. graminearum.* Inoculation studies performed with mutated strains of *F. graminearum* on wheat and corn have indicated that strains lacking the ability to produce trichothecene have reduced the incidence of disease in these crops (Desjardins et al., 1996; Harris et al., 1999). Similarly, a strategy to modify the site of action of mycotoxins within host plant cells may be expected to lead to improved tolerance to this pathogen in crops sensitive to ear rot disease. Following this approach, in the present study, the corn plants were transformed with a modified rice gene (*Rpl3*) that codes for a ribosomal protein L3, which is a primary target of deoxynivalenol, DON (Feinberg and McLaughlin 1989). This gene was previously used to increase the tolerance of transgenic tobacco to the presence of DON. However, over-expression of the modified rice *Rpl3* gene led to growth abnormalities in transgenic tobacco plants. This was attributed to an incompatibility between the RPL3 protein of rice and tobacco (Harris and Gleddie, 2001). In contrast to this finding, we did not observe any phenotypic or genetic abnormalities in the transformed corn plants containing rice RPL3 protein. This is likely because the rice RPL3 protein shares 94% of its amino acid sequence identity with the corn RPL3 protein. Establishing infection levels for effective screening is a challenge with *F. graminearum* due to the pathogens' sensitivity to environmental conditions and plant development stage (Parry et al., 1995; Doohan et al., 2003). In the current study, consistent levels of infection and high disease scores were observed for the non-transgenic WT plants infected by silk or kernel inoculation. This proved that the disease screen was effective and that the lower disease scores observed in the transgenic populations were not escapes and rather reflected the effectiveness of the *Rpl3* gene in transgenic plants (Table 1 and Table 2). The transgenic plants that were tested in field conditions reported in this paper were T2 segregating populations developed from independent transgenic events (T0) of *Agrobacterium* mediated transformation with PML14 (35S CaMV: *Rpl3*) and PML16 (ZmGRP5: *Rpl3*) constructs. Due to the heterogeneous nature of these populations, we expected that they would contain plants with a range of disease symptoms. This was evident by the distribution of disease severity recorded for individual T2 transgenic plants (Table 1). The genetic constructs used for the transformation included the 35S promoter, which has been demonstrated to drive gene expression in a variety of corn tissues (Ishida et al., 1996). The silk-specific promoter was used from the *ZmGRP5* gene that encodes a novel glycine-rich cell wall localized protein of corn silk tissues (Tao et al., 2006). A similar promoter from a *Myb* transcription factor *pSH64* gene was previously used to express cDNA for secondary metabolite production in corn that showed higher resistance to corn earworm (Johnson et al., 2007). Corn silk tissues are the primary site of natural infection for *F. graminearum* (Reid et al., 1994), and they are also the target of foraging insects such as the western corn rootworm (Hoffmann et al., 2000), european corn borer, corn earworm and fall armyworm (Hazzard et al., 2003). Entry sites caused by insect pests facilitate access of *F. graminearum* to corn ears. Therefore, expression of the modified *Rpl3* gene in silk tissue could decrease pathogen entry sites and reduce crop damage. In fact, our results showed that transgenic corn plants expressing the *Rpl3* gene with the silk promoter had higher levels of tolerance than those produced with the constitutive promoter. Higher tolerance was observed in the PML16

