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Original Contribution

TRANSFORMED TOBACCO EXPRESSING *ScAYT1* DETOXIFIES DEOXYNIVALENOL IN EXTRACT OF dsRNA AND CURED ISOLATES OF *Fusarium graminearum*

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ABSTRACT

Fusarium head blight (FHB), has been the most destructive disease of wheat, and producing the mycotoxin deoxynivalenol (DON), a protein synthesis inhibitor, which is harmful to humans and livestock. Previous studies have indicated that over-expression of yeast acetyl transferase gene (*ScAYT1*) encoding a 3-OH trichothecene acetyl transferase that converts DON to a less toxic acetylated form, leads to suppression of the DON sensitivity. In this study, we tested whether over-expression in tobacco transgenic plants can also increase toxin resistance. Thus, we introduced *AYT1* into the model tobacco plants through Agrobacterium-mediated transformation in an attempt to detoxify DON. *In vitro* tests with extraction of normal and dsRNA carrying isolates of *F. graminearum* and 10 ppm of DON indicated variable resistance levels in transgenic plants and formation of 3AcDON upon DON treatment was confirmed by TLC method.

Key Words: Fusarium head blight, Detoxification, DON, 3ADON, *ScAYT1*, *In vitro* screening, TLC

INTRODUCTION

Gibberella zeae (Schwein.) Petch (anamorph: *Fusarium graminearum* Schwabe) is an important pathogen of cereal crops, and causes head and seedling blight of small grains, such as wheat and barley (1) Available options of managing FHB include use of fungicides, cultural practices, resistant cultivars and biological agents (2). Resistance mechanisms involved in wheat to FHB are not well understood, but quantitative trait loci associated with resistance have been identified (3). Resistance to FHB is classified into five types and resistance to mycotoxin accumulation is one of them. However, no wheat cultivar is completely resistant to FHB (4).

Head blight is a disease of contaminated grains; it is a mycotoxin, such as 8-ketotrichothecene (including deoxynivalenol (DON), nivalenol (NIV), as well as an oestrogenic mycotoxin, zearalenone (5). Trichothecene mycotoxins such as deoxynivalenol are potent protein synthesis inhibitors for eukaryotic organisms. Trichothecene biosynthesis genes are localised in a gene cluster, and include those encoding trichodiene synthetase (*Tri5*) (6), P450 oxygenases (*Tri4* and *Tri11*) (7, 8), a cytochrome P450 monooxygenase required for oxygenation of position C-8(*Tri1*) (9), acetyl transferase (*Tri3*) (10), a toxin efflux pump (*Tri12*) (11), and two regulatory genes, *Tri6* (12) and *Tri10* (13). *Tri7* controls C-4 acetylation but is a non-functional pseudo gene in *F. graminearum* strains that produce DONs that lack C-4 hydroxyl or acetoxy groups (14) and *Tri8* encodes an esterase responsible for removal of the C-3 acetyl group from trichothecenes as a final step in biosynthesis (15). Other studies indicate that *Tri16* encodes a C-8 acetyl transferase and establishes the presence of a second trichothecene gene cluster (16). Another acetyl transferase (*Tri101*) is unlinked to the cluster, and encodes the trichothecene C-3 acetyltransferase (17).

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As a group, trichothecenes are characterized by the presence of a 12, 13-epoxy-trichothec-9-ene nucleus, a feature that is necessary for toxicity (18) and addition of different combinations of side groups to this trichothecene core nucleus plays an important role in determining the level of toxicity of each trichothecene (19). The 3-*O*-acetyl derivatives of these toxins were shown to reduce their *in vitro* activity significantly. Introduction of an *O*-acetyl group at the C-3 position in the biosynthetic pathway works as a resistance mechanism for *Fusarium* species that produce t-type trichothecenes: trichothecenes synthesized via the precursor trichotriol (20). Thus, transgenic expression of *Tri101* is expected to protect cereals from the phytotoxic effect of trichothecenes and to reduce the disease severity. In model tobacco plants, *Tri101* was expressed and found to protect the transgenic lines from phytotoxic effects of trichothecene DAS(4,15-diacetoxy scripenol) (21), transformed wheat plants with *Tri101* from *Fusarium sporotrichioides* and obtained a transgenic line with partial resistance to *F. graminearum* in greenhouse trials, although this line did not show resistance to FHB in the field (22); it generated also transgenic rice plants expressing *Tri101*, and was less susceptible to floral infections of *F. graminearum* (23).

