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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 10 (14): 2327-2333, 2007
ISSN 1028-8880

Double Mutation in Tomato Ribosomal Protein L3 cDNA Confers Tolerance to Deoxynivalenol (DON) in Transgenic Tobacco

[1,2,3]Akbar Safipoor Afshar, [3]Amir Mousavi, [1]Ahmad Majd, [3]Renu and [4]Gerhard Adam
[1]Department of Biology, Islamic Azad University (Science and Research Branch), Tehran, Iran
[2]Department of Biology, Islamic Azad University (Neyshabour Branch), Neyshabour, Iran
[3]Department of Plant Molecular Biology, National Research Centre for Genetic Engineering and Biotechnology, P.O. Box 14155 6343, Tehran, Iran
[4]Centre of Applied Genetics, BOKU-University of Natural Resources and Applied Sciences, Muthgasse 18, A-1190 Vienna, Austria

Abstract: The contamination of mycotoxins associated with head blight of wheat and other grains caused by *Fusarium graminearum* is chronic threat to crop, human and animal health throughout the world. Deoxinevalenol (DON), produced by the fungus, belonging to class trichothecene is believed to act as a virulence factor in fungal pathogenesis by inhibiting eukaryotic protein synthesis, thereby blocking or delaying the expression of defense related proteins induced by host plant. The putative site of action of DON is 60s ribosomal protein L3 (RPL3). In order to reduce the effects of DON in the host plants, we modified tomato RPL3 (LeRPL3) to introduce $W^{258}R/H^{259}Y$ mutations so that amino acid residue 258 is changed from tryptophan to arginine and 259 from histidine to tyrosine. Transgenic tobacco plants expressing these modified LeRPL3 cDNAs were tested for growth pattern of T1 seedlings in presence of DON. When seedling of these transgenic tobacco plants were compared for growth in the presence of DON, a significant difference in growth rate and the ability to undergo differentiation was observed among those plants expressing the modified version of the Rpl3 gene, compared to those expressing the wild-type Rpl3 gene. Expression of the tagged gene product indicates that is was not due to somaclonal variation. These results indicate a possible mechanism of host plant resistance to the fungal pathogen *F. graminearum* among the susceptible cereal species based on the expression of modified Rpl3 genes.

Key words: *Fusarium graminearum*, Deoxinevalenol, Ribosomal protein L3, *Nicotiana tabacum*, transgenic, Fusarium head blight

INTRODUCTION

The fungal pathogen *Fusarium graminearum* Schwabe [teleomorph *Gibberella zeae* (Schweinitz) Petch], is the most common causal agent of *Fusarium* Head Blight (FHB) in many parts of the world (Goswami and Kistler, 2004). This destructive disease, commonly but perhaps inappropriately known as scab, affects wheat, barley and other small grains both in temperate and in semitropical areas. The disease has the capacity to destroy a potentially high-yielding crop within a few weeks of harvest (McMullen *et al*., 1997). The threat posed by this fungus is multifaceted. It causes yield and quality losses due to sterility of the florets and formation of discoloured, withered and light test-weight kernels. These characteristics cause difficulties for marketing, exporting and processing infected grain. Additionally, infected grains may contain significant levels of trichothecenes and the oestrogenic mycotoxin, zearelanone, which are hazardous to animals, thus making the grain unfit for food or feed (McMullen *et al*., 1997). Trichothecene toxins such as deoxynivalenol (DON), commonly known as vomitoxin, are sesquiterpenoids that are potent inhibitors of eukaryotic protein biosynthesis. In humans, *F. graminearum* has been linked to alimentary toxic aleukia and akakabi toxicosis, illnesses characterized by nausea, vomiting, anorexia and convulsions. Perhaps as expected for inhibitors of protein synthesis, chronic exposure to trichothecenes has wide-ranging effects, including neurological disorders and immunosuppression (Bennett and Klich, 2003). Plant cultivars highly resistant to the disease or tolerant to the toxin currently are not available and the use of fungicides for controlling the disease is limited by cost, difficulty in efficient

Corresponding Author: Akbar Safipoor Afshar, Department of Biology, Islamic Azad University (Neyshabour Branch), Neyshabour, Iran

application to wheat heads and an incomplete understanding of factors that influence disease development (McMullen *et al*., 1997; Pirgozliev *et al*., 2003). Increased resistance against toxic substances can result from several mechanisms, ranging from reduced uptake to bypass, overexpression, or mutation of the toxin target.