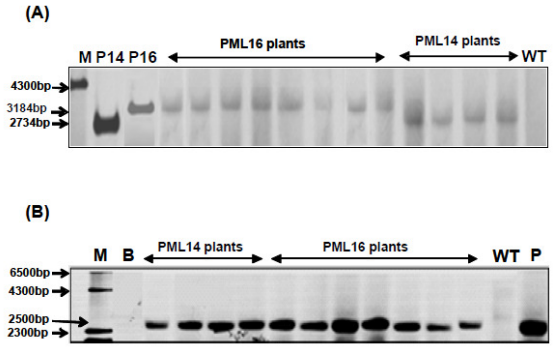

Fig 3. Southern hybridization analysis of transgenic corn plants having *Rpl3* and *BAR* genes. A- To confirm *Rpl3* gene presence, DNA was digested with *Pst1* restriction endonuclease and hybridized with *Rpl3* probe. In PML16 transgenic plants hybridization band of 3184 bp was detected and in PML14 transgenic plants hybridization band of 2734 bp was detected. B- To confirm *BAR* gene presence, DNA was digested with *Dra1* restriction endonuclease and probed with *BAR* gene. Both PML14 and PML16 transgenic plants had hybridization band of 2500 bp. P14 and P16- Plasmid DNA, WT- DNA from the non-transgenic plants, M- marker ladder, Bblank.

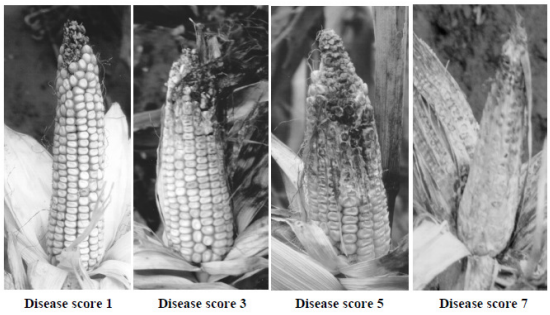

Fig 4. Disease scores in corn ears after inoculation with *F. graminearum*. The disease scores represents, 1 = no visual signs of disease, 3 = 4-10%, 5 = 26-50%, and 7 = 76-100% disease infection.

transgenic plants compared to the PML14 plants after kernel inoculation. This might be ascribed to the fact that all of the PML16 plants were developed from self-pollination of the T0 plants, whereas two of the three PML14 plants were obtained from crosses between the T0 and WT plants, resulting in a higher proportion of heterozygous plants. Cross-pollination rather than selfing is sometimes required to recover plants regenerated from the multi-shoot tissue culture system as these plants develop ears instead of tassels. The appearance of ears instead of tassel was reported on corn plants following extended exposure of plant material to cytokinins in tissue culture that causes the conversion of the tassel to an ear (Li et al., 2002). In subsequent generations all the transgenic plants were normal in morphology, producing both tassels and ears.

The silk inoculation imposed higher disease symptoms in WT and transgenic plants (Table 1). There are many inbred lines that have shown sensitivity of silk tissues to *F. graminearum* (Reid at al., 1992) and in our study inbred CG62 corn line was no exception with the development of severe disease symptoms following silk inoculation in WT plants compared to kernel inoculation (Table1 and 2). In natural field conditions, infection via silks appears to be the most common route for fungal spores (Koehler 1942; Munkvold et al., 1997). After spores attach to silk tissues, infection occurs via growth of hyphae from the silks to the kernels. This mode of infection is most likely conducive for faster disease spread as most silks traverse several kernels before terminating at their own kernel. Such disease development patterns in corn were reported by Miller et al., (2007) using a fluorescence tagged *F. graminearum* strain. Additionally, hundreds of silks that are exposed per ear represent a large conduit for spore capture and transmission of the disease to the kernels. This might be a reason why both PML14 and PML16 transgenic plants had more plants with higher disease scores (>4) following silk inoculation than kernel inoculation (Table 1 and 2). Transgenic plants developed with the silk promoter had higher disease resistance after *F. graminearum* inoculation via the kernel. The improved effectiveness of the silk promoter in transgenic plants expressing the *Rpl3* gene within the developing silk may be attributed to a broader activity of the silk promoter in the seed pericarp tissue. This extended activity of another silk promoter of the *pSH64* gene in pericarp tissues was also observed by Johnson et al., (2007) with high levels of gene expression in the silk and pericarp tissues of transgenic corn plants. The putative protein coding regions of *ZmGRP5* and *pSH64* are 97% identical at the genomic level. Therefore, it is likely that the *ZmGRP5* and *pSH64* encode similar types of proteins in the silk tissues.