The *AYT1* gene, a homologue of *FsTri101* (*Fusarium sporotrichioides*) with 45% identity and 70% similarity, was found in wild type baker's yeast. *ScAYT1* located on chromosome XII of *Saccharomyces cerevisea* and encodes a functional 3-*O*-acetyltransferase; it was capable of trichothecenes 3-O-acetylation (24). Previous results demonstrated that over-expression of *AYT1* in sensitive yeast strains to DON suppressed the hypersensitivity of pdr5 yeast strains (25). In addition *ScAYT1* over-expression in transgenic tobacco plants exhibits increased DON resistance (26).

Double – stranded RNA (dsRNA) mycoviruses have been described in a wide variety of fungi and yeasts (27). In the first report of dsRNA infection in *F. graminearum* isolates, results showed that the dsRNA causes morphological changes, including reduction in mycelial growth, increased pigmentation, reduced virulence towards wheat and decreased (60-fold) production of trichothecene mycotoxin (DON). The presence or absence of dsRNA was correlated with the changes in pathogenicity and morphology (28).

To identify whether *ScAYT1* over-expression in transgenic tobacco plants can deal with mycotoxin (DON) in fungal extract and detoxifying them, we have treated T1 *AYT1* transgenic tobacco seedlings with complete extracts of normal *F.graminearum* isolate and isolate carrying dsRNA metabolites.

MATERIALS AND METHODS

Strains, media and culture conditions

F. graminearum isolates (F38, F42 originally isolated from wheat grains collected in different wheat production areas in Iran) were used for mycotoxin production. According to Lauren and Agnew method (29), twice autoclaved rice substrate was employed for DON production culture medium, and were inoculated with 2-3 plugged from the margin of 4 day old growing colony of each isolate on PDA (potato dexterous agar) and then incubated for 4 weeks at 25 º C.

Extraction and clean up

The mycelial mass and substrate were dried at 50 º C. 15 grams of dried substrate was ground in a blender and extracted (30). Each ground sample was extracted with 60 ml acetonitrile: methanol: water (16: 1: 3) in a 250 ml Erlenmeyer flask for 3h on a rotary shaker (170 rpm), and the extract was filtered through Whatman (Maidston, Kent, England) No. 1 filter paper. A glass tube (30 cm +1 cm id) was plugged with glass wool and dry packed with alumina/carbon (20:1 ; 1g) and then 2g pre-washed cation exchange resin added to form a mini- clean up column.

The filtrate was applied to this column and allowed to drain under gravity and the eluent collected. For further clean up 10 ml of the eluent was made to pass through the first column, a mini column made by packing glass tube (30 cm +1cm id) with small glass wool plug and alumina/carbon (1: 1 ; 1g). It was drained by gravity. The second column eluent was evaporated at 70 º C until dry. Residues were dissolved in methanol/water (10: 90; 2ml) and maintained at rest for 1 h at room temperature. Then residues were dissolved in methanol/water (5:95; 2 ml) and acetonitrile/methanol (3:1; 2 ml) and evaporated at 70 º C. Final residues were dissolved in methanol/water (5:95; 2 ml) and kept in the refrigerator at 4 º C until analysis. All cultivation and extraction experiment were done thrice. Flasks with rice, albeit without fungus inoculum, received same culture and extraction treatment as samples (controls) for

the exclusion of interfering compounds that might be confused with the mycotoxins under analysis.

DON analysis by HPLC

The HPLC system consisted of a Waters™ 600 pump system with a Waters 2487 UV detector. Chromatographic separation was performed on a Luna™ C_{18} column (25 cm, 4.5 µm particle sizes) connected to a guard column security Guard™ (4 × 3.0 mm). The mobile phase consisted of methanol/water (5: 95, v/v) at a flow rate of 1 ml min^{-1}. Detector was set at 220 nm with an attention of 2 AUFS. Injection volume was 50µl and the retention time of DON was 96s. Quantification was relative to external standards of 1-4µg/ ml in methanol/water (5:95).

