MtNOOT HETEROLOGOUS EXPRESSION WITH *FaWRKY1* OVER-EXPRESSION *CONFER STRAWBERRY* RESISTANCE AGAINST *Macrophomina phasiolena*

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Strawberry (*Fragaria x ananassa*) is a cultivated member of the family *Rosaceae*, and globally, it is one of the most important fruits (Parikka, 2004). Wild strawberries were consumed by the native people since the Stone Age and they are native to Asia and Europe. Strawberry plants have distinctive varieties regarding their genetic makeup and most Strawberry species are diploid species and the hybrids can be varied from tetraploid to decaploid. It has a high nutritional value (Giampieri *et al*., 2017) as they are favorable for people suffering from dysentery, arthritis, gout, high cholesterol, premature aging, high blood pressure, diabetes, a weak immune system, liver damage, respiratory infections, high toxicity, cancer risk, and constipation. Environmental condition including abiotic and biotic stresses is affecting such economically important fruits. Some varieties display high tolerance against various stresses (Phukan *et al*., 2015; Garrido-Bigotes *et al*., 2018). Worldwide Strawberry production threat mainly is due to numerous black root rot (charcoal rot) diseases caused by *Macrophomina phaseolina* (Browne *et al*., 2002; Millner 2006; Chamorro *et al*., 2015; and Burkhardt *et al*., 2019) and *Rhizoctonia solani* (Sharma and Bhardwaj, 2001; Timudo-Torrevilla *et al*., 2005). *Macrophomina phaseolina* is of high abundance and wide distribution in strawberry plants in Egypt (Hussein *et al*., 2012). This article, aimed to produce some valuable resistant traits to the cultivated strawberry to enhance its ability to tolerate

some destructive fungi and hence promote its high productivity.

Plants normally respond to the perceived signal molecules of any external hazard by stimulating specific genes that are associated with the biotic and abiotic stress as well. The resistance to pathogens attacking is regularly harmonized by plants *via* a complex molecular defense network (Robert-Seilaniantz *et al*., 2011). The particular defense genes include transcription factors (TFs) like; WRKY, NAC, ERF, and MADS. WRKYs are involved in both biotic and abiotic stress response as well as in physiological and developmental processes (Robatzek and Somssich, 2002; Desveaux *et al*., 2005; Jiang *et al*., 2015; Seo *et al*., 2015; Liu *et al*., 2016). WRKYs is achieving cellular homeostasis by recognition of W-box existing in target genes' promoters and inducing their expression. Diqiu *et al*. (2001) presented that W-box found in the promoter sequences of the NPR1 (Nonexpresser of PR genes 1) are recognized particularly by SA-induced WRKY DNA binding protein in Arabidopsis and thus, WRKY genes act upstream of the NPR1 genes and positively control their expression during plant defense actions. Previous data proved that overexpression of NPR1 genes leads to enhance disease resistance in various plant species like; rice, *Arabidopsis*, wheat, tobacco, and apple (Spoel and Dong, 2008; Chern *et al*., 2005, Makandar *et al*., 2006).

Cauliflower mosaic virus (CaMV) promoter P35S, the most efficient plant promoter, was used (Odell *et al*., 1985; and Benfey *et al*., 1989) to drive the expression of *FaWRKY* gene in *Escherichia coli*. Assaad and Signer (1990) presented evidence that *P35S* successfully directed the expression of neomycin phosphotransferase II (NPTII) gene in *E. coli* as the translation nucleotide sequence start site is strongly homologous to that of the consensus sequence of a prokaryotic promoter. In this article, *Macrophomina phaseolina* and *Rhizoctonia solani* were subjected to the effect of both *FaWRKY* transformed *E. coli* to assess the antagonistic ability of *E. coli* ecologically expressed the two chimeric gene in enhancing defense activity.

