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Original Contribution

INCREASE RESISTANCE TO DEOXYNIVALENOL IN TRANSFORMED TOBACCO EXPRESSING ENGINEERED TOMATO RIBOSOMAL PROTEIN L3 (*LERPL3*)

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ABSTRACT

In order to reduce the effects of DON, amino-acid residue 258 is changed from tryptophan to arginine and 259 from histidine to cyctein in tomato ribosomal protein L3 (LeRPL3) cDNA through Site Directed Mutagenesis (SDM). Transgenic tobacco plants expressing these modified LeRPL3 cDNAs were tested for ability of leaf discs to regenerate and produce callus in the presence of DON. Significant differences in callus induction and ability to undergo regeneration was seen in transformed lines as compared to non-transformed tobacco plants in DON assays. Among the mutant types, marked difference with respect to resistance against DON was observed and plants expressing LeRPL3H^{259}Y giving better response than transforments expressing LeRPL3W^{258}C. The results indicate the possibility of increase in DON tolerance (and Fusarium head blight resistance respectively) among the plants based on expression of engineered RPL3.

Key words: *Fusarium graminearum*, Fusarium head blight, Deoxinevalenol, Ribosomal protein L3, Site-directed mutagenesis, modification of target site.

INTRODUCTION

Fusarium graminearum Schwabe. (*Gibberella zeae* Schwein) and *F. culmorum* (W.G. Smith) Sacc. are causal agents of Fusarium head blight (FHB) which can reduce yield of wheat and decrease the value of harvested grain by accumulation of trichothecene mycotoxins (1). The most efficacious and economical management strategy for this problem is to breed genetic disease resistance into adapted cultivars. Breeding for resistant cultivars is difficult given the complexity of FHB resistance, the need to screen host plants at maturity, and the large environmental effects on disease expression (2). Lightweight Fusarium-damaged kernels (FDK) may contain high concentrations of mycotoxins, such as deoxynivalenol (DON), rendering the grain unsuitable for food or feed (3). DON play an important role as a virulence factor during the development of FHB, binding to *RPL3* (ribosomal protein L3) and inhibited eukaryotic protein biosynthesis, therefore resistance to it considered to be an important component of the complex and polygenic resistance against FHB (4). One of the several resistance mechanisms against DON is alteration of the mycotoxin target site (*RPL3*). Single amino acid change in RPL3 (located at the peptidyltransferase center in ribosome) has been described in yeast *(Saccharomyces cerevisiae)* (5). In mutant screening, (*TCM1)* a semi dominant allele of *RPL3* was identified (6) which differs from wild type in a single nucleotide alteration (leading to a change from tryptophan to cysteine at position 255) (7). Cells carrying *RPL3W^{255}C* are viable but show growth retardation, and in a heterozygous*RPL3/RPL3W^{255}C* strain the mutant protein can only be detected in DON-treated cells (8).

Expression of an engineered tomato RPL3 (*LeRPL3W^{255}C*) cDNA, improved the ability of

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transgenic tobacco plants to adapt to the trichothecene deoxynivalenol (DON), but did not result in constitutive resistance (8). It was reasoned that there is post-transcriptional effect leads to the utilization of mutant Rpl3 in a toxin-dependent manner. Besides this, Mitterbauer and his coworkers also identified other amino-acid alterations $W^{255}R$ and $H^{256}Y$ conferring DON resistance in the C-terminal half (8).

The goal of our work is to increase plant resistance to FHB by modification of host target site of action of the mycotoxin DON i.e. *Rpl3* by introducing the other identified changes in DON resistant yeast RPL3 mutants viz., $W^{255}C$ and $H^{256}Y$ equivalent to amino acid residues 258 and 259 in tomato *Rpl3*, respectively, so as to reduce its susceptibility. In the present study, we have modified tomato *LeRPL3* gene and introduced into tobacco model plants, 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

Isolation of *LeRPL3* gene: Young leaves of tomato (*Lycopersicon esculentum* c.v. Nun-hemz 6108*)* were used for total RNA extraction by RNeasy kit (QIAGEN). The cDNA encoding *LeRPL3* was constructed using expand reverse transcriptase (Roche Molecular Biochemicals, Germany) and amplified with TL3-ATG1/TL3-*Xho*I primers **(Table 1)**. Amplification were performed in a thermal cycler (Eppendorf, Inc., Waltham, Mass.) set to the following: initial denaturation at 95 ^{0}C for 2 min, 30 cycles of denaturation at 95 ^{0}C for 1 min, annealing at 52 ^{0}C for 1 min and extension at 72 ^{0}C for 1 min. Final extension was done at 72 ^{0}C for 7 min. The amplified RT-PCR product was analyzed by agarose gel electrophoresis. Products of the expected sizes (~ 1.2 kb) were cloned into pBluescript SK (Strategen) and used as a template for site-directed mutagenesis (pRPL3).