Isolation of AYT1 gene

After extraction of genomic DNA from *Saccharomyces cerevisiae* by Hoffman and Winston method (31), the *AYT1* gene was amplified. For PCR, about 50 ng of genomic DNA was used as a template in a 50-µl reaction mixture containing Ex*Taq* PCR buffer, which contains 2 mM $MgCl_2$ (Cinna Gen, Tehran, Iran), deoxynucleoside triphosphate mixture (0.2 mM each), a 2 µM concentration of each primer (AYT1Fw, AYT1Re, **Table 1**), and 1.25 U of Ex *Taq* (Cinna Gen, Tehran, Iran). PCR were performed in a thermal cycler (Eppendorf, Inc., Waltham, Mass.) set to the following: denaturation at 94°C for 5 min; 30 cycles of 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min; and a final extension step at 72°C for 10 min.

The amplified PCR products were analysed by agarose gel electrophoresis. PCR products of the expected sizes were cloned into pBluescript SK (Strategen). DNA sequencing was done at MWG -Biotech (Ebensburg, Germany). Sequences were assembled using the SeqMan program (DNASTAR, Inc.) and analysed with the MegAlign and MapDraw programs (DNASTAR, Inc.). BLAST Ver. 2.2.10 searches were done against the NCBI and GenBank databases.

Leaf-disk Transformation of Tobacco plants

The *AYT1* gene was recovered as *BamH*I/*Sac*I fragment from pBluescript SK derivates and cloned into *BamH*I/*Sac*I site of the binary plant transformation vector pBI121 (CLONTECH) and named pAYT1 (**Figure 1**). The binary plasmid was 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 pAYT1 and reporter gene (GUS) were selected on regeneration medium: MS +0.1mg/ml NAA +2mg/ml BA containing 100mgL^{-1} kanamycin. Plantlets were regenerated from regeneration medium and were grown in a greenhouse under 16 h light/8 h dark at 25-28°C.

Molecular analysis of transformed plants

The presence of the transgene was tested by PCR using primers AYT1Fw/AYT1Re. Transcription of the transgene in positive lines on PCR analysis was tested by reverse transcriptase PCR using the same primers. Transformed plants lines were selected for *in vitro* screening against extraction of normal and dsRNA carrying isolates of *F. graminearum*.

In vitro screening of transformed plants

Selfed seeds from transformed lines as well as non transformed and transformed plants with reporter gene as control were germinated on 1XMS medium without vitamin, 2%sucrose, 7% phytoagar and 100mgL^{-1} kanamycin, on mesh in Petri plates (approximately 30 seeds/mesh). At two leaf stage, transgenic seedlings with the same shoot length (2 mm) were transferred to liquid 20ml MS containing 5ppm of DON, after carefully removing agar and were kept on orbital shaker with 16 h light (60 μE m−2 s−1)/8 h dark at 25◦C for adaptation. One week later, seedlings were transferred to fresh flasks having 100ml of MS medium with 0 or 10 ppm of DON, 10 ppm 3ADON, and extraction of dsRNA-containing and free isolates(F38, F42) of *F. graminearum* (containing 10 ppm of DON measured by HPLC). After three weeks average height of plants and roots and weight of the seedlings was recorded.

Detection of in vitro trichothecene 3-O-acetyltransferase activity in transgenic lines

Tobacco leaves (approximately 0.1 g each) were ground in liquid nitrogen and suspended in 200μl of lysis buffer (100 mM Tris-HCl, pH 8.0 with 0.8% 2- mercaptoethanol, 5 mM DTT, 25% glycerol and 1% polyvinyl pyrrolidone). After homogenisation in the lysis buffer, the soluble protein extract was recovered by centrifugation (21,600×*g*) and

the supernatant was used to analyse the expression of the recombinant protein.

For detection of acetyl transferase activity, DON (40μg) was mixed with 100μg of total leaf protein extract and acetyl-CoA, and the volume was adjusted to 250μl with 50 mM Tris-HCl, pH 7.5 (final concentration of acetyl-CoA, 1mM). The reaction was allowed to proceed at 37◦C for 12 h. Ethyl acetate-extracted reaction mixtures were separated on TLC papers (Ridel-deHaen™, Cat. No. 37368) with ethyl acetate/toluene (3:1) as the developing solvent. Trichothecenes were visualized with fluorescent indicator 254 nm.

RESULTS

DON analysis

In our study rice substrate was employed and fungus cultures had to pass through extraction and purification process prior to identification by High performance liquid chromatography (HPLC) and similar graphs were obtained (**Figure 2**). DON production levels by dsRNA free and dsRNA containing *F. graminearum* isolates on rice substrate are shown on **Table 2**. These data showed that DON was detected at significantly lower levels in dsRNA containing derivatives than the dsRNA free isolates.

