Multiple fungal diseases resistance induction in C*ucumis melo* through co-transformation of different pathogenesis related (PR) protein genes

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Abstract

Achieving multiple resistance to fungal diseases, major threat in cucurbits production, is challenging due to the quick rate of variation of fungi species and races, the lack in the knowledge of resistance genes or their complexity, and dragging undesired genesin traditional breeding. Here, we generate polygenic resistance to fusarium and powdery mildew by introducing a transgene cassette of three antifungal pathogenesis related (PR) protein genes (*chitinase, glucanase* and *PR1*) into melon (*Cucumis melo* L.) as a single locus. The presence of three PRs was confirmed by PCR in eight transgenic plantlets among 130 regenerants grown in selective medium. Southern blot hybridization confirmed integration of the transgene cassette in the genome of three lines . Enzyme activity assay demonstrated that chitinase and b-1,3-glucanase activity were higher in transgenic lines than in wild type plants. *In-vitro* and *in-vivo* bioassay tests showed that transgenic lines were also more resistant to fungal diseases. Finally, to evaluate the stability and heritability of the acquired resistance, selected transgenic lines were self-pollinated and T_1 generation was further evaluated for disease resistance. After artificial inoculation by *Fusarium oxysporum* f. sp. *melonis* (FOM), more than 80% of wild types seedlings were infected. By contrast, on avarage 87% of T_1 transgenic seedlings did not

show disease signs. Moreover, powdery mildew infection was strongly delayed in transgenic plants.

Key words: *Agrobacterium tumefaciens*, pathogenesis related (PR) protein genes, Melon, Fungal resistance, Genetic transformation, Molecular analysis

INTRODUCTION

Melon (*Cucumis melo* L.; Cucurbitaceae; 2n=2x=24), is a popular crop cultivated worldwide, especially in hot and dry climatic zones. China, Turkey and Iran are the main producers of melons (FAO 2019). Iran is considered as a secondary diversity center of melon (Luan et al., 2008), where several local landraces and hybrids are extensively cultivated for their unique flavor and shape. Susceptibility to fungal diseases is a strong limit, causing large yield loss (Etebarian, 2002). *Fusarium solani*, *Macrophomina phaseoli*, *Fusarium oxysporum*, *Phytophthora capsici*, and *Monosporascus cannonballus* are the most widespread pathogenic fungi in the Varamin area in Iran, one of the main centers of melon production (Shahi-Bajestani and Dolatabadi, 2016). Although there is a high diversity rate in the melon genetic background (Pavan et al., 2017), with a considerable variability in the Iranian germplasm (Danesh et al., 2015; Maleki et al., 2018) and several resistance genes are known in this species (Brotman et al., 2002; Frantz and Jahn, 2004; Mascarell-Creus et al., 2009; Dogimont, 2011; Gao et al., 2015; Li et al., 2017; Howlader et al., 2020), the lack of highly resistant germplasm, the reproductive barriers for interspecific crosses, and the time-consuming work for traditional breeding, hinder the development of genotypes with multiple fungal diseases resistance.

Conversely, new methods such as genetic engineering can be quick and effective way to transfer genes of interest to plants (Vahdati et al., 2002; Garcia Almodovar et al., 2017). So far, there is only one report for the transfer of resistance gene to fungal diseases (chitinase) in melon (Bezirganoglu et al., 2013). However, a single gene is often not sufficient to create a stable full resistance. In the plant-pathogen interaction, new races of pathogens evolve overcoming single resistance genes (McGrath et al., 2001). For example, in 2008, a new race of powdery mildew was controlled in Georgia by using resistant melon genotypes, however the resistance to this disease decreased within the following two years (McGrath et al., 2001). Therefore, plant breeders need to transmit several genes with different mechanisms of resistance by horizontal resistance to susceptible genotypes to create a long-lasting resistance in melons (Wang et al., 2020). To our knowledge, no study has ever been conducted on pyramiding resistance genes in melon using the transformation system.