In yeast, resistance to trichothecene trichodermin was found to be conferred by a single gene known as *tcm1* (Grant *et al*., 1976; Schinder *et al*., 1974), the wild type name as RPL3. RPL3 has been identified as an essential protein for peptidyl transferase activity in prokaryotic ribosome (Green and Noller, 1997). Studies by Harris and Gladdie (2001) showed that modified rice 60s ribosomal protein L3 (leading to alteration W258C), which mimics a RPL3 mutation in *Saccharomyces cervisiae* strain (leading to amino acid alteration W255C) (Schultz and Friesen, 1983; Threadgill *et al*., 1986) carrying transgenic tobacco calli and protoplasts showed significantly greater regeneration and viability in presence of DON as compared to those from tobacco expressing the unmodified gene. Expression of engineered tomato Rpl3 (LeRpl3) having corresponding W258C change, improved the ability of transgenic tobacco plants to adapt to DON, but did not result in constitutive resistance (Mitterbauer *et al*., 2004). Besides this, Mitterbaurer and his coworkers (2004) also identified other amino-acid alterations conferring DON resistance in the N-termainal half to be S2P and P9L and in C-terminal half W255R and H256Y.

In the present study, we have modified tomato *LeRPL3* gene and introduced into close relative genus, tobacco, in order to test whether improved toxin resistance can be engineered in plants expressing modified tomato *LeRPL3* cDNA, into which mutation conferring DON resistance has been introduced.

MATERIALS AND METHODS

Site-directed mutagenesis and vector construction: Total RNA was isolated from tomato (*Lycopersicon esculentum* cv Super strain B) leaves using RNeasy kit (QIAGEN) and used for RT-PCR. The cDNA encoding *RPL3* (*LeRPL3*) was amplified with primers TL3-ATG1 (5′-AGGATCCAACAATGTCACAGGAAGTTTGA) and TL3-*Xho*I(5′-TACTCGAGGTGGCTTATGAGTATTTCTT CCAG) which were constructed based on the sequence of tomato *Rpl3* in the GenBank. The ~ 1.4 kb *Bam*HI/*Xho*I fragment was cloned into plasmid pBluescript KS$^+$ (Stratagene). The resulting plasmid was used for sequencing and as a template for site-directed mutagenesis.

Site-directed mutagenesis was done in LeRPL3 to introduce $W^{258}R/H^{259}Y$ mutations by overlap extension PCR. Mutations were generated using following overlapping mutant primer pairs: W258R (5′-GCTTGTATTGGTGCCCGGCATCCTGCTAGAGTTTC)/ W258R-RV(5′-GGATGCCGGGCACCAATACAAGCAA) for $W^{258}R$ and H259Y (5′-GCTTGTATTGGTGCCTGGTAT CCTGCTAGAGTTTC)/H259Y-RV (5′-GGATACCAGGCA CCAATACAAGCAA), for $H^{259}Y$ where mutated nucleotides are underlined. The 5' and 3' receptor fragments were used as megaprimers in PCR including the flanking primers TL3- ATG1 and TL3-*Xho*I to generate the cDNA containing the entire translated region of mutated LeRPL3.

A single copy of the c-Myc epitope EQKLISEEDL(Stop) was added to the C-terminus of the double mutated *LeRPL3* in a two step procedure. First, the overlapping primers TomMYC-FW (5′-TATGGACGCTGAAGGCTGAACAAAAGCTTATTTC TGAA) and TomMYC-RV (5′-TTGAGCTCAAAGATCTTCTTCAGAAATAAGCTTT TGTTC) were annealed and filled in to produce a short product, which was subsequently used in a fusion-PCR with double mutated LeRPL3 fragment. The final cDNA constructs were cloned into plasmid pBluescript KS$^+$ (Stratagene). The introduction of mutation as well as the absence of the undesired spontaneous mutations was checked by sequencing. Modified as well as unmodified form of RPL3 gene was cloned into the binary plant transformation vectors (pZGA22 and pBI121) behind cauliflower 35S promoter. The resulting plasmids were named as pSA50 (LeRPL3$^{WR/HY}$) and pSA51 (LeRPL3^{WR-HY}-Myc). Two other plasmids were used as control in our studies viz., pRM588 (LeRPL3-Myc) and pRA101 (LeRPL3^{H259Y}). Empty vector pZGA 22 was used as GUS control.