For additional insight into the biotic function of *FaWRKY1* as well as *MtNOOT* genes within the pathogen resistance mechanism, we have transiently overexpressed both genes in strawberry tissue. The two genes were introduced separately and in combination with each other into strawberry leaves *via Agrobacterium*-mediated transient transformation approach (Mangano *et al*., 2014). *FaWRKY* gene was previously isolated from the stressed strawberry tissues by applying jasmonic acid, salicylic acid, and *Macrophomina phasiolena* fungal homogenate (Hussein *et al*., 2016). *MtNOOT*

encodes BTB/POZ-ankyrin repeat protein specific for NPR1 family. NPR1 family are triggered during plant's attack *via* Systemic Acquired Resistance (SAR) (Klessig and Malamy 1994; Fu and Dong, 2013) and has a fundamental role in signaling activation of PR-mediated protection against various pathogens through salicylic acid SA signaling pathway (Yan and Dong, 2014). *MtNOOT* was isolated through investigating *noot*, the symbiotic mutant of *Medicago truncatula* (Couzigou *et al*., 2012).

This investigation proposed that susceptibility to *Macrophomina phasiolena*, is significantly decreased in the strawberry plant where *FaWRKY1* & *MtNOOT* were transiently overexpressed separately or in combination. This study highlights the positive regulation of resistance establishes after overexpression of *FaWRKY* with the ectopic expression of *MtNOOT* in strawberry due to the defense response network between the two genes.

MATERIALS AND METHODS

Plant Materials and Fungal Strains

Strawberry (*Fragaria* × *ananassa* cv. Camarosa) plants were kindly provided from the modern company (PICO). *Macrophomina phasiolena* and *Rhizoctonia solani* were preserved on potato dextrose agar (PDA) (Altindag *et al*., 2006) at 20ºC with 16/8 light/dark photoperiod. To rise the fungal infectivity prior to inoculation with the pathogen, the conidia stock suspensions were prepared by rubbing the four week old fungal mycelia surface in dH_2O supplied by 0.03% Tween80, then they were filtrated with glass wool. The conidia suspensions concentrations were quantified with a Neubauer Chamber Cell Counting and adjusted with dH_2O to be 10^6 conidia/ml (Higuera *et al*., 2019). The fungal spores' suspensions with 10^6 spore/ml concentration were autoclaved.

Binary Vectors

Modified pCambia 1390 binary vector was used for *FaWRKY1* construction under the control of S35 promoter and nos terminator, while pCP 42 was used for cloning of *MtNOOT* under the control of its promoter region and nos terminator. Modified pCambia 1390 and pCP 42 binary vectors were kindly provided from Dr. Pascal Ratet, Institut des Sciences des Plantes de Paris Saclay, IPS2BATIMENT 630 PLATEAU DU MOULON RUE NOETZLIN.

Plasmid Construction for Fungal Application and Strawberry Transient Transformation

For transient overexpression of *MtNOOT* gene in strawberry leaves, a fragment of 4700 bp including; 2811 bp of *NOOT* gene plus 1889 bp of its promoter fragment was amplified using the two previously designed primers (Couzigou *et al*., 2012); Pnoot 5'-GGACCGACGAATGTATTAA-GCCTTAAA-3' & nootR 5'-TTAGTAGTCATGACCATGAGAGT-3'

was cloned in pGEM-Teasy and double digested by *Eco*RI/*Sal*I after destruction *of Eco*RI site toward the SP6 promoter of pGEM-Teasy and sub-cloned in pCP42 binary vector to generate pCP42::*MtNOOT* under the control of *MtNOOT* gene promoter. For preparing pCambia1390::FaWRKY1 (35S::FaWRKY1) expression cassette, *Bam*H1/*Eco*R1 restriction sites were added to the start and the end of *FaWRKY1*, respectively *via* the two oligonucleotide primers*;* WR-F 5' C*GGATCC*ATGGATACCTACCCAGCATTC '3 & WR-R 5' G*GAATTC*TCACAAAGAAGTGTAGATTTGCAT '3 using pGEM-Teasy-FaWRKY1 clone (Hussein *et al*., 2016) as a DNA template to amplify a fragment of 575bp. The amplified FaWRKY1 fragment and the modified pCambia 1390 binary vector were double digested by *Bam*H1/*Eco*R1 and ligated together. pCP42::MtNOOT and pCambia1390::*FaWRKY1* were transformed into *E. coli* competent cells and the transformed colonies were selected on 100 mg/ml ampicillin LB plates and confirmed by the proper colony PCR protocol.