Material and methods

Plant material for transformation

Corn (*Zea mays*) CG62 inbred line was used for developing transgenic plants. This corn line is tissue culture responsive (Li et al., 2002) and susceptible to *F. graminearum* (Prof. Lana Reid personal communication). The multiple shoot cultures from the corn apical meristems were developed from seedlings which were used as plant material for the transformation. The procedure for preparation of multiple shoot cultures from the corn was described previously by our laboratory (Li et al., 2002). In brief, surface sterilized corn seeds were germinated and coleoptile nodes without expanded leaves were removed and cultured onto multiple shoot induction medium (MSM) composed of MS basal medium (Sigma, St. Louis, USA) supplemented with 1 mg/l 2,4 D and 2 mg/l BAP (Li et al 2002). The cultures were incubated in a growth chamber at 23 ± 2 ºC under constant illumination (40-50 µmol m^{-2} s^{-1}). Multiple shoot clumps appeared after 3 - 4 weeks of culture on MSM medium.

Plasmid construction

Two vectors were developed (Fig 1) containing the modified *Rpl3* gene of rice (replacement of tryptophan with cysteine at amino acid residue 258). The modified *Rpl3* gene was fused with (i) a 35S CaMV promoter and named plasmid PML14 (RB-UBI-*BAR*-/intron-E35SCaMV-*Rpl3:c258*-LB), or (ii) a silk-specific promoter, *ZmGRP5* from corn glycine-rich protein (Tao et al., 2006; Johnson et al., 2007) and named plasmid PML16 (RB- UBI -*BAR*/intron-*ZmGRP5*-*Rpl3:c258*-LB). Both PML14 and PML16 vectors were constructed in the background of plasmid pBU-35S.IG (Trifonova et al., 2001), which contains a herbicide resistant phosphinothricin acetyl transferase (*BAR*) gene driven by the *Ubiqutin* promoter to allow selection of transgenic shoots in tissue culture. It also contains a spectinomycin resistance gene

outside of the T-DNA region for the bacterial selection. The regions between the left and right borders of both plasmids were sequenced to confirm the correct orientations of the cloned genes. The PML14 and PML16 plasmids were transferred into *Agrobacterium* strain EHA101 by electroporation.

Agrobacterium- mediated transformation of corn

Agrobacterium tumefaciens cultures were grown from -80 °C stocks in Yeast Peptone medium (5 g/l yeast extract, 10 g/l peptone, 5 g/l NaCl) supplemented with 50 mg/l rifampicin and 50mg/l Spectinomycin for 48 h at 28 °C. *Agrobacterium* culture was diluted to OD of 0.6 with liquid infection medium (MS basal 4.4 g/l, sucrose 3%, L-proline 0.7g/l, acetosyringone 200µM, 2,4-D 0.5 mg/l, BAP 3 mg/l , pH 5.6). Actively growing corn multiple shoot cultures were used for co-cultivation with the *Agrobacterium* strain EHA101 containing PML14 or PML16 plasmid. The multiple shoot cultures were immersed in the bacterial suspension and vacuum infiltrated for 20 min. The inoculated explants were cultured on solidified infection medium (MS basal 4.4 g/l, sucrose 3%, L-proline 0.7g/l, acetosyringone 200µM, 2,4-D 0.5 mg/l, BAP 3 mg/l and 8 g/l agar, pH 5.6) in the dark at 25 °C for 4 days for co-cultivation. For selection of transgenic corn tissues, the co-cultivated tissues were cultured onto selection medium consisting of MSM medium supplemented with 1 mg/l phosphinothricin herbicide. Co-cultivated tissues were selected onto selection media for 2 months, transferring newly growing tissues onto new selection media every 15 days. Regeneration was performed by transferring selected multi-shoots on MS media without phytohormones. The roots developed in 1-2 months on regeneration medium. To decrease the number of untransformed plantlets the concentration of phosphinothricin herbicide was increased from 1 to 3 mg/l in regeneration medium. The plantlets of approximately 10 cm in height were transferred to Turface® (heat treated montmorillonite clay mineral, Profile Products LLC, Buffalo Grove, IL) and maintained in a greenhouse until maturity with 16 h light 26 °C/ 8 h dark 22 °C growing conditions.