Plant transformation and characterization of transgenic plants: The binary plasmids were individually transformed into *Agrobacterium tumefaciens* strain LBA4404 which was subsequently used to transform *Nicotiana tabacum* cv. Xanthi. Transform lines of *N. tabacum* were selected on regeneration medium containing 100 mg L^{-1} kanamycin. The presence of the transgene was verified by PCR using primers 35SFW (AGA TAC ATG CTC AGA AGA CCA) and W258R-RV. Transcription of the transgene was tested by reverse transcriptase PCR using primers TL3-ATG1 and TL3-*Xho*I.

For screening DON tolerance, selfed seeds from three representative lines obtained by transformation with pSA50, pRA101, pZGA22 and wild type tobacco were

germinated on 1XMS medium without vitamin, 2% sucrose, 0.7% phytoagar on mesh in petriplates (approximately 70 seeds/mesh). For mutated as well as GUS control constructs, seedlings were selected on kanamycin (100 mg L^{-1}). At 2 leaf stage seedlings along with mesh were transferred to liquid MS medium containing 4 ppm DON after carefully removing agar and were kept on orbital shaker. After one week, seedlings were removed and transferred to fresh flasks having 100 mL of fresh liquid MS medium containing 0 or 10 μg mL^{-1} DON. After 3 weeks, wet weight of plants was determined to which average weight of 70 seedlings was subtracted to obtain the biomass increase. Also, average height of the seedlings after 3 weeks was recorded.

For immunodetection of *c-Myc*-tagged mutated RPL3, about 200-500 mg of plant material were homogenized in liquid nitrogen. 500 μL of extraction buffer (200 mM Tris-HCl pH 8.9; 200 mM KCl; 35 mM $MgCl_2$; 12.5 mM EGTA; 15 mM DTT; 0.6 mM sorbitol) were added to the still frozen samples and the mixture was incubated under vigorous shaking for 15 min at 4°C. After centrifugation (14.000 rpm for 15 min at 4°C), 200 μL aliquots of the supernatants were transferred into fresh tubes and stored at -20°C. Equivalent amounts of protein (50 μg) were used for Western blot analysis which was carried out using primary anti c-Myc antibody purified from hybridoma supernatant (clone 9E10).

RESULTS

Generation of transgenic tobacco plants carrying mutated version of LeRPL3 genes: In the systematic screening of DON- resistant yeast mutants, the changes $W^{255}R$ and $H^{256}Y$ were found at C-terminal region of RPL3 (Mitterbaurer *et al*., 2004). Alignment of RPL3 amino acid sequences showed that changes corresponding to $W^{255}R$ and $H^{256}Y$ in yeast are $W^{258}R$ and $H^{259}Y$ in tomato, respectively (Fig. 1). These alterations were introduced into LeRPL3 cDNA by PCR based site-directed mutagenesis and confirmed by sequencing. Double mutant LeRPL3 was recovered and successfully cloned into binary vector.

In presence of hormones BAP (2 mg L^{-1}) and NAA (0.1 mg L^{-1}) in the selection medium, direct shoots regeneration was achieved and the callus phase was totally omitted in this protocol. In presence of kanamycin (100 mg L^{-1}) the emerging shoots gave high percentage of transgenic lines which is proved in our studies later on. Multiple shoots established from independent transformations were rooted and grown in for seedset by self crossing.

Characterization of transgenic plants: At least 30 independent transgenic tobacco plants were obtained with each vector construct. Transgenic plants with either of the genes appeared vegetatively and reproductively indistinguishable from non-transformed plants of tobacco which indicated that expression of LeRPL3 produced no noticeable morphological effects.