***Table 1**: Primers used for Isolation of AYT1 gene*

AYT1Fw	5'–ATCGAATTCGAAGGTAGATGGATGTTTAGAG- 3'
AYT1Re	5'–TAGTCGACATATCATCATCCTATATGTGTAG- 3'

***Table 2**. Effect of dsRNA infection on DON production by different F. graminearum isolates on rice substrate*

Isolate	DON production (ppm)	DON reduction (%)
dsRNA- containing F-38	290	50
dsRNA- free F-38	445	
dsRNA- containing F-42	1172	60
dsRNA- free F-42	1853	

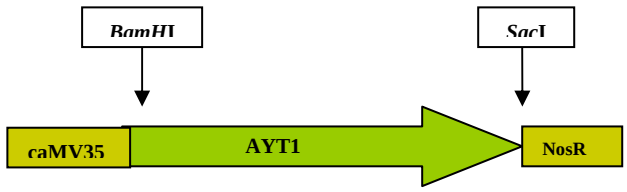

***Figure 1**: Schematic figure of pAYT1 construct*

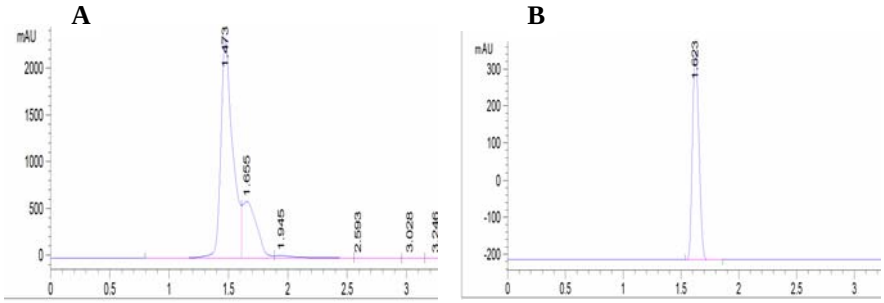

***Figure 2**. HPLC analysis of A: isolate F42 F.graminearum extraction and B: standard DON*

Isolation and cloning of *AYT1*gene

The presence of inserted gene (*AYT1*) in pBluescript SK (Strategen) was confirmed by PCR of plasmid isolated from transformed *E. coli* (DH5α) cultures (**Figure 3**). The primer pairs AYT1Fw/AYT1Re were used to amplify 1.4-kb *AYT1* gene. For additional confirmation, *BamH*I/*Sac*I digestion was done and fragment in expected size (1.4kb) was observed on agarose gel electrophoresis.

Generation of transgenic tobacco carrying *AYT1*

For Agrobacterium-mediated transformation of tobacco, leaves were used as explants. Leaves' strips after inoculation with transformed Agrobacterium and blot drying

placed upside down on co-cultivation medium (without antibiotic selection) were kept in the dark for 48hrs. After washing with cefotaxim (500mg/L) and blot drying it was transferred to direct regeneration medium (0.1mg/ml NAA+ 2mg/ml BA containing 100mgL^{-1} kanamycin) and two weeks later regenerated shoots, especially from the mid-vein, were obtained (**Figure 4**). The callus phase was totally omitted in this protocol.

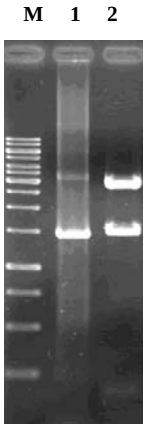

Figure 3. *Isolation of AYT1 gene .lane 1: Amplification of AYT1gene at 1.4-kb and lane 2: BamHI/SacI fragment digested cloned into pBluescript SK. M: 1Kb Ladder (Fermentas)*

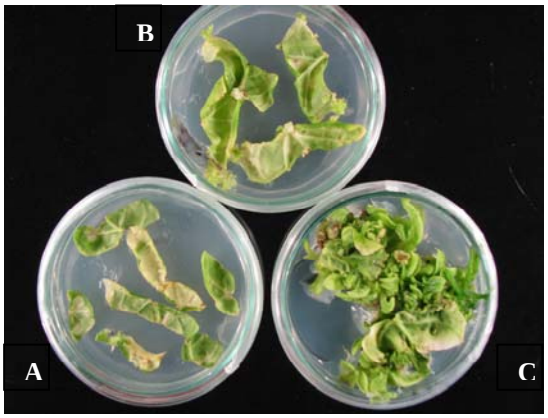

Figure 4*. Leaf- disk Transformation of Tobacco plants. A: Tobacco Leaves' strips after 4days(untransformed leaves became white), B: Leaves' strips after one week and direct regeneration from the cut ends, C: Direct regeneration of transformants from the cut ends after two weeks*