FaWRKY1 Antagonistic Activity

Antifungal activity of *FaWRKY1* was assayed by estimating the inhibition initiated by two parasitic fungi on potato dextrose agar plates. *E. coli*-containing *FaWRKY1* gene cloned in pGEM-Teasy was inoculated on the edges of both *Macrophomina phasiolena and Rhizoctonia solani* PDA plates. *Macrophomina phasiolena, Rhizoctonia solani* grown separately on PDA plates were used as a positive control and were inoculated at the edge with non-transformed *E. coli* cells to eliminate the effect of *E. coli* on the fungal growth. The plates were incubated at 28 ºC for seven days and fungi inhibition were determined.

Construction *FaWRKY* and *MtNOOT* and Agrobacterium Transformation

pCP42::MtNOOT and pCambia1390::FaWRKY1 constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 (Koncz *et al.*, 1989) by electroporation protocol (Sukharev *et al*., 1992) at 25 mF, 2.5 kV and 400 Ω. The transformed cells were selected on LB- agar media containing 50 mg/ml streptomycin and 50mg/ml kanamycin. Agro-infiltration experiment was carried out using the two constructs separately and together.

Agro-infiltration in Strawberry Leaves

Strawberry plant leaves were used for transient expression by Agro-infiltration (Kapila *et al*., 1997; Yang *et al*., 2000). One hundred microliters of transformed frozen cells stock was inoculated in 5 ml LB broth supplemented with 50 mg/ml streptomycin and 50 mg/ml kanamycin. The culture was incubated at 28ºC at 210 rmp shaking overnight. 500 µl of transformed *Agrobacterium* was used to inoculate 50 ml of LB medium and the cells were shaking at 28ºC and 210 rpm until the culture reached the O.D.600=0.6. The cells were harvested at 6000 rpm by

centrifugation and re-suspended in 50 ml MES buffer (10 mM MES; pH 5.5, 10 mM $MgCl_2$). MES buffer was incubated at room temperature for two hours with 100 µM acetosyringone. The leaves of two months old strawberry plants were pressure injected into the lower epidermis of the leaf through a 3 ml disposable syringe. Post-infiltrated plants were kept in the growth chamber with a 16/8 light/dark photoperiod at 25ºC.

Mechanical Injury and Strawberry Leaves Infection

Two days post-agro-infiltration process, strawberry leaves were infected mechanically by *Macrophomina phasiolena* parasitic fungus, a wound was formed by a red hot pinhead touched the epidermis of the infiltrated strawberries leaves gently (De Meyer and Hofte, 1997). After wounding the leaves, the spores' suspensions were sprayed exogenously over the strawberries plants. Then the pots with the infected and control plants were incubated in closed chamber at 25ºC separately. The data were taken three, five, and seven days post fungal inoculation.

RNA Extraction and RT-PCR Conditions

RNA extraction was carried out from *MtNOOT* and *FaWRKY1* transformed leaves using RNeasy Mini Kit according to the manufacturer's instructions. All DNA contaminates were removed *via* Thermo Scientific, DNase1 RNase free kit. Recovered RNAs were measured by spectrophotometry at A260 and A280 nm. SuperScript-III, the one-step RT-PCR was carried out according to the manufacturer's instruction. NanoDrop Spectrophotometer (Thermo scientific) was used for quantifying the Purified RNA. Oligonucleotide pair; nootF 5'-ATGTCCCTTGAAGACTCACTAA-GATCT-3' & nootR and oligonucleotide pair WR-F & WR-R were used for *NOOT* and *WRKY* expression detection respectively in strawberry leaves.

RESULTS AND DISCUSSIONS

Strawberry is the major popular fruit and its health benefits were intensively indicated by many researchers worldwide, but its yield and quality are extremely limited due to various pathogen attacks. *Macrophomina phasiolena and Rhizoctonia solani* are seriously limiting parasites that are thriving strawberry productivity. Thus, the strategies to expand resistance in such important crop will be the most relevant economically.