To test the presence of stable integration of transgene, genomic PCR was carried out using DNA from all the kanamycin resistant regenerants. Transgenic plants having integrated the T-DNA of pSA50 and pSA51, amplified 1183 bp DNA fragment covering 35S promoter region and part of RPL3 gene (Fig. 2a). The result of genomic PCR was positive for most of the regenerant lines again indicating the efficiency of the direct shoot regeneration protocol of tobacco transformation. To check the transcription of the mutant transgene, RT-PCR of the RNA isolated from transformed lines was carried out using primer pair TL3-ATG1/TL3-*Xho*I. The RT-PCR reaction yielded strong PCR product of 1.4 kb while control reaction without reverse transcriptase was negative showing that there was no contamination of genomic DNA (Fig. 2b). Although this analysis did not reveal the level of expression, but it was apparent that

Sc RPL3	742	AAG	GTT	GCT	TGT	ATT	GGT	GCT	TGG	CAT	CCA	GCC
Sc RPL3 WC	742	AAG	GTT	GCT	TGT	ATT	GGT	GCT	**TGC**	CAT	CCA	GCC
Le RPL3	784	AAG	GTT	GCT	TGT	ATT	GGT	GCC	TGG	CAT	CCT	GCT
Le RPL3 HY	784	AAG	GTT	GCT	TGT	ATT	GGT	GCC	TGG	**TAT**	CCT	GCT
Le RPL3 WR	784	AAG	GTT	GCT	TGT	ATT	GGT	GCC	**CGG**	CAT	CCT	GCT
Le RPL3 WR/HY	784	AAG	GTT	GCT	TGT	ATT	GGT	GCC	**CGG**	**TAT**	CCT	GCT

Sc RPL3	248	K	V	A	C	I	G	A	W	H	P	A
Sc RPL3 WC	248	K	V	A	C	I	G	A	**C**	H	P	A
Le RPL3	251	K	V	A	C	I	G	A	W	H	P	A
Le RPL3 HY	251	K	V	A	C	I	G	A	W	**Y**	P	A
Le RPL3 WR	251	K	V	A	C	I	G	A	**R**	H	P	A
Le RPL3 WR/HY	251	K	V	A	C	I	G	A	**R**	**Y**	P	A

Fig. 1: cDNA and deduced protein sequence comparison of *S. cerevisiae RPL3* and *tcm1-2 RPL3* and *Le RPL3*. The mutant nucleotide and relevant amino acids are in bold

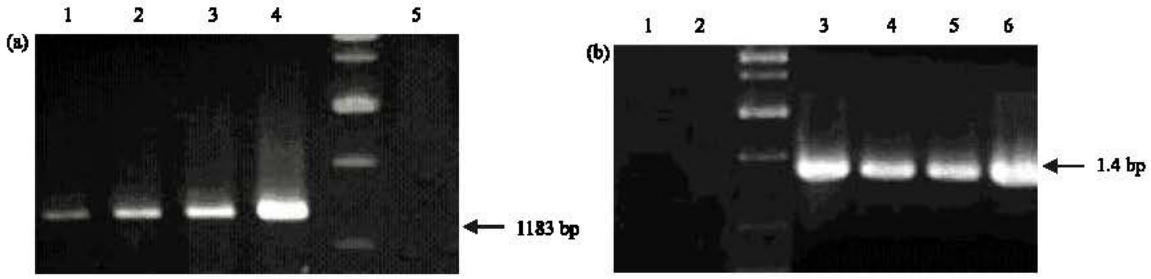

Fig. 2: (a): Molecular characterization of putative transformants via PCR : lanes 1-3 are transgenic lines amplifying 1183 bp gene fragment constituting 35 S promoter region and part of RPL3 gene. Lane 4 is binary plasmid having WR/HY mutant allele of *LeRPL3*. Lane 5 is negative control, untransformed line. (b): RT-PCR of mRNA isolated from LeRPL3 $^{WR/HY}$ transformed lines (lanes 3-6) amplifying 1.4Kb cDNA. Lane 1 is wild type untransformed control and lane 2 is negative control without reverse transcriptase

Fig. 3: DON tolerance assay of T1 transgenic lines. A expressing LeRPL3 $^{WR/HY}$, B expressing LeRPL3 ^{H259Y} and wild type untransformed tobacco (C) in presence of 10 μg mL^{-1} of DON

transcripts from LeRPL3 gene are present in transgenic leaf mRNA. Also, co-integration of transgenes along with kanamycin resistance gene was checked by germinating T1 seeds on medium containing kanamycin. It was found that transgenes were stable and co segregating with kanamycin resistance gene.