Molecular characterization of transgenic plants

Genomic DNA was isolated from corn leaves using the CTAB method (Saghai-Maroof 1984). The presence of the *Rpl3* gene in transgenic plants was confirmed with PCR. Forward primers were designed in the 35S promoter region of plasmid PML14-F (5'-ATAATTTCGGGAAACCTCC-TCGG-3') and in actin intron of plasmid PML16-F (5'GTAGGTAGACGAtAAGCTTGATC-3'). They were used with a common reverse primer RPL3-R (5'AGCCATAGTCCACCTTGATCGGC) designed in the *Rpl3* gene (Fig 1). The presence of the *BAR* gene in transgenic plants was tested by PCR using *BAR* gene specific primers, BAR-F (5'TGCACCATCGTCAACCACTA) and BAR-R (5'GGTACCGGCAGGCTGAAGTCCAGC). The PCR reactions were carried out in a program: initial denaturing at 94 °C for 2 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 min, and the final extension was done at 72 °C for 5 min.

For Southern hybridization analyses, the gene specific probes as shown in Fig 1 were prepared with a PCR digoxigenin probe synthesis kit from Roche Diagnostics (Roche Molecular Biochemicals, Mannheim, Germany). The *Rpl3* probe was synthesized by amplifying first 200 bases of *Rpl3* gene (accession number D12630). Hybridization was performed at 42 °C for overnight and washing was done with 1x 20 min in 1x SSC, 0.1% SDS at 45 °C. The blots were further washed under high stringency conditions (0.5x SSC, 0.1% SDS at 65 °C, 3x 20min each) to eliminate hybridization signals from endogenous corn *Rpl3* sequences. All procedures were followed as described by Sambrook and Russell (2001). DNA of plasmids was used as a positive control and the DNA from non-transformed WT corn was used as a negative control.

Experimental design for field evaluation of transgenic plants

The experiments were conducted in a confined field experiment at the Field research station, Elora, University of Guelph, Canada to determine the response of the transgenic plants expressing *Rpl3* gene to ear rot disease development after inoculation with *F. graminearum*. The experiment was sown in a randomized complete block design with three replications. In each replication 15 seeds from each genotype were sown in a row. Genotypes transformed with PML14 or PML16 plasmids were analyzed in two different plots. Each plot included *F. graminearum* inoculation via either kernel or silk (Reid et al., 1996a, b). Progeny of three PML14 progenies (PML14-102, PML14-110 and PML14-133) and two PML16 progenies (PML16-53 and PML16-61) transgenic plants were used for evaluation in the field for *Fusarium* ear rot disease after *F. graminearum* inoculations.

Plant inoculation and disease scoring

F. graminearum macroconidial suspension was produced in the laboratory as described by Reid et al., (1996a). For the kernel inoculation, 2 ml of *F. graminearum* suspension ($2.5x10^5$ spores/l) was injected using a syringe needle into the kernels of primary ears 10-15 days after pollination. For silk inoculation, the same amount of spore suspension was injected into the silk 2-3 days after the onset of pollination when the silks were still slightly moist. The inoculated ears for each treatment combination were hand-picked and dehusked and assessed for disease scores at harvest depending upon the disease symptoms developed on the ears. Ear rot disease symptoms were scored as, 1 = no visual signs of disease infection, 2 = 1-3%, 3 = 4-10%, 4 = 11-25%, 5 = 26-50%, 6 = 51-75% and 7 = 76-100% of the kernels showing visible symptoms of ear rot infection and mycelial growth. Pictures of the ears with scores of 1, 3, 5 and 7 are shown in Fig 4.

Statistical analysis

Data was statistically analyzed by Fisher's protected least significant difference (LSD) test using SAS statistical software (SAS Institute). P values of less than 0.05 were considered to be statistically significant. Data shown in results are mean ± SD.