Expression Performance of *FaWRKY1* as an Antagonistic to Fungal Growth

Promoter elements of prokaryotes and eukaryotes developed differently through evolution with regard to their structure and sequences. Previously many researchers demonstrated that the eukaryotic promoter sequences can't drive an efficient gene expression in prokaryotes upon its translocation. However, Assaad and Signer (1990) used cauliflower mosaic virus constitutive promoter *p35S* to di-

rect the expression of neomycin phosphotransferase II gene in *E. coli.*

In this study *FaWRKY1* was designed an experiment to evaluate the effect of transgene that is produced into *E. coli* transformed cells on limiting the growth of the harmful parasitic fungi. pCambia1390::FaWRKY1 (p35S::FaWRKY) transformed colonies were selected and confirmed by PCR. *Macrophomina phasiolena*, and *Rhizoctonia solani* growth plates were subjected to the effect of *FaWRKY1* as described in materials and methods section. Effectively inhibition zones were obviously detected on both *Macrophomina phasiolena*, and *Rhizoctonia solani* PDA plates treated with p35S::FaWRKY transformed *E. coli*. The detected Inhibition zones were almost similar in case of the two parasitic fungal growth. *Macrophomina phasiolena* and *Rhizoctonia solani* growing on PDA plates and inoculated with un-transformed *E. coli* were used as positive control as it hasn't any effect on fungal growth performance (Fig. 1). This result is a preliminary evidence that *FaWRKY1* can positively limit harmful fungal growth and inhibits its activity.

Previously, Jacob *et al*., (2002) succeeded to prove that constructs designed to be for transformetion into plants are able to be expressed in *E. coli* as well. They demonstrated that plants promoter contains elements that are recognized by eubacteria transcription machinery even though the promoter sequences are considerably less specific than of commonly expected. To prove their findings, they evaluated lux gene expression driven by ten diverse plant-specific promoters, mainly the *P35S* promoter, in five diverse bacteria species, including *E. coli*. Our finding presented that the designed constructs, P35S-*FaWRKY-3'nos* transformed into *E. coli* were thrived to generate an inhibition zone in *Macrophomina phasiolena,* and *Rhizoctonia solani* fungal growth plates. This result is great standing for our knowledge around the organization of gene expression evolution and on construction of various expression vectors. In a similar manner, many studies used *P35S* to drive gene expression in *E. coli* and in *Agrobacterium tumefaciens* -mediated transformation as well. Glutenin genes as an example were isolated and their expression were drived by *p35S* using pCAMBIA-1304 (Pandey and Somssich, 2009).

Transient Overexpression of *FaWRKY1* & *MtNOOT* Genes in Strawberry Leaves Reduced Tissue Damage post *Macrophomina phasiolena* Inoculation

On the other hand, we evaluated the performance of our two genes; *FaWRKY1* and *MtNOOT* when transformed separately and in combination into Strawberry plant's tissue. Hence, the transformed plants were subjected to mechanical fungal infection. In such case, the expression of each gene is influenced by other transcription factor proteins in a definite pathway inside a complete defense network system.