To test the ability to tolerate DON in mutated RPL3 transformed tobacco plants, seeds from transformants containing either LeRPL3 $^{WR/HY}$ or LeRPL3 ^{H259Y} or LeRPL3 ^{W258R} and untransformed tobacco were germinated on MS medium on mesh. The seedlings at 2 leaf stages were transferred into liquid medium containing low amount of DON (4 μg mL^{-1}) for adaptation and were subsequently transferred to liquid medium containing 0 or 10 μg mL^{-1} DON, respectively. After 3 weeks as seen in Fig. 3, untransformed plants as well as GUS control transformant, showed inhibitory growth in shoot development in presence of DON in the medium, where as seedlings from transformant lines having mutated RPL3 showed almost normal and healthy shoot and root development. It was observed that transformant containing LeRPL3 $^{WR/HY}$ (double mutant) showed much higher increase in biomass and much higher increase

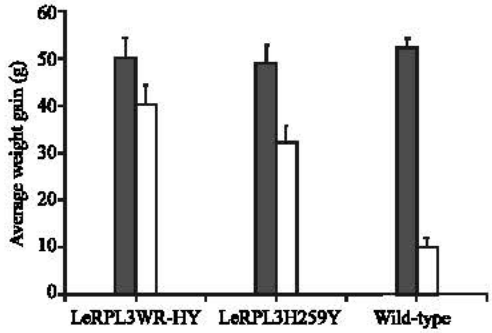

Fig. 4: The effect of DON on transgenic tobacco seedling in liquid medium supplemented with DON as described in Materials and Methods. The weight gain of seedling after 3 weeks in culture (■, plants in media without DON; □, plants in media with 10 μg mL^{-1} DON). Bars represent standard errors based upon three replicates (one plant line per genotype)

in shoot length as compared to transformant having LeRPL3 ^{H259Y} (single mutant) (Fig. 4). The phenotypic assays clearly demonstrated the ability of introduced

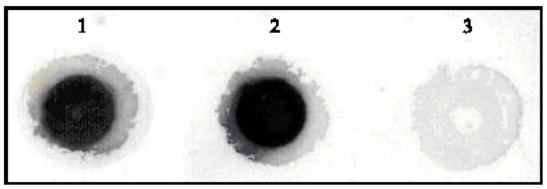

Fig. 5: Immuno blot of the c-Myc epitope-tagged protein of the WR/WY mutated LeRPL3 allele (1), HY mutated LeRPL3 (2) and wild-type LeRPL3 of tomato in transgenic tobacco

genes to give better resistance to DON. The results are also in consistent with the results obtained in the yeast complementation assays to check the functionality and resistance of mutated alleles towards DON (data not shown). To obtain clear evidence that the LeRPL3$^{WR/HY}$ transgene was indeed causing the phenotype and that is was not due to somaclonal variation between transformants, we attempted to detect expression of the tagged gene product. We extract protein from the plant material and detect the c-Myc-tagged protein on dot blots (Fig. 5).

DISCUSSION

Since several studies have proved DON to have phytotoxic properties and as an important virulence factor in head blight (Bai *et al*., 2002; Procter *et al*., 2002; Harris *et al*., 1999; Gang *et al*., 1998; Mesterhazy *et al*., 2002), hence we reasoned that engineering trichothecene tolerance in transgenic crop can be an attractive strategy for restricting disease susceptibility by imparting increased resistance to colonization of fungus. There are several mechanisms imparting increase in resistance against toxic substances viz., reduced uptake of toxin or bypass, metabolic transformation or detoxification of toxin, overexpression or mutation of toxin target. One of the strategies that is already in advance and is at field testing stage is detoxification of DON to less toxic 3-Acetyl-DON by fungal acetyletransferase (Syngenta, US patent 60346.655 B1). Besides this, detoxification by glucosyltransferase from *Arabidopsis thaliana* which converts DON into DON-3O-glucoside (Poppenberger *et al*., 2003) has also been reported. Work is also being conducted on reducing toxin uptake by ABC transporter proteins, PDR5- like genes by Mitterbaurer *et al*. (in progress).