Characterization of transgenic plant

Fifty-seven transgenic plants were obtained and to test for the presence of stable integration of transgene, genomic PCR was carried out using DNA from all the kanamycin resistance regenerants. Transgenic plants amplified 1.4Kb DNA fragment *AYT1* with the primer pair AYT1Fw/AYT1Re. The results of genomic PCR was positive for all the regenerated lines indicating the efficiency of the direct shoot regeneration

Protocol for tobacco transformation (Figure 5 A).

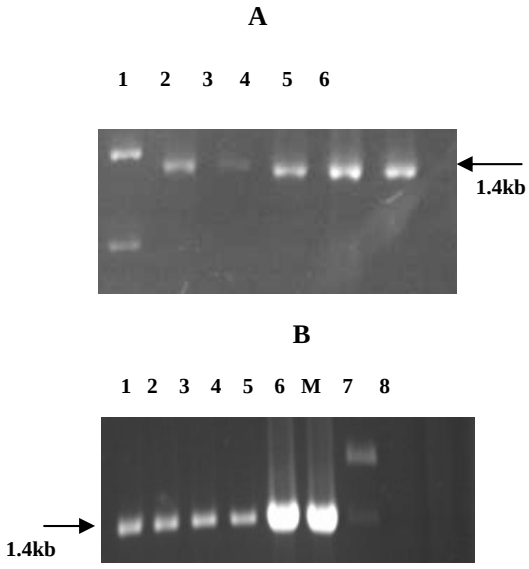

Figure 5*. Molecular characterization of putative tobacco transformants via PCR and RT-PCR.* ***A****: PCR of gDNA isolated from transformed lines with AYT1Fw/AYT1Re primers. (M: 1Kb Ladder, lanes1-4 are transgenic lines amplifying 1.4-kb fragment (ScAYT1), lane 5 is pAYT1 as positive control, lane 6 is untransformed line as negative control).* ***B****: RT-PCR of mRNA isolated from transformed lines with AYT1Fw/AYT1Re primers: (lanes1-4 are transgenic lines amplifying 1.4-kb fragment, lane 5: recombinant pSK and lane 6: pAYT1 are positive controls which amplifying 1.4-kb fragment (ScAYT1), M: 1Kb Ladder, lane 7 is untransformed plant and lane 8 is negative control without reverse transcriptase)*

To check the transcription of transgene, RT-PCR of the total RNA isolated from transformed lines were carried out using primer pairs AYT1Fw/AYT1Re. The RT-PCR reaction yielded strong PCR product of 1.4Kb, while in wild type and negative control without reverse transcriptase, no band was amplified (**Figure 5 B**).

In vitro **screening for DON sensitivity assays in transgenic seedlings**

To evaluate the ability of *AYT1* transgenic tobacco plants tolerance against DON, we recorded plant length and root growth in addition to total weight of plants in the presence of DON, 3ADON and extraction of dsRNA containing and free isolates (F38, F42) of *F. graminearum* and MS medium without toxin. While weight and length of wild-type plants was severely inhibited by

both DON and fungal extractions of dsRNA free isolates, they have less phytotoxic affects to the transgenic lines (**Figure 6**). The results suggested that *AYT1* activity in transgenic plants leads to DON resistance. Control plants (transformants with reporter gene GUS) did not differ from non-transgenic plants (data not shown).

As we expected, lower inhibition on root growth and plant length were observed by both 3ADON and extraction of dsRNA containing isolates. Apparent toxicity of 3-ADON observed with non-transgenic plants may be attributed to C-3 deacetylation inside the cells. Because of less mycotoxin (DON) production in dsRNA containing isolates (**Table 2**), extraction of dsRNA containing isolates significantly had less inhibitory function on plant growth in screening tests and supported the role of DON as a virulence factor of *F. graminearum* (**Figure 7**).