pCP42::MtNOOT and pCambia1390::FaWRKY1 constructs; *MtNOOT* is cloned under the control of its own promoter and *FaWRKY* is cloned under the control of p35S (Pnoot:: MtNOOT& *p35S*::FaWRKY), were transiently transformed into strawberry leaves *via Agrobacterium tumefaciens* followed by PCR confirmation (Fig. 2). The two expression cassettes were introduced into strawberry leaves *via* agrobacterium-infiltration procedure separately and in compnation with each other while the *Macrophomina phasiolena* mechanical infection was carried out two days post the agrobacterium-infiltration. The *FaWRKY1* & *MtNOOT* fragments and transcripts viability were confirmed by PCR and RT-PCR respectively third, fifth, and seventh days post mechanical infection with *Macrophomina phasiolena* (Fig. 3). The consequent symptoms were monitored on transiently transformed strawberry leaves and compared to the two controls; the healthy plant as negative control and the untransformed-infected strawberry leaves as a positive control for the disease. The control healthy plant was injected with an empty *Agrobacterium tumefaciens* to eliminate the effect of agrobacterium on strawberry leaves. While, the positive control strawberry plants were mechanically injured and sprayed with *Macrophomina phasiolena* spores but were not transiently transformed with any of the constructs or an empty agrobacterium. The data showed that three days post parasitic infection, welting and drying symptoms were observed in the positive control plants, and moreover the plants were eventually died after five days compared with uninfected control healthy plants (Fig. 4 A, B and C). Strawberry leaves transiently expressing *MtNOOT* and *FaWRKY* genes accompanied to each other displayed the highest resistance activity at the third, fifth, and seventh days post-inoculation with *Macrophomina phasiolena*, followed by the leaves transiently expresseing *MtNOOT* however, leaves transiently overexpresseing *FaWRKY1* were the most susceptible to the fungal infection but they were still more resistant than the positive control leaves (Fig. 4 D, E, F, G, H, I, J, K, and L).

Our data revealed that the overexpression of *FaWRKY1* gene in strawberry plants considerably enhance defense mechanism against the fungal infection three, five, and seven days post mechanical infection. Likewise, ectopically expression of *Medicago truncatulla NOOT* gene in strawberry tissue drived a more protection pattern than that of *FaWRKY1*. In a previous data, the role of *FaWRKY1* in defense response against various pathogen attacks was proposed by dramatically up-regulated *FaWRKY1* transcript in strawberry (Casado-Diaz *et al*., 2006; Encinas-Villarejo *et al*., 2009). Similarly, *VvWRKY1* overexpression enhanced resistance against downy mildew disease caused by *Plasmopara viticola* in grapevines, *via* JA-pathway related genes induction (Marchive *et al*., 2013).

WRKY transcription factor family contains the WRKYGQK main sequence upstream a zinc-finger motif, have various

roles during plant defense response and it can control its own expression positively and negatively selfregulation upon parasitic infection and in health condition, respectively due to the presence of a W-box within its promoter region (Asai *et al*., 2002; Robatzek and Somssich, 2002). WRKY is modulating plant defense either positively or negatively (Eulgem and Somssich, 2007). Contrarily to our result, *FaWRKY1* silencing is enhancing resistance against the pathogen *Colletotrichum acutatum* in strawberry (*Fragaria* _ *ananassa* cv. Primoris), so in that case *FaWRKY1* acts as a negative regulator of resistance activity (Higuera *et al*., 2019). In a similar way, the *GbWRKY1* silencing in cotton promoted plant resistance against *Verticillium dahlia* and *Botrytis cinerea* (Li *et al*., 2014).

The members of WRKY family can bind to the consensus sequence [(T)TGAC(C/T)] of the W-box found in the promoter of various stress-specific genes sequences, including PR proteins that responded to pathogens attacks and wounding (Ülker and Somssich, 2004). Thus, we transformed *MtNOOT*, the NPR1-family related gene, which can be influenced and positively activated by the overexpression of *FaWRK1* transcription factor during the parasitic attacks into strawberry tissue. NPR1 family compresses a BTB/POZ as well as ankyrin repeats domains in its sequence (Aravind and Koonin, 1999; Sedgwick and Smerdon, 1999), SAR is the plant defense action that is essentially regulated by NPR1. When the plants were subjected to biotic or abiotic stress, redox potential elicited by salicylic acid (SA) warning sign molecules accumulation is enhancing NPR1 to be converted from the oligomeric form to the monomeric form and hence can be translocate of to the nucleus from the cytosol in order to interact with TGA transcription factors, (the TGACG specific binding protein sequence) that binds to SA-responsive elements. Various WRKYs were well-defined as SA-dependent responses regulators through the NPR1-dependent SAR initiation (Ishihama and Yoshioka, 2012).