Defense response is activated by interaction of pathogen effectors with receptors on the surface of the plant cell, products of the R genes (Shirley et al., 2020). Subsequently, a signaling cascade (ROIs, SA, NO, ethylene) transmits the message received by the receptors to the cell nucleus (Ruan et al., 2019; Noman et al., 2020; Imran and Yun, 2020). There, transcription factors (e.g. NPR1: non-expresser of PR genes) are activated, increasing the expression of

resistance genes (Clinckemaillie, 2017). The genes products eventually interfere with the development of resistance to disease (Andersen et al., 2018). Pathogenesis-related (PR) genes are considered the most important plant resistance genes to fungal diseases (Jain and Khurana, 2018). PR proteins are relatively small and have a significant amount of cysteine residues, forming disulfide bonds and stabilizing three dimensional structures (Prasannath, 2017). PRs are divided into seventeen groups (PR1-17), each of them having a specific mechanism to defend against the fungal diseases, affecting directly pathogen integrity, and/or inducing plant defense related pathways (Boccardo et al., 2019). The product of each group's genes are different to make a range of resistance in diverse hosts (Iqbal et al., 2019).

Among 17 known PR groups, the most important ones belong to groups 3, 4, 8 and 11, which code for chitinase proteins (*Chi*) and cause chitin degradation of fungi cell wall (Seidl et al., 2005). Additionally, PR-2 genes produce glucanases that break down the cell wall glucans of fungi, while PR-1 genes are involved in the pathogen plasma membrane damage (Boccardo et al., 2019). Interestingly, different mechanisms of synthesis for each group of PR are known, therefore the production and subsequent accumulation of specific PRs can increase at the same time the resistance level to different pathogens in the hosts (Saboki et al., 2011; Akbudak et al., 2020).

The main goal of the present research was to regenerate resistant plants of melon by simultaneous transfer of chitinase (*Chi*), glucanase (*Glu*) and *PR1* via *Agrobacterium tumefaciens* in two Iranian cultivars, 'Khatooni' and 'Samsoori', in order to confer them a broad and multi long lasting resistance against fungal diseases and to prevent breakdown of resistance acquired.

MATERIALS AND METHODS

Plant material

Two Iran local landraces of *C. melo* were used in this study, namely 'Khatooni' and 'Samsoori' belonging to Inodorus and Cantalupensis groups, respectively. Uncoated seeds were sterilized and cultured in MS medium (Murashige and Skoog, 1962) and cotyledons were dissected from one-week-seedlings. To select the best regenerating plant growth regulator (PGR) combination, cotyledon explants from the two genotypes were cultured on MS medium supplemented with different concentrations of PGR, as described in previous study (Raji et al., 2021).

Agrobacterium-mediated transformation

The *A. tumefaciens* LBA4404 strain, harboring the binary construct pBI121 with three PR fungal resistance genes (*Chi* - M13968.1 -, *Glu* - AF515785.1 - and *PR1* - X06361.1 -, selected from bean (*Phaseolus vulgaris*), tobacco (*Nicotiana tabacum*) and barley (*Hordeum vulgare*), respectively) and a neomycin phosphotransferase II gene (*nptII*) as selective marker (Figure 1) was utilized for inoculation. The three target genes were under the control of CaMV35S promoter and Nopaline Synthase (NOS) Terminator with different directions, for selecting the transformed plants by their resistance to kanamycin. The bacterial colonies were grown overnight at 28°C in 5 ml of Luria Bertani Broth (LB) media, containing 50 mg l^{-1} kanamycin and 100 mg l^{-1} rifampicin, on a shaker incubator at 220 rpm, to have an exponential growth phase at an optimum density (OD600 = 0.5 to 1). The bacterial cells were collected by centrifuging at 5000 rpm for 5 min and the pellet was re-suspended in MS medium. Explants were incubated in Agrobacterium suspension with OD=0.6 for 2–3 min and dried on a sterile Whatman filter paper and then transferred on MS hormone-free co-culture medium for 48 h. Co-cultured explants (with bacteria) were then placed on MS medium supplemented with kanamycin (50 mg l^{-1}) and cefotaxime (250 mg l^{-1}) along with 600 µg/l BAP and 25 µg/l NOA. Regeneration of plants was carried out according to the method of Raji et al. (2021). The inoculated explants were kept in a growth chamber (KK 1450 FIT P) with 40-Watt lumichrome fluorescent bulbs (100 µmol m^{-2} s^{-1} light) under 16/8 h and 26°C/20°C day/night cycles. After three weeks, elongated and live shoots were transferred to a PGR-free medium with the same concentration of antibiotics for root formation. The rooted plants were transferred to the jiffy pots and acclimatized in greenhouse (Raji et al., 2018).