Figure 6*. In vitro screening for DON sensitivity assays in transgenic seedlings. Left: Plant growth in A: control plant (untransformed) in MS, B: transgenic tobacco+10ppm DON, C: control plant+10ppm DON after 3 weeks. Right: compared the length of transgenic (C) and control plants (A) exposed to 10ppm DON after 3 weeks with normal growth of control plant (B) in MS medium*

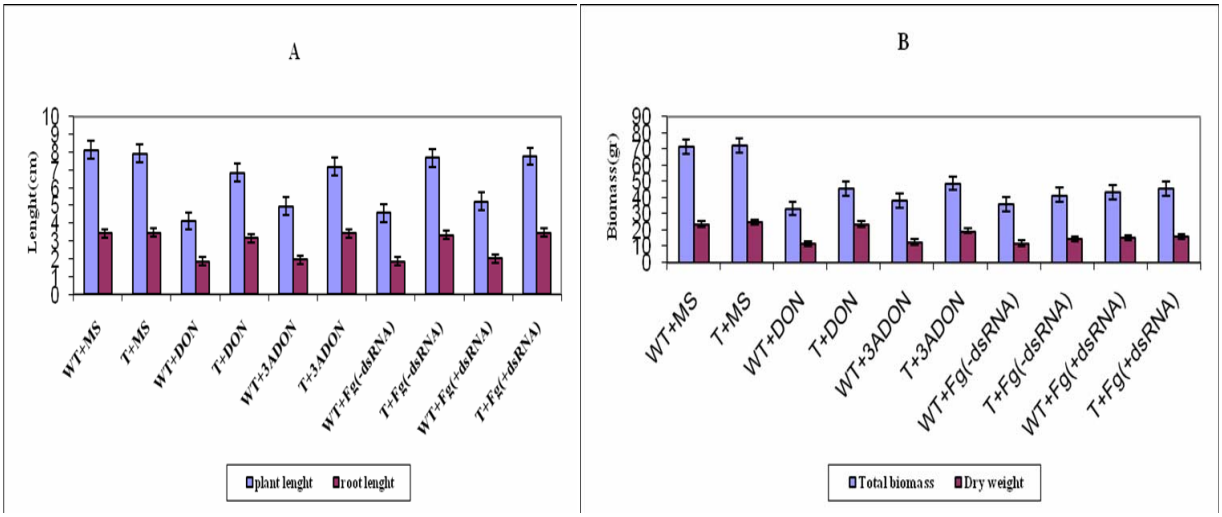

Figure 7: *The effect of mycotoxin on transgenic plants in liquid medium supplemented with DON, 3ADON, normal and dsRNA carrying isolate of F.graminearum as described in Materials and Methods. A: The plant and root length gain of seedling after 3 weeks in culture, B: The total and dry weight gain of seedling after 3 weeks in culture (WT: untransformed control plants, T: transformed lines). Bars represent standard errors based upon four replicates*

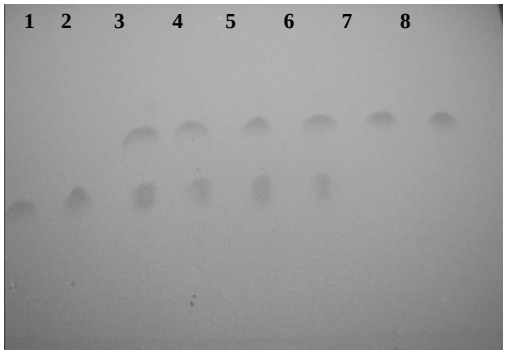

Figure 8. *Trichothecene 3-O-acetyltransferase assay by TLC. 1: Standard samples of DON, 2: protein extract non-transformed tobacco+10ppm DON, 3-6: protein extracts transformed tobacco+10ppm DON: showed 3ADON indicated 3-O-acetyltransferase activity of AYT1 in transgenic plants, 7: protein extract non-transformed tobacco+10ppm3A DON indicated deacetylation is insignificant, 8: Standard samples of 3A DON*

Acetylation of DON in transgenic plants

3ADON was detected only in transgenic lines extracts, not in control plants, thus confirms the C-3 acetylation function of *AYT1* in transgenics. However, acetylation was not completed and DON was detected as well (**Figure 8**).