The protection proposed by *FaWRKY*1 overexpression combined by heterologous overexpression of *MtNOOT* in strawberry plants was ultimately better than when introducing each gene separately into plant's tissue. This is due to the fact that the sequences of W-box in promoter region of the *MtNOOT* are recognized precisely by SA-induced *FaWRKY* DNA binding proteins and hence *MtNOOT* expression was activated. The results proved that constitutive overexpression of *FaWRKY1* in strawberry tissue positively affected the ectopic expression of the *MtNOOT*-NPR1 related gene upon fungal infection. In a similar manner, Choi *et al*., (2004) conferred high resistance against fungus pathogen *A. alternata* and bacterial pathogen *P. syringae* in tobacco by fusing a promoter region of pea DRR206 gene, which contains the W-box and the wound/pathogen-inducible box (Palm *et al*., 1990), with *F. solani* DNase elicitor gene. Mutations in W-box sequence eliminated their recognition *via*

WRKY DNA binding protein, and hence the promoter will not be able to activate the downstream protective genes (Yu *et al*, 2001). In addition, Viejobueno *et al*., (2021) induced defense response against *M. phaseolina* by treatments with *Azospirillum brasilense* bacterial, the Plant Growth-Promoting Rhizobacteria (PGPR). This bacterium has a native antagonistic effect against parasitic agents and increased tolerance of strawberry plants to charcoal rot disease. This way of elevating the innate immune system of the plants is an ecological and environmentally friendly approach.

CONCLUSION

The results demonstrated that the *FaWRKY* transcript can be confirmed directly in *E. coli*, despite the fact that the main transcription sites seem not to be similar as in plants. The enhanced resistance through overexpression of *MtNOOT* & *FaWRKY1* genes in strawberry-transiently transformed leaves suggests that both genes activate plant defense mechanisms upon pathogen attack comparing to untransformed strawberry plants. Network regulation between *FaWRKY1* & MtNOOT were dedicated to ultimately increase strawberry defense response to *Macrophomina phasiolena* attack.

SUMMARY

Strawberry (*Fragaria x ananassa*) is one of the favorite fruit worldwide due to its wide health benefits, and distinct flavor and aroma. *FaWRKY1* gene was suggested as a significant element intermediating defense response against various pathogens attack in strawberry. Because of the influencing role of WRKY family involving in defense network, it has become a favorable candidate for improving crops quality. WRKY can precisely recognize and bind to the downstream promoters of transcription factors activating defense cascades. The ability of FaWRKY to enhance resistance against *Macrophomina phasiolena* was investigated by performing Agrobacterium-mediated transformation protocol for transient overexpression of *FaWRKY1* gene in strawberry leaves (Fragaria *x* ananassa cv. Camarosa) to evaluate its function upon the fungal infection. However, *MtNOOT* gene were transiently hetero-expressed in strawberry leaves separately and in compintion with the *FaWRKY*1. We demonstrated that the existence of W-box sequences within the *MtNOOT* (NPR1-like gene) promoter region, which are recognized definitely by SA-induced FaWRKY DNA binding protein, increased strawberry resistance activity when the two genes are transformed combined to each other .The severity of leaf injury was observed at three, five, and seven days post pathogen inoculation on *FaWRKY1*, *MtNOOT*, and *FaWRKY1* & *MtNOOT* combination-transformed strawberry plants compared to the control un-transformed infected plant as positive control and a healthy untransformed non-infected plants as negative control . Susceptibility to fungal infection was obviously detected and showing that the two genes combination (*FaWRKY* & *MtNOOT*) revealed the best

resistance against the pathogen fungal attack followed by *MtNOOT* and finally by the *FaWRKY1* transcription factor. Our results evidence that *FaWRKY1* gene acts upstream of the heterologous *MtNOOT* (*NPR1-like gene)* and positively regulates its expression throughout plant defense activation during pathogen attack.