In the present work, we introduced two amino-acid changes conferring DON resistance phenotype in yeast (Mitterburer *et al*., 2004), together into tomato RPL3 and tested for their toxin resistance properties in transgenic tobacco plants. Although it is suggested that editing endogenous RPL3 genes of the crop plants and introduction of mutations would lead to resistance (Beetham *et al*., 1999; Zhu *et al*., 1999), but sometimes such alterations could lead to some unwanted and deleterious changes in traits. This has happened while changing amino-acid at position 255 in endogenous yeast RPL3 protein from tryptophan to cysteine which although conferred trichothecene resistance but side by side slowed the growth of yeast (Mitterburer *et al*., 2004). Furthermore, in case of wheat which is hexaploid, there are 6 endogenous RPL3 genes and bringing changes in all of them would be a difficult task.

The fact that the eukaryotic ribosome is composed of over 70 proteins which must be assembled correctly with ribosomal RNAs to form a functional unit makes the engineering of resistance to mycotoxins a challenge (Harris and Gladdie, 2001). Transgenic tobacco containing the modified LeRPL3$^{WR/HY}$ gene should contain a mixed population of ribosomes-DON-tolerant ribosomes with the modified tomato RPL3 protein and DON-sensitive ribosomes with the wild-type tobacco RPL3 protein. Perhaps this mixed population of ribosomes within transgenic LeRPL3$^{WR/HY}$ plants would not produce as clear a case of trichothecene resistance as one would obtain if the endogenous sensitive genes were eliminated. Considering how highly conserved all RPL3 proteins are, it appears that a strong bias against mutations (such as amino acid substitutions) is exerted upon this region of this protein. We did not, however, see any phenotypic or genetic abnormalities with any of the transgenic lines expressing the modified LeRPL3$^{WR/HY}$ gene. The over-expression of the wild-type tomato Rpl3 gene in tobacco did not confer resistance to the mycotoxin DON upon tobacco seedlings (data not shown). This suggests that the reason for resistance in the plants which expressed LeRPL3$^{WR/HY}$ was the specific substitution of the cysteine for tryptophan in the plant ribosomal protein L3 and not simply an over expression of RPL3. Since the ribosomal protein L3 is so highly conserved between almost all eukaryotic organisms in the region between amino acids 240 and 263, this must be an important domain of this ribosomal protein for normal ribosomal function. Presumably most mutations in this region are lethal and mutations are very rarely if ever found. RPL3 is also the receptor site for an antiviral ribosome-inactivating protein, PAP, isolated from pokeweed (Hudak *et al*., 1999). The mak8-1 mutant, which confers resistance to PAP upon

yeast, maps to the same position as tcm1-2 and mak8-1 yeast produce a RPL3 protein with two amino acid substitutions, a proline to serine at position 257 and the same tryptophan to cysteine at position 255 as tcm1-2 (Peltz *et al*., 1999). Therefore, this region of Rpl3 has a strong influence on or is the binding target for two very different ribosome-inhibiting compounds. By modification of the target site of the fungal mycotoxin DON we have shown that transgenic plants have elevated tolerance to this toxin. Since it has been shown in several studies that *F. graminearum* strains which lack the ability to produce trichothecenes have reduced pathogenicity on cereal crops under field conditions (Desjardins *et al*., 1996; Harris *et al*., 1999) it is reasonable to assume that a strategy which attempts to modify the site of action of this Mycotoxin within plant cells would lead to a strategy of engineered resistance to this pathogen. The production of trichothecenes by *Fusarium* may allow the fungus to overcome host plant defenses by inhibiting protein synthesis in host cells. The results of this study suggest that engineering the target site of trichothecenes in host plants may allow Fusarium-infected plant cells to continue to synthesize proteins in the presence of trichothecenes and therefore mount an effective defense against the fungus.

ACKNOWLEDGMENT

This research was funded by Technical Cooperation Organization of Iranian Presidency Office (TCO) and carried out under project No. 159 allocated by National Institute for Genetic Engineering and Biotechnology, Iran. We also acknowledge Dr. Forough Sanjarian for her excellent technical help.

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