Table 1. *Primers used for cloning, site-directed mutagenesis, sequencing, transgene detection and RT-PCR of LeRPL3.*

Primer	Sequence (5' to 3')
1. TL3-ATG1	AGGATCCAACAATGTCACAGGAAGTTTGA
2. TL3-3 *Xho*I	TACTCGAGGTGGCTTATGAGTATTTCTTCCAG
3. W258C	GCT TGT ATT GGT GCC CGG CAT CCT GCT AGA GTT TC
4. W258C-RV	GGA TGC CGG GCA CCA ATA CAA GCA A
5. H259Y	GCT TGT ATT GGT GCC TGG TAT CCT GCT AGA GTT TC
6. H259Y-RV	GGA TAC CAG GCA CCA ATA CAA GCA A

Site-directed mutagenesis in *LeRPL3*: Site-directed mutagenesis was done in LeRPL3 to introduce $W^{258}C$ and $H^{259}Y$ mutations by overlap extension PCR. Mutations were generated using overlapping mutant primer pairs: W^{258}CF/W^{258}C-RV for $W^{258}C$, and H^{259}YF/H^{259}Y-RV, for $H^{259}Y$ (mutated nucleotides are underlined, **Table 1**). The final cDNA constructs were cloned into *Bam*HI/*Xho*I site of plasmid pBluescript KS (Stratagene). The introduction of mutation as well as the absence of the undesired spontaneous mutations was checked by sequencing. DNA sequencing was done at MWG -Biotech (Ebensburg, Germany), Sequences were assembled using the SeqMan program (DNASTAR, Inc.) and analyzed with the MegAlign program (DNASTAR, Inc.). BLAST ver. 2.2.10 searches were done against the NCBI and GenBank databases.

Leaf-disk Transformation of Tobacco plants: Modified *LeRPL3* gene (LeRPL3 $W^{258}C$ and LeRPL3 $H^{259}Y$) were recovered as *Bam*HI/*Xho*I fragments from pBluescript SK derivates and cloned into *Bam*HI/*Sac*I site of the binary plant transformation vector pBI121 (CLONTECH) behind cauliflower 35S promoter and named pWC and pHY **(Fig. 1)**. The binary plasmids transformed into *Agrobacterium tumefaciens* (strain LBA4404) by freeze and thaw method which was subsequently used to transform *Nicotiana tabacum* cv. *Xanthi*. Transformed lines of *N. tabacum* with pWC and empty vector were selected on regeneration medium: MS (9) +0.1mg/ml NAA +2mg/ml BA containing 100mgL^{-1} kanamycin. Plantlets were regenerated from regeneration medium were grown under 16 h light/8 h dark at 25°C.