Conclusions

The ear rot disease symptoms were lower in transgenic plants compared to WT plants. This lower disease occurrence reflected reduced fungus development in the transgenic plants and theoretically less fungal DON production. Although the disease control in transgenic plants was not absolute, nevertheless the disease symptoms were significantly lower in these plants. The results suggest that

transgenic plants expressing the *Rpl3* gene with a tissue specific promoter could be an effective strategy for decreasing corn yield losses and reduce the level of contamination of corn grains caused by *F. graminearum*. The results shown in this study clearly emphasis that genetic engineering is an effective strategy to develop disease resistance in crop plants and such genes could be used in cereal crops to enhance resistance against *F. graminearum*.

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References

Ali LM, Taylor JH, Lie J, Sun G, William M, Kasha KJ, Reid LM, Pauls KP (2005) Molecular mapping of QTLs for resistance to Gibberella ear rot, in corn, caused by *Fusarium graminearum*. Genome 48:52-533

Anand A, Zhou T, Trick HN, Gill BS, Bockus WW, Muthukrishnan S (2003) Greenhouse and field testing of transgenic wheat plants stably expressing genes for thaumatin-like protein, chitinase and glucanase against *Fusarium graminearum*. J Exp Bot 54 (384):1101-1111

Buerstmayr H, Ban T, Anderson JA (2009) QTL mapping and maker-assisted selection for *Fusarium* head Blight resistance in Wheat: a review. Plant Breeding 128:1-26

Desjardins AE, Procter RH, Bai G, McCormick SP, Shaner G, Buechley G, Hohn TM (1996) Reduced virulence of trichothecene-nonproducing mutants of *Gibberella zeae* in wheat field tests. Mol Plant Microbe In 9:775-781

Doohan FM, Brennan J, Cooke BM (2003) Influence of climatic factors on *Fusarium* species pathogenic to cereals. Eur J Plant Pathol 109:755-768

Fried HM, Warner JR (1981) Cloning of yeast gene for trichodermin resistance and ribosomal protein L3. P Natl Acad Sci USA 78:238-242

Feinberg B, McLaughlin CS (1989) Biochemical mechanism of action of trichothecene mycotoxins. *In*: Trichothecene Mycotoxinsis: Pathophysiologic effects. V. R. Beasley (ed.), CRC Press, Boca Raton FL, USA. Vol 1, p. 27-35

Harris LJ, Desjardins AE, Plattner RD, Nicholson P, Butler G, Young JC, Weston G, Proctor RH, Hohn TM (1999) Possible role of trichothecene mycotoxins in virulence of *Fusarium graminaream* on maize. Plant Dis 83:954-960

Harris LJ, Gleddie SC (2001) A modified *Rpl3* gene from rice confers tolerance of the *Fusarium graminearum* mycotoxin deoxynivalenol to transgenic tobacco. Physiol Mol Plant P58:173-181

Hazzard RV, Schult BB, Groden E, Ngollo ED, Seidlecki E (2003) Evaluation of oils and microbial pathogens for control of *Lepidopteran* pests of sweet corn in New England. J Econ Entomol 96:1653-1661

Higa A, Kimura M, Mimori K, Ochiai-Fukuda T, Tokai T, Takahashi-Ando N, Nishiuchi T, Igawa T, Fujimura M, Hamamoto H, Usami R, Yamaguchi L (2003) Expression in cereal plants of genes that inactivate *Fusarium* mycotoxins. Biosci Biotechnol Biochem 67:914-918

Hoffmann MP, Kirkwyland JJ, Gardner J (2000) Impact of western corn rootworm (*Coleoptera: Chrysomelidae*) on sweet corn and evaluation of insecticidal and cultural control options. J Econ Entomol 93:805-812

Igawa I, Takahashi-Ando N, Ochiai N, Ohsato S, Shimizu T, Kudo T, Yamaguchi I (2007) Reduced contamination by the *Fusarium* mycotoxin zearalenone in maize kernels through genetic modification with a detoxification gene. Appl Environ Microb 73:1622-1629