DISCUSSION

Our results showed that isolates carrying the dsRNA produce much less mycotoxin (DON) than the dsRNA free isolates on rice culture (**Table 2**) and extraction of dsRNA containing isolates significantly had less inhibitory function on plant growth in *"in vitro* screening" tests. These observations are similar to results of previous studies which showed that the dsRNA causes reduced virulence towards wheat and decreased (60 fold) production of trichothecen mycotoxin DON (28). Trichothecene production has a role in pathogenicity as virulence factor (32); therefore dsRNA containing *F.graminearum* isolates caused much slower disease development on infected wheat plants. If the dsRNA fragment were transferable to dsRNA free isolates through hyphal fusion with a high incident, and if the virulence level and the mycotoxin production are reduced due to the dsRNA as described above, biological control of diseases caused by *F.graminearum* could be achieved.

Besides, detoxification of trichothecenes (such as deoxynivalenol) *in planta* could be an attractive approach for FHB resistance. Researchers found *FsTri101* gene which converts 3-hydroxy trichothecenes (such as DON) into less toxic 3-O-acetyl forms, (such as 3ADON) (17) and correlation between the acetylation of the C-3 hydroxyl group by *Tri101* and reduced toxicity of DON has been demonstrated in previous researches (33). In the present study, we attempted to detoxify DON in tobacco model plants by introducing *ScAYT1* from *Saccharomyces cerevisiae* which has highly similar structure and function to the *Tri101* (24). Previous results demonstrated that over-expression of the yeast *AYT1* gene, encoding a putative trichothecene 3-O-acetyltransferase suppressed DON sensitivity of *pdr5* mutants (26). In addition *AYT1* over-expression in transgenic tobacco plants exhibits increased DON resistance (25). To identify whether this transformation can deal with fungus extract and detoxifying them, we have treated *ScAYT1* transgenic tobacco seedlings with complete extraction of *F. graminearum* metabolites and DON. Our results from the *in vitro* tests indicated that *AYT1* could reduce DON and DON containing extractions at 10 ppm concentration. 3ADON has approximately a 10-fold lower effect on human leukocytes than does DON (34), and also was less phytotoxic than DON based on cell culture experiments in wheat (35). Similarly, in our study we observed that 3ADON has less toxic effect on biomass production and growth on plants.

Previous studies revealed that DON significantly reduced root growth of barley, while their toxic effects on callus growth and seed germination were rather weak (36). Similar phenotypic effects in the assays with *Tri101* transgenic rice plants have been observed (23). We used reduction of root growth in addition to other indexes (such as biomass production and plant growth) and observed weight and length of wild-type plants as well as root growth; this was severely inhibited by DON while *AYT1* activity in transgenic plants, leads to DON resistance, and C-3 acetylation activity of *AYT1* blocks inhibitory action of DON.

The use of *In vitro* screening method with culture medium extraction of *F. graminearum* or mycotoxin DON would avoid the difficulties that arise from time-consuming and low sensitive greenhouse or field screening analyses to find resistance. In this study, we demonstrated that *in vitro* screening method with culture medium extraction of the fungus provides a rapid and reliable method to find out the resistance level of transformed plants with yeast acetyltransferase gene (*AYT1*). The wide

range substrate specificity of Ayt1 makes it an interesting alternative for production of transgenic wheat. This is important for areas like Iran where the DON and NIV producing strains co-occur (25). However, identified acetyl transferase, which converts DON into 3ADON does not seem to be a prime candidate for a biotechnological application. The toxicity of 3ADON towards mammals is only slightly lower than that of DON (37). Furthermore, most *Fusarium* strains seem to initially produce acetylated compounds. Plant carboxylesterases, in addition to fungal enzymes, may play a significant part in the conversion of the acetylated precursors to DON. Consequently, the effectiveness of over-expression of an acetyl-transferase in plants could be severely limited by futile cycling (38).Therefore studying additional candidate detoxification enzymes from fungi and other sources, which, hopefully will lead to a more pronounced and irreversible detoxification, for example, detoxification by *DOGT1* glucosyltransferases from *Arabidopsis thaliana* which converts DON to DON-3-O-glucoside(39) and degradation of ZEN by *ZHD101* from *Clonostachys rosea* (40) could be focused.

To develop an efficient transgenic strategy to control FHB, isolation of fungal molecules essential for the pathogenesis and affected target proteins of host plants are indispensable (41). With such knowledge in molecular plant pathology, GM crops may offer a possible contribution to the problems of FHB in the future. For the moment, however, the problems associated with FHB are most likely to be dealt with natural genetic resistance, management practice, and/or fungicide application.

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