Kanamycin Assay

To investigate the effect of antibiotic on survival and regeneration of explants, viability and regeneration of non-inoculated cotyledon disks were evaluated using different concentrations of kanamycin (0, 25, 50, 75 and 100 mg l^{-1}) added in MS media (Table 2). The concentration of kanamycin that completely prevented the growth of non-inoculated explants was chosen as selective medium.

Molecular Analysis

PCR analysis

Young fresh leaves from kanamycin-resistant plants were collected and ground into a fine powder in liquid nitrogen. Total DNA was extracted using the CTAB method (Doyle and Doyle, 1987) and the Polymerase Chain Reaction (PCR) was performed using specific primers

to amplify the inserted PR transgenes. To test for Agrobacterium contamination, the transformed plants were also analyzed by *virG* specific primers. PCR was performed in 25 µl reaction volume including 100 ng DNA as template, 10 pm of each primer and 1U Taq DNA polymerase. PCR reaction was performed under the following cycle program: 5 min at 94 °C (hot start), followed by 35 cycles at 94 °C for 45 sec (denaturation), then at annealing temperature (depending on the primers used) for 1 min, and finally at 72 °C for 1 min (extension), followed by a final extension step at 72 °C for 10 min.

Reverse Transcription-PCR (RT-PCR) analysis

Total RNA was isolated from PCR-positive melon plants with TRIzol® reagent (Invitrogen™ -Thermo Fisher Scientific, Wilmington, DE) according to the manufacturer's instruction. The extracted RNAs were treated with DNase and utilized for cDNA synthesis using 2-steps RT-PCR KiT (RTPL-12) (Sinaclon, Iran) by following the manufacturer's protocol. After preparing the cDNA, PCR was performed with primers for PRs gene (Niazian et al, 2019). Gel Red was added to the PCR products and runed in electrophoresis system (PowerPac, BIO-RAD, USA) for 30 min on a 1% agarose gel and then observed under UV irradiation.

Real time RT-PCR analysis

The Real-Time RT-PCR primers were designed with the assistance of primer 3 web software (Whitehead Institute for Biomedical Research, MA, USA) and Gen runner software (version 3.05) and manufactured by MWG-Biotech (Germany). All Primers were blasted against nucleotide sequences in National Center for Biotechnology Information (NCBI) database to ensure that their sequences are specific to each gene (Table 1). The mRNA expression levels were quantified by using a LightCycler 96 System (Roche, Basel, Switzerland). Quantitative PCR was conducted in a total volume of 20 µl, with 2 µl of cDNA (50 ng), 1 µl (0.2 µM) of each primer, 10 µl 2x Maxima SYBER Green/Flourescein qPCR Master Mix (Fermentas, Germany) and 6 µl sterile distilled water, with the following conditions: initial denaturation at 95 °C for 5 min and then 40 cycles of 95 °C for 20 sec (denaturation), annealing at 58°C for 20 sec and finally 72 °C for 40 sec (extension). Each experiment was repeated three times independently as biological replicates and two technical replicates were carried out for each biological replication. *ADP ribosylation factor* (*ADP*) gene was used as a reference gene (Kong et al., 2014). The delta-delta Ct method was used to calculate the relative expression levels (Livak and Schmittgen, 2001)

Southern blot analysis

Fifty µg of DNA extracted from young leaves was completely digested with *Eco*RI at 37ºC, overnight .The digested DNAs were electrophoresed on 1% (W/V) agarose gels and then DNA fragments blotted onto N^+ Hybond membrane (GE Healthcare UK Ltd, Buckinghamshire, UK). Coding sequence of the *Chi* gene (*Eco*RI fragment ~1200bp) was labeled with the DIG DNA labeling kit (Roche) and used as a probe. Detection was carried out using the DIG detection kit according to the manufacturer's instructions (Roche, Germany).

Collecting the T_1 seeds

Three confirmed transgenic lines (K44, K58 and S5) by PCR assay and Southern blot analysis were successfully self-pollinated and the seeds of their T_1 progenies (100 seeds from each line) were collected.

Enzyme assays

To compare the enzymatic defense response of transgenic and wild-type plants, CHI and GLU enzymes activity of seedlings was evaluated according to Tohidfar et al. (2005). Twenty transgenic plants regenerated *in vitro* from each line were sampled 20 days after inoculation by *F. oxysporum* f. sp. *melonis* (FOM). Statistical analysis was performed by SPSS software (Niazian et al, 2017).

Disease bioassays test

Bioassay was performed in three steps. In the first experiment, leaf extracts from transgenic and control plants were utilized to assay the in vitro growth inhibition of FOM induced by PR genes, according to Tohidfar et al. (2005). Briefly, young leaves from putative transgenic lines as well as untransformed plants were flash frozen in liquid nitrogen and were ground to fine powder. The soluble proteins were then extracted in 10 mM sodium acetate buffer (pH 5.0), as leaf extracts. The assay for each sample was performed three times. The resistance of T_1 transgenic progenies to FOM and powdery mildew (PM) were assessed in greenhouse condition in the second and the third experiment, respectively, following the procedure described in Haegi et al. (2017). The fungal spores were gently removed from the PDA media with a scalpel and added to 5 ml of water. The resulting mixture was transferred to a falcon tube and shaken with a shaker to mix the spores evenly with water. Ten microliters of the suspension were poured onto a homocytometer slide and counted under a microscope at 40x magnification to a concentration of $4*10^4$ spores per milliliter. The resulting suspension was sprayed on the leaves of transgenic and control plants. Resistance to PM was measured

according to complete infection of the leaf surface by the fungus 20 days after inoculation. The data were analyzed based on one-way ANOVAS, including the non-transgenic control plants in the analyses and performing Dunnett's test for pairwise comparisons between each transgenic line and the non-transgenic control.

RESULTS

Kanamycin test

The bud formation from cotyledon explants of non-inoculated plants was completely inhibited at kanamycin concentration ≥ 50 mg l^{-1} and the plants died after three weeks. Since 50 mg l^{-1} was the lowest selective kanamycin concentration for both *C. melo* landraces used in the present work (Table 2), this condition was applied to select the transformants.

Plant cell transformation and regeneration

The regeneration percentage of inoculated and co-cultured explants from the two melon genotypes (Figure 2) were evaluated by their bud formation ability in the selective medium (SM). In total, 58 kanamycin-resistant plantlets (51%) were obtained from 114 explants of the two landraces, with 58% and 44% of 'Khatooni' and 'Samsoori' transformed plants showing bud formation, respectively (Table 2).

Molecular and enzymatic evaluation

Molecular analysis confirmed co-transfer of the three PR genes inserted in 8 out of 58 kanamycin resistant plants selected. The PCR assay showed clear and specific bands with the expected size for *Chi* (870 bp), *Glu* (680 bp) and *PR-1* (588 bp) transgenes in 3 and 5 plants of 'Khatooni' and 'Samsoori', respectively) (Figure 3). As expected, no amplicons were obtained in non-transformed plants and water, used as negative control. Expression of *Chi*, *Glu* and *PR-1* in the eight transgenic lines selected was subsequently tested by RT-PCR. Qualitative PCR analysis, using c-DNA as template, showed clear amplicons for all the insertedgenes , while as expected no fragments were obtained in non-transformed plant used as negative control (Figure 4-A). However, southern blot analysis for the *Chi* gene only showed gene integration in three out of the eight transgenic plants, two lines from 'Khatooni' (K44, K59) and one from 'Samsoori' (S5) landrace, respectively (Figure 4-B).

Real-time PCR (RT-qPCR) analysis was performed to evaluate the expression levels of the three genes in the transformed lines (Figure 5). Untransformed control lines showed no expression.Quantitative PCR underlined the expression of each gene transferred in the three transgenic plants. Interestingly, the expression levels of *Chi*, *Glu* and *PR-1* were significantly

higher in S5 than K44, and K59 lines. By contrast, no significant differences were showed between K44 and K59 cultivars.

The expression pattern was paralleled by the enzymatic activity of the gene products, with a significantly higher activity of CHI and b-1,3- GLU in S5, K59 and K44 transgenic lines than in wild type plants (Table 3).

The highest Chi (45.75 mg protein-1) and b-1,3- Glu (25.63 mg protein-1) activity was obtained in K44 in comparison with other transgenic plants and control. The transgenic lines had Chi and b-1,3- Glu activity levels more than 400 to 500-times higher than the control plants (Table 3).

Bioassay of lines with fungal causal agents

Leaf extracts from transgenic lines (S5, K44, and K59 lines) and wild type were used in the bioassays to verify their effect on fungal hyphae growth. A clear effect of FOM inhibition growth was observed for all concentration tested (25, 50, 75, and 100 μg) of the extracts from transgenic line while, as expected, no inhibitory effect was highlighted by the leaf extract of non-transgenic lines (Table 4).

We finally tested the plant for resistance to two pathogenic fungi. All transgenic lines showed a significant higher rate of survival (92%, 83% and 91% for T_0 and 84%, 79% and 83% for T_1 plants belonging to K44, K59 and S5 lines, respectively) than non-transgenic plants (18%), after FOM inoculation (Figure 6-A). Plants showed increased resistance also to PM infection, as measured as by hyphae extension. Leaves from non-transgenic plants were colonized within a week (control), whereas the transgenic lines were completely infected only after 20 (S5) or 22 (K49, K55) days (Figure 6-B, Table 5). The growth rate of the fungus was higher on older leaves than young leaves (not shown). Qualitative evaluation confirmed high resistance of transgenic plants compared to non-transgenic lines, with a mean of 3.5 vs 9.1, respectively, in the susceptibility scale.

Discussion

Pathogenesis-related (*PR*) genes are among the most important resistance genes to fungal diseases. *PR* genes are sorted into 17 groups (Xu et al., 2016), each with a specific mechanism for confronting the disease (Saboki Ebrahim et al., 2011) . PR proteins show a protective role in plants through their accumulation both locally in the infected and also in remote uninfected tissues. A general role of this family of proteins in response to biotic/abiotic stress has been underlined in many plant species (Lawrence et al. 2000; Yamamoto et al. 2000; Anguelova-

Merhar et al. 2001; Sharma 2013). They are also involved in hypersensitive response (HR) and systemic acquired resistance (SAR) against infection (Jain and Khurana (2018). SAR is a broad-spectrum long-lasting defense where the first signaling of a pathogen attack in a primary site can generate a wide answer in other tissue against several kinds of pathogens. After the pathogen infection, a inducible defense mechanism is activated that include the secondary metabolites accumulation, callose deposition with cell wall lignification, ROS (Reactive Oxygen Species) generation, and also the PR production, including chitinase, glucanase and PR1genes (Yang et al. 1997; Sels et al. 2008; Bernardino et al. 2020). HR can induce SAR reaction which consists in a broad spectrum systemic enhanced resistance to pathogenic infection following a localized infection by a fungal pathogens (Balint-Kurti, 2019). It is dependent on the phytohormone salicylate and associated with the accumulation of pathogenesis-related (PR) proteins such as PR genes (Fu and Dong, 2013). It is assumed that co-expression of *Chi*, *Glu* and *PR1*, genes with three different resistance mechanisms, could enhance resistance to various plant fungal diseases in melon (Jain and Khurana, 2018). Therefore, in the present study, we transformed melon with *A. tumefaciens* LBA4404 strain, harboring the binary construct pBI121 with three *PR* fungal resistance genes (*Chi*, *Glu* and *PR1*). After co-cultivation with Agrobacterium, explants were transferred to selective medium supplemented with 50 mg l^{-1} kanamycin in order to select transgenic explants. In our experimental conditions this level of kanamycin completely inhibited the growth of non-transformed plant material. The same concentration had been used in melon previously (Chovelon et al., 2011) but in some studies higher concentrations such as 75 mg l^{-1} (Fang and Grumet, 1990; Zhang et al., 2014), 100 mg l^{-1} (Valles and Lasa, 1994), 150 mg l^{-1} (Nora et al., 2001) and 200 mg l^{-1} (Papadopoulou et al., 2005) of kanamycin were utilized for selection of transformed cultures. These differences in concentrations may be related to the different tolerance of cultivars to kanamycin (Hao et al., 2011). In the present study, 51% of the explants were able to produce regenerants on the selective medium, a value higher than earlier published reports (Vallés and Lasa, 1994; Nunez-Palenius et al., 2006), but lower than others (Fang and Grumet, 1990; Chovelon et al., 2011).

Since survival of non-transgenic melons had been observed on selective medium by some researchers (Ren et al., 2012), in order to identify the transgenic plants, the genomic DNA from the in vitro regenerated shoots was subjected to PCR and Sothern blotting analysis. We observed that eight plants were positive to PCR and three plants were positive to Southern blot, with a transformation rate of 5.8%, similar to Choi et al. (2012) and Garcia-Almodóvar et al. (2017), and higher than Bezirganoglu et al. (2014) and Hsin-Mei Ku et al. (2011). These

different results, can be related to transformation, regeneration conditions and regenerative potential of different genotypes (Raji et al., 2018).

RT-PCR or real-time PCR is usually utilized to evaluate mRNA expression in many transformation reports (Hao et al., 2011; Ren et al., 2013; Zhang et al., 2014). We observed that semi-quantitative RT-PCR detected transcripts of all the PR transgenes. The expression levels of PR transgenes in S5 line were higher than the two other lines, possibly because of cultivar, copy number and position of gene insertion.

In bioassay analysis, the activities of two critical enzymes (CHI and GLU) in transformed lines were higher than in wild type melon. This had been observed in other transgenic plants including tomato (Wu and Bradford, 2003), potato (Chye et al., 2005; Khan et al., 2008), cucumber (Moravcikova et al., 2007; Kishimoto et al., 2002), rice (Nishizawa et al., 2003; Kim et al., 2003), soybean (Li et al., 2004) and tobacco (Rohini and Rao, 2001). Transgenic cotton plants, harboring *Chi* gene, showed higher degree of CHI activity and resistance to verticillium (Tohidfar et al., 2005).

The inhibition of FOM fungal growth by transgenic melons agrees with what observed by Bezirganoglu et al. (2013) and in many other transgenic plants such as tobacco (Carstens et al., 2003), rice (Kumar et al., 2003; Coca et al., 2004, 2006), peanut (Chye et al., 2005), finger millet (Latha et al., 2005), oilseed rape (Melander et al., 2006), pearl millet (Girgi et al., 2006), Indian mustard (Mondal et al., 2007), citrus (Figueiredo et al., 2011), avocado (Chaparro-Pulido et al., 2014) and salvia (Figlan and Makunga, 2017). Similar to previous studies, it seems that the degree of disease resistance directly correlated to the protein expression levels of the transgenes and number of genes responding to the disease (Esfahani et al, 2010). FOM is one the most important diseases in melon (Gonzalez et al., 2020) causing yield and quality loss (Xue et al., 2020). Bezirganoglu et al. (2013) examined the resistance of melon transgenic lines overexpressing *Chi* to FOM3, while in present study T_0 and T_1 transformed melon lines with three *PR* genes were tested against race 1 and 2 of FOM. The results revealed that differently from wild-type, transgenic plants survived effectively against both FOM races. Moravcikova et al. (2004) showed that plants transformed with either *Chi* gene or a b-1,3-*Glu* gene were more sensitive to FOM than plants expressing both genes. The expression of PR genes in other transgenic plants, such as pearl millet (Girgi et al., 2006), wheat (Mackintosh et al., 2007), tobacco and peanut (Anuradha et al., 2008), Acacia (Gonzalez-Teuber et al., 2009), Salvia (Figlan and Makunga, 2017), also enhanced resistance against FOM.

PM caused by *Podosphaera xanthiiis* often infects muskmelon, limiting its production (Zhu et al., 2018). Some research (Ishak and Daryono, 2020; Cui et al., 2020) has identified PM

resistance genes in melons, but this resistance is apparently not complete. Some resistance genes, such as barley chitinase II and ß-1,3-glucanaseII (Dohm et al., 2001) were transferred to rose. Barley chitinase (Eissa et al., 2017) and wheat Pm3e (Koller et al., 2019) were transferred to wheat to increase resistance against PM. Similar to previous studies (Dohm et al., 2001; Eissa et al., 2017; Koller et al., 2019) we observed that resistance of transgenic melon plants to PM was higher than control plants. In our study, after one week of inoculation, fungal hyphae covered the leaf surface of the control plants, but fungus growth was inhibited up to six weeks after inoculation in transgenic plants. On the other hand, older lower leaves were more infected than young upper leaves.

Expression of PR genes and production of antifungal enzymes is one of the common SAR reactions (Kamle et al., 2020). In our study, as the result of the expression of transformed genes in melons, *Chi*, *Glu* and *PR1* enzymes were produced and possibly mimicked a SAR reaction, leading to resistance to the two diseases FOM and PM. On the other hand, we never observed HR lesions in any transgenic melons.

Conclusion

Transgenic expression of *chitinase*, *glucanase* and *PR1* enhanced resistance of two melon cultivars to the two fungal pathogens FOM and PM. Resistance was maintained in the second generation. The simple monogenic inheritance of this transgenic multigene locus greatly simplifies its use in breeding programs.

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Conflict of interest

The authors declare no competing interests.

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Tables and Figures

Table 1. Primer sequence for real-time PCR

Primers names	Sequence	Amplified size (bp)
Glucanase	F-5'CGTAGGAACAGCAGCGAAT-3'	97
	R-5'CACCAGATACTCCGTGTGTG-3'	
Kitinase	F-5'TTGCCATAACCGACTCCAAG-3'	111
	R-5'GAACATCATCAAGGGAGGCC-3'	
PRP-1	F-5'CCGTGCCCAAAATTCTCAAC-3'	182
	R-5'CAGCTAGGTTTTCGCCGTAT-3'	
ADP	F-5' ATATTGCCAACAAGGCGTAGA -3'	132
	R-5' TGCCCGTAAACAAGGGATAAA -3'	

Table 2. Survival explants (%) of different melon genotypes after two weeks of culture on MS medium containing five kanamycin concentrations and data of plants transformed incubated on Kanamycin.

	0 mg/l kanamycin	**25** mg/l kanamycin	**50** mg/l kanamycin	**75** mg/l kanamycin	**100** mg/l kanamycin	Inoculated explants in MS with **50** mg/l kanamycin	Resistant explants (%)in MS with **50** mg/l kanamycin	Regenerated plants in MS with **50** mg/l kanamycin
'Khatooni'	96	72	0	0	**0**	60	58	35
'Samsoori'	78	22	0	0	**0**	54	44	24
Explant color	green	green	yellow	yellow	yellow			

Table 3. Chitinase and glucanase specific activity in leaf tissues of transgenic melon lines (T0) and untransformed control plant.

Transgenic line (T0)	Specific activity of Chitinase (mg protein$^{)-1}$)[a]	Specific activity of Glucanase (mg protein$^{)-1}$)[a]
K44	45.75*	25.63*
K59	36.66*	23.38*
S5	33.45*	23.12*
Control	0.08	0.05

Stars above the columns indicate significant difference between each transgenic line and the control analyzed by Dunnett's test.

Table 4. Bioassay of leaf extract inhibition effect on FOM hyphae growth. 100 µg of non-transgenic leaf extract, extraction buffer, and several concentrations (25, 50, 75 and 100 µg) of transgenic plant leaf extract (S5, K44, and K59) were tested.

Leaf extract (µg)			Transgenic line (T0)		
	Extraction buffer	Non-transgenic leaf extract	K59	K44	S5
Blank	15.5				
100		15.6	11.4*	12.2*	12*
75			11.4*	12.2*	12.1*
50			12.1*	12.2*	12.2*
20			12.4*	13.2*	12.7*

Stars above the rows indicate significant difference between each transgenic line and the control analyzed by Dunnett's test.
Fungal growth rate in millimeters. Smaller numbers indicate more inhibitory material in the wells. As the amount of transgenic plant extract in the well increased, it further reduced hyphae growth and increased fungal growth inhibition.

Table 5. Resistance evaluation of T0 transgenic melon lines to powdery mildew (PM) fungus.

Evaluated parameters	K44	K59	S5	Control
Days for a PM complete infection of leaf surface	22*	22*	20*	6
Fungal growth (mm) at two weeks after PM inoculation	6.7*	5.4*	6.1*	10 (Completely covered)
Qualitative evaluation of fungal growth on leaf surface [a]	3.25*	3.5*	3.75*	9.6

Stars above the rows indicate significant difference between each transgenic line and the control analyzed by Dunnett's test. [a] A scale from 1 (resistant) to 10 (susceptible) were defined.