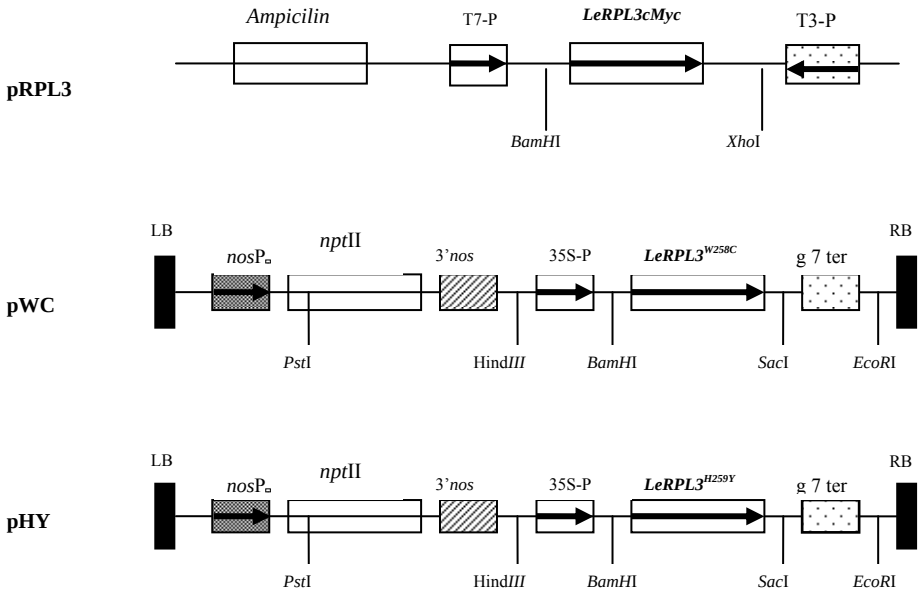

Fig. 1. Schematic presentation of pRPL3, pWC and pHY plasmids used for cloning and plant transformation.

Molecular analysis of transformed plants: The presence of the transgenes was tested by PCR using primers TL3-ATG1/TL3-*Xho*I. The amplification conditions were same as isolation LeRPL3 gene conditions. Untransformed tobacco plants were kept as negative and pRPL Plasmid was taken as positive control for PCR. Transcription of the transgenes in positive lines on PCR analysis was tested by reverse transcriptase PCR using the same primers and conditions. Transformed plants lines were selected for DON resistance assays.

DON assay by leaf regeneration and callus induction ability: In order to determine whether modified LeRPL3 gene can confer tolerance to FHB, the ability to regeneration and callus production on medium supplemented with 10ppm DON were evaluated. Young leaves from randomly selected T0 lines from PCR and RT-PCR positive transgenic plants were taken and cut into strips on sterile Petri dishes as well as untransformed ones. Total number of regenerated shoots on shoot regeneration medium (MS+2mg/l BAP +0.1 mg/l NAA, 100mgL^{-1} kanamycin) supplemented with either 0 or 10ppm DON and callus production on callus induction medium (MS+0.1mg/ml NAA+10mg/ml Kinetin, 100mgL^{-1} kanamycin) supplemented with either 0 or 10ppm DON from each explants were recorded.

RESULTS

Isolation and Site-directed mutagenesis in *LeRPL3*: The presence of inserted genes (modified and wild type forms of *LeRPL3*) in pBluescript SK (Strategen) was confirmed by PCR of plasmids isolated from transformed *E. coli* (DH5α) cultures. The primer pair TL3-ATG1/TL3-*Xho*I was used to amplify 1.2-kb *LeRPL3* gene that was observed on agarose gel electrophoresis **(Fig. 2)**.The $W^{258}C$ and $H^{259}Y$ alterations introduced into LeRPL3 cDNA by PCR based site-directed mutagenesis and corresponding amino acid changes were confirmed by sequencing **(Fig. 3)**.

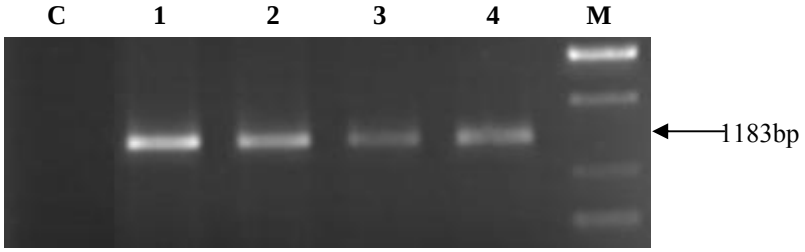

Fig. 2. Amplification of 1183 bp fragment using TL3ATG1/TL3-3 *Xho*I primer pair. Lane 1: *LeRPL3* gene (the amplified RT-PCR product), lane 2: *LeRPL3* gene (positive control), lane 3: *LeRPL3*$W^{258}C$, 4: *LeRPL3*$H^{259}Y$, cloned into pBluescript SK, C: untransformed pSK, M: 1Kb Ladder (Fermentas)

A:

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                *           680                 *           700                 *           720
Le-RPL3         :ATTGGTGTCACCAAGGGTAAGGGTTATGAAGGTGTTGTTACTCGTTGGGGTGTGACACGT    :          720
LeRPLWC         :ATTGGTGTCACCAAGGGTAAGGGTTATGAAGGTGTTGTTACTCGTTGGGGTGTGACACGT    :          720
LeRPL3HY     :ATTGGTGTCACCAAGGGTAAGGGTTATGAAGGTGTTGTTACTCGTTGGGGTGTGACACGT   :    720

                *           740                 *           760                 *           780
Le-RPL3         :CTTCCTCGCAAAACCCACAGGGGTCTACGTAAGGTTGCTTGTATTGGTGCCTGGCATCCT    :          780
LeRPLWC         :CTTCCTCGCAAAACCCACAGGGGTCTACGTAAGGTTGCTTGTATTGGTCCCTGGCATCCT    :          780
LeRPL3HY     :CTTCCTCGCAAAACCCACAGGGGTCTACGTAAGGTTGCTTGTATTGGTGCTTGGCATCCT   :    780

                *           800                 *           820                 *           840
Le-RPL3         :GCTAGAGTTTCATTCACAGTGGCTCGTGCTGGTCAAAATGGATACCATCACCGTACTGAG    :          840
LeRPLWC         :GCTAGAGTTTCATTCACAGTGGCTCGTGCTGGTCAAAATGGATACCATCACCGTACTGAG    :          840
LeRPL3HY     :GCTAGAGTTTCATTCACAGTGGCTCGTGCTGGTCAAAATGGATACCATCACCGTACTGAG   :    840
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B:

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            *        80          *       100          *       120
RPL3     :PGSKLHKKETCEAVTIVETPPMVIVGVVGYVKTPRGLRCLNTVWAQHLSEDIKRRFYKNWCK : 124
RPL3WC   :.............................................................. : 124
RPL3HY   :.............................................................. : 124

            *       140          *       160          *       180
RPL3     :SKKKAFLKYSKKYETDEGKKDIQAQLEKLKKYACVIRVLAHTQIRKMKGLKQKKAHLMEIQV : 186
RPL3WC   :.............................................................. : 186
RPL3HY   :.............................................................. : 186

            *       200          *       220          *       240
RPL3     :NGGSIAQKVDFAYGFFEKQVPVDAVFQKDEMIDIIGVTKGKGYEGVVTRWGVTRLPRKTHRG : 248
RPL3WC   :.............................................................. : 248
RPL3HY   :.............................................................. : 248

            *       260          *       280          *       300
RPL3     :LRKVACIGAWHPARVSFTVARAGQNGYHHRTEMNKKVYKLGKVGQESHTALTEFDRTEKDIT : 310
RPL3WC   :.........C.................................................... : 310
RPL3HY   :.........Y.................................................... : 310

            *       320          *       340          *       360
RPL3     :PIGGFPHYGVVKEDYLLIKGCCVGTKKRVVTLRQSLLNQTSRVALEEIKLKFIDTSSKFGHG : 372
RPL3WC   :.............................................................. : 372
RPL3HY   :.............................................................. : 372
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Fig. 3. cDNA (A) and deduced protein sequence (B) alignment of *LeRPL3*, *LeRPL3* $W^{258}C$, *LeRPL3* and $H^{259}Y$. The mutant nucleotide and relevant amino-acids are colored.

Generation and Characterization of transgenic tobacco lines carrying modified versions of *LeRPL3*: Two weeks after Agrobacterium-mediated transformation, regenerated shoots especially from midvein were obtained and direct shoot regeneration was achieved on leaves' strips (callus phase was totally omitted in this protocol)**.** Regenerated shoots were rooted on root induction medium (0.1mg/ml NAA containing 100mgL^{-1} kanamycin) and grown to seed production. At least 25 transgenic lines were produced with each pWC and pHY vector constructs. Transgenic plants showed no vegetative differences from untransformed ones indicated that transformation produced no noticeable morphological effects.

Stable integration of transgenes was tested by genomic PCR using gDNA from all the kanamycin resistant regenerants. Transgenic plants amplified 1.2Kb fragment with the primer pair ATG1/TL3-*Xho*I. The results of genomic PCR was positive for all the regenerated lines indicating the high efficiency of the direct shoot regeneration protocol for tobacco transformation **(Fig. 4)**. Transcription of the transgenes was checked by RT-PCR of the total RNA isolated from PCR positive transformed lines, using primer pair TL3-ATG1/TL3-3*Xho*I. The reaction yielded strong 1.2kb RT-PCR products while control reaction without reverse transcriptase no band was amplified showing that there was no contamination of genomic DNA **(Fig. 5)**.