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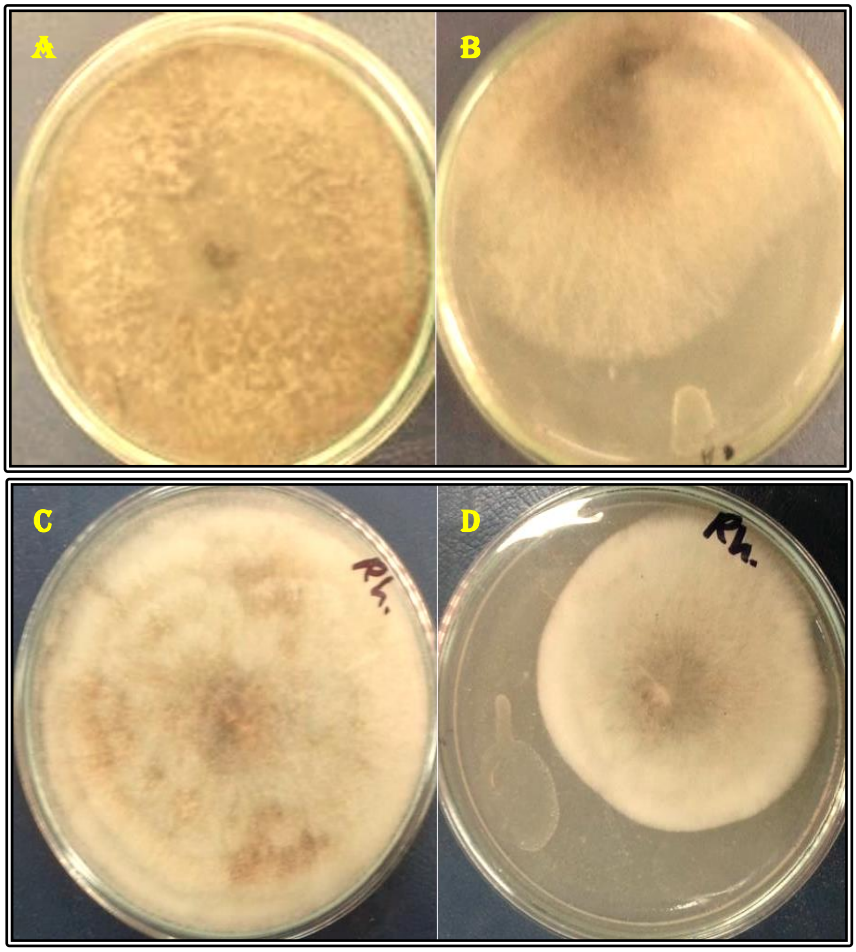

Fig. (1): Potato dextrose agar (PDA) plates inoculated with parasitic fungi; *Macrophomina phasiolena* grown on PDA as positive control (A), an inhibition zone was clearly observed on *Macrophomina phasiolena* PDA plates when inoculated with p35S::FaWRKY1 (B), *Rhizoctonia solani* grown on PDA as positive control (C), and an inhibition zone was clearly observed on *Rhizoctonia solani* PDA plates when inoculated with p35S::FaWRKY1 (D).

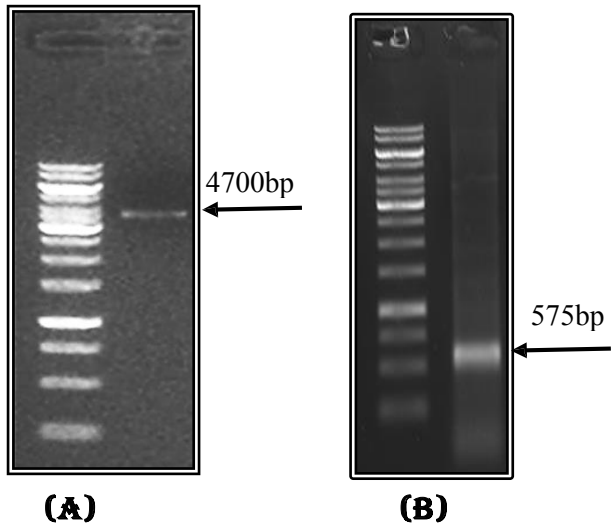

Fig. (2): Amplified fragment of 4700 bp including; 2811 bp of *MtNOOT* gene plus 1889 bp of its promoter region using Pnoot & nootR oligonucleotides primer (A), Amplified fragment of *FaWRKY1* giving a size of 575 bp using specific oligonucleotides primers*;* WR-F & WR-R (B).The two PCR were carried out to confirm the transformation step of the two genes into *Agrobacterium tumefaciens.* GeneRuler 1kb DNA ladder was used as DNA marker.