Ishida Y, Sait H, Ohta S, Hiei Y, Komari T, Kumashiro T (1996) High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nature Biotechnol 14:745-750

Johnson ET, Berhow MA, Dowd PF (2007) Expression of a corn Myb transcription factor driven by a putative silk-specific promoter significantly enhances resistance to a *Helicoverpa zea* in transgenic maize. J Agr Food Chem 55:2998-3003

Koehler B (1942) Natural mode of entrance of fungi into corn ears and some symptoms that indicate infection. J Agric Res 64:421-442

Li W, Masilamany P, Kasha KJ, Pauls KP (2002) Developmental, tissue culture, and genotypic factors affecting plant regeneration from shoot apical meristems of germinated *Zea mays* L. seedlings. In Vitro Cell Dev-Pl 38:285-292

Logemann J, Jach G, Tommerup H, Mundy J, Schell J (1992) Expression of a barley ribosome-inactivating protein leads to increased fungal protection in transgenic tobacco plants. Nature Biotechnol 10:305-308

Mackintosh CA, Lewis J, Radmer LE, Shin S, Heinen SJ, Smith LA, Wyckoff MN, Dill-Macky R, Evans CK, Kravchenko S, Baldridge GD, Zeyen EJ, Muehlbauer GJ (2007) Overexpression of defense response genes in transgenic wheat enhances resistance to *Fusarium* head blight. Plant Cell Rep 26:479-488

Makandar R, Essig JS, Schapaugh MA, Trick HN, Shah J (2006) Genetically engineered resistance to *Fusarium* head blight in wheat by expression of Arabidopsis *NPR1*. Mol Plant Microbe In 19:123-129

Miller SS, Reid LM, Harris LJ (2007) Colonization of maize silk by *Fusarium graminearum*, the causative agent of gibberella ear rot. Can J Bot 85:365-376

Mitterbauer R, Poppenberg B, Raditschn A, Lucyshyn D, Lemmen M, Gloss J, Adam G (2004) Toxin-dependent utilization of engineered ribosomal protein L3 limits trichothecenes resistance in transgenic plants. Plant Biotechnol J 2:329-340

Munkvold GP (2003) Cultural and genetic approaches to managing mycotoxins in maize. Annu Rev Phytopathol 41:99-116

Munkvold GP, McGee DC, Carlton WM (1997) Importance of different pathways for maize kernel infection by *Fusarium moniliforme*. Phytopathology 87:209-217

Parry DW, Jenkinsen P, McLeod L (1995) *Fusarium* ear blight (scab) in small grain cereals- a review. Plant Pathol 44:207-238

Reid LM, Mather DE and Bolton AT (1992) Genotypic differences in the resistance of maize silk to *F. graminearum*. Can J Plant Pathol 14:211-214

Reid LM, Mather DE, Bolton AT, Hamilton RI (1994) Evidence for a gene for silk resistance to *Fusarium graminearum* Schw. ear rot of maize. J Hered 85:118-121

Reid LM, Hamilton RI, Mather DE (1996a) Screening maize for resistance to Gibberella ear rot. In: Agriculture and Agri-food Canada Technical Bulletin, 5th edn. AAFC, Ottawa

Reid LM, Stewart DW, Hamilton RI (1996b) A 4-year study of the association between Gibberella ear rot severity and deoxynivalenol concentration. J Phytopathol 144:43-436

Saghai-Maroof MA, Soliman KM, Jovgensen RA, Allard RW (1984) Ribosomal DNA spacer-length polymorphisums in barley: Mendelian inheritance, chromosomal location, and population dynamics. P Natl Acad Sci USA 81:8014-8019

Sambrook J, Rusell DW (2001) Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Tao TY, Ouellet T, Dadej K, Miller SS, Johnson DA, Singh J (2006) Characterization of a novel glycine-rich protein from the cell wall of maize silk tissues. Plant Cell Rep 25:848-858

Trifonova A, Madsen S, Olesen A (2001) *Agrobacterium*-mediated transgene delivery and integration into barley under a range of *in vitro* culture conditions. Plant Sci 161:871-880