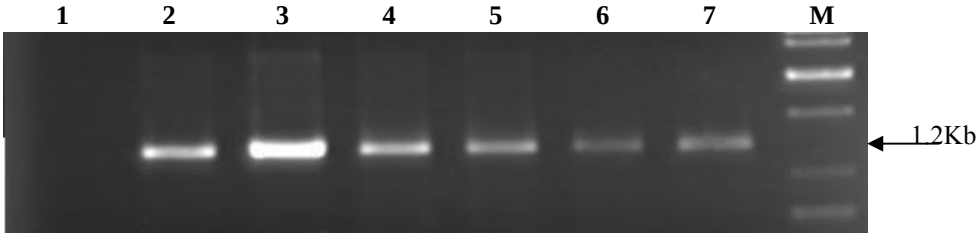

Fig. 4. Molecular characterization of putative transformants via PCR using TL3ATG1/TL3-3 *Xho*I primer pair. Lane 1: negative control (untransformed plant), Lane 2: pWC plasmid (positive control), Lane 3,4: Transformed line with pWC, Lane 5: pHY plasmid (positive control), Lane 6,7: Transformed line with pHY, transformed lines amplifying 1.2 Kb fragment. M: 1Kb Ladder (Fermentas)

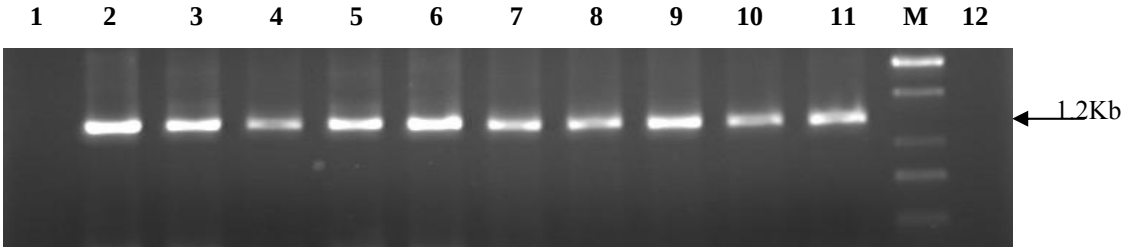

Fig. 5. Transcription confirmation of transgenes via RT-PCR of mRNA isolated from transformed lines. Lane 1: negative control (untransformed plant), Lane 2: pWC plasmid (positive control), Lane 3-6: Transformed line with pWC, Lane 7: pHY plasmid (positive control), Lane 8-11: Transformed line with pHY, M: 1Kb Ladder (Fermentas) and lane 12: is negative control without reverse transcriptase.

DON tolerance assays in transgenic plants: To evaluate the ability of transgenic tobacco plants tolerance against DON, total number of regenerated shoots and callus productions on medium containing either 0 or 10µg/ml DON were recorded. Leaf explants from transgenic lines transformed with either of the mutated RPL3 gene (LeRPL3 $W^{258}C$ and LeRPL3 $H^{259}Y$), empty binary vector as well as non transgenic plants were cultured on shoot regeneration and callus induction mediums containing either 0 or 10µg/ml DON. It was seen that leaf explants from plants transformed with mutated RPL3 genes were able to undergo both direct regeneration into shoots and callus production in presence of DON. The frequency of undergoing regeneration and callus production in *LeRPL3* $H^{259}Y$ transformants was more than in the explants from the transgenic lines transformed with *LeRPL3* $W^{258}C$ **(Fig 6)**.

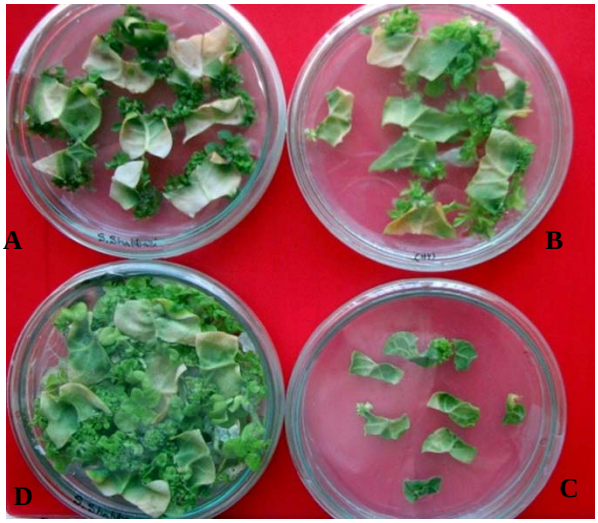

Fig. 6. Direct regeneration from leaf explant in transformed tobacco plants having LeRPL3H^{259}Y (A), LeRPL3W^{258}C (B) and untransformed tobacco (C) in presence of 10 ppm of DON, and untransformed tobacco (D) without DON.

Additionally, both indexes (number of regenerants and callus production) showed higher levels of resistance to DON in explants from the transgenic lines transformed with *LeRPL3* $H^{259}Y$ construct as compared with those transformed with *LeRPL3* $W^{258}C$ **(Fig. 7)**.

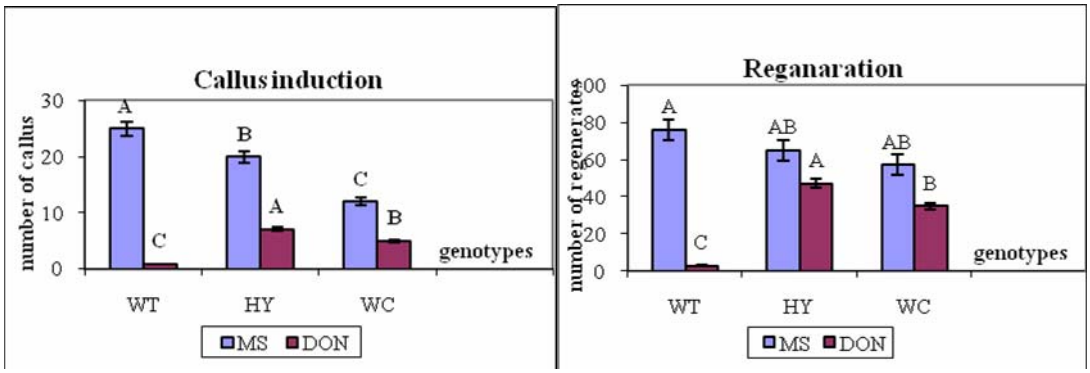

Fig. 7. Comparison the effect of 10 ppm of DON mycotoxin on the rate of regeneration (left) and callus formation (right) in transgenic plants after 3 weeks. (WT: untransformed control plants, HY: transformed lines having LeRPL3H^{259}Y and transformed lines having LeRPL3W^{258}C. Bars represent standard errors based upon 8 replicates.

It was seen that in presence of DON, control explants from transgenic tobacco lines having empty binary vector and untransformed ones underwent chlorosis and were not able to undergo regeneration or callus production. In absence of DON, explants from transgenic lines as well as non transformed control, produced calluses and regenerants at almost equal frequency. Shoot regeneration of transgenic lines in presence of kanamycin in the medium showed the stability of transgenes **(Fig. 6)**.

DISCUSSION

The mycotoxin deoxynivalenol (DON) produced by *Fusarium graminearum* is a potent inhibitor of eukaryotic protein synthesis and it been believed to play role in fungal pathogenesis (10, 11). Engineering trichothecene tolerance in transgenic crop plants is attractive disease management strategy (12). Several mechanisms imparting increase in resistance against toxic substances: efflux toxins by ABC transporter proteins (*PDR5*-like genes) (13), detoxification by fungal acetyltransferase that converts DON into less toxic 3-Acetyl-DON and plant glucosyltransferase, which detoxifies DON by formation of DON-3O-glucoside (14) and over expression of toxin target Ribosomal Protein L3 (RPL3), as well as modification of this protein by specific amino acid change (8).

Trichothecenes (such as DON) were found to interact with peptidyltransferase site of eukaryotic ribosome and inhibit eukaryotic protein synthesis. Ribosomal protein L3 (RPL3) participates in the formation of the peptidyl-transferase centre (15).

It has been found that region between 240-263 amino- acids in ribosomal protein L3 in almost all eukaryotic organisms is highly conserved; hence it must be an important site for normal ribosomal function. Besides these trichothecenes, RPL3 is also found to be receptor site for ribosome-inactivating protein (RIP), PAP, isolated from pokeweed, *Phytolacca americana,* (16) that inhibits viral and fungal infections. PAP resistant mutant yeast, mak 8-1, produces RPL3 protein with two amino-acid substitutions, proline to serine at 257 and tryptophan to cysteine at 255, similar to tcm1-2 mutant allele of yeast conferring trichothecene resistance. Mutation identified in yeast tcm1-2 mutant allele of RPL3 have been introduced into rice RPL3 cDNA, the mutation imparting change from tryptophan to cysteine at residue 255 in yeast and at 258 in rice RPL3 protein. This modified RPL3 gene was found to give significant difference in growth rate and ability to undergo differentiation as compared to those expressing the wild-type RPL3 gene in transgenic tobacco plants (7).

Hence, this region of RPL3 which is binding site of two different ribosome inhibiting compound should play a crucial role in resistance. The mutations which we introduced into LeRPL3 also fall in this region i.e. at

position 258 and 259. Alignment of RPL3 amino acid sequences showed that changes corresponding to $W^{255}C$ and $H^{256}Y$ in DON-resistant yeast mutants (8) are $W^{258}C$ and $H^{259}Y$ in tomato, respectively. In our study, these alterations were introduced into *LeRPL3* cDNA by PCR based site-directed mutagenesis and confirmed by alignment with *LeRPL3* sequence in data base.

The mutated RPL3 was individually transformed into tobacco. Transformation was mediated by Agrobacterium taking leaf as explants and selected on medium containing kanamycin. The initial step for the successful transformation is attributed to successful tissue culture and organogenic plant regeneration system. It was seen that in our tissue culture protocol auxin (0.1mg/l NAA) and cytokinin (2mg/l BAP) combination in the selection medium allowed shoots to regenerate directly from the cut ends, especially midvein, of the leaf totally omitting the callus phase. It is believed that adventitious shoot regeneration derived from an initial callus phase may result however in somaclonal variation (17) while direct shoot regeneration from leaf or stem explants may eliminate such an undesirable (18).

The transgenic plants having mutated *LeRPL3* were tested for toxin resistance. It was found that some level of increase in resistance to DON was imparted upon regeneration of transgenic explants in regeneration medium supplemented with DON. The increase in resistance was more prominent in the transgenic plants transformed with *LeRPL3HY* and clear cut phenotypic differences were observed at the DON concentration of 10 ppm.

Harris and Gleddie (7) were also able to show some level of resistance in $W^{258}C$ mutate rice RPL3, but in our case mutation at $W^{258}C$ position corresponding to $W^{255}C$ in yeast was not able to show relevant increase in toxin resistance as compared to LeRPL3 $H^{259}Y$ mutant allele. It was phenotypically evident that *LeRPL3* $H^{259}Y$ gave better resistance than *LeRPL3* $W^{258}C$. Mitterbauer while in search for single amino-acid alterations conferring DON resistance in yeast in C-terminal half, isolated 3 times $W^{255}R$ and 12 times $H^{256}Y$(8). In their study with transgenic tobacco plants expressing *LeRPL3* $W^{258}C$ mutant allele, they also found that mutation at amino acid position 258 did not result in convincing difference in DON resistance when tested by germination of seeds from selfed transgenic plants on media containing variable amount of DON, nor callus/shoot formation from leaf disks (8). In our case also alteration of amino-acid at 258 position performed poorly as compared to H to Y alteration at 259 position in regard to toxin tolerance.

It must be mentioned that however mutations in ribosomal protein L3 have been shown to confer resistance against trichothecenes in yeast (6) and plants (8) but post-translational processes such as differential ribosome assembly or differences in protein turnover may affect the incorporation of allelic variants of Rpl3p into ribosome, and that the presence of the toxin can affect this process (8). On the other hand, competition of engineered *RPL3* genes with the endogenous ones is very difficult and may be lead to unwanted phenotypes and inadequate agronomic traits (5), thus, finding of natural resistance sources within breeding programs seems to be a more promising strategy.

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