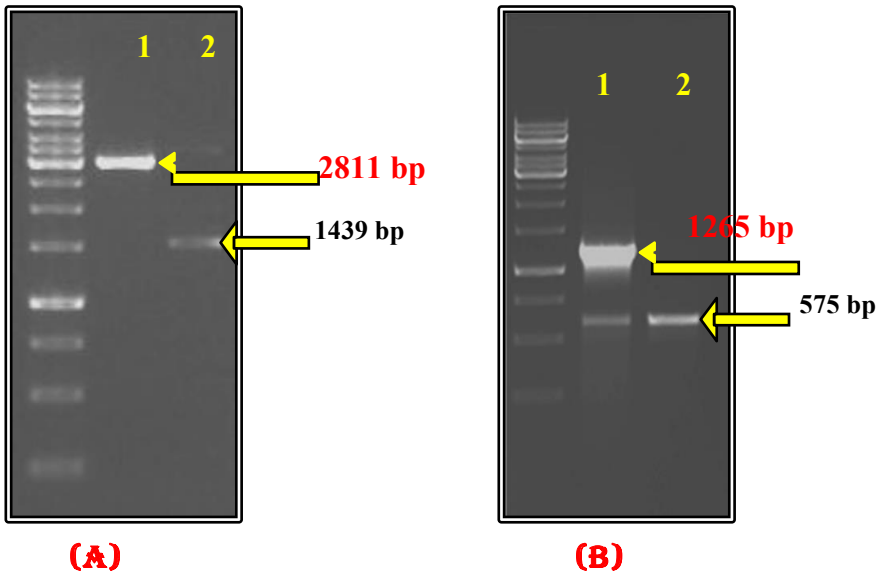

Fig. (3): PCR amplified fragment of 2811 bp for *MtNOOT*-ORF, RT- PCR amplified fragment of 1439 bp for *MtNOOT*, nootF&nootR oligonucleotides primer pair was used for PCR and RT-PCR (A1&2), Amplified two sizes fragments of *FaWRKY1*; 1265 bp fragment for the genomic *FaWRKY1* gene that is natively existing in strawberry plants, and 575 bp fragment for the transiently transformed *FaWRKY1* gene, RT-PCR amplified fragment of 575 bp for *FaWRKY1*, oligonucleotides primer pair WR-F&WR-R was used for PCR and RT- PCR (B1&2). The PCR and RT-PCR were carried out 3, 5, 7 days post mechanical infection with *Macrophomina phasiolena* to confirm the transient transformation and transcripts viability of the two genes in strawberry plants. GeneRuler 1kb DNA ladder was used as DNA marker.

Fig. (4): Transient overexpression of *MtNOOT* & *FaWRKY1* genes in strawberry was substantially altered susceptibility to *Macrophomina phasiolena* infection. Control healthy plant injected with empty agrobacterium (A). Disease symptoms appears strongly on the mechanically infected strawberry leaves three days post *Macrophomina phasiolena* infection (b). Strawberry infected plants were eventually died after five days post *Macrophomina phasiolena* infection compared with uninfected control healthy plants(C). Strawberry plants agro-infiltrated with (pCambia1390::FaWRKY1) displayed moderate infection three, five, and seven days post *Macrophomina phasiolena* inoculation (D, E, and F). Plants agro-infiltrated with (pCP42::MtNOOT) exhibited low to moderate symptoms three, five, and seven days post parasitic infection (G, H, and I). Plants agro-infiltrated with (pCambia1390::FaWRKY1& pCP42::MtNOOT) showed very mild disease symptoms three, five, and seven days post *Macrophomina phasiolena* inoculation compared to pCambia1390::FaWRKY1 and pCP42::MtNOOT separately-injected plants(J, K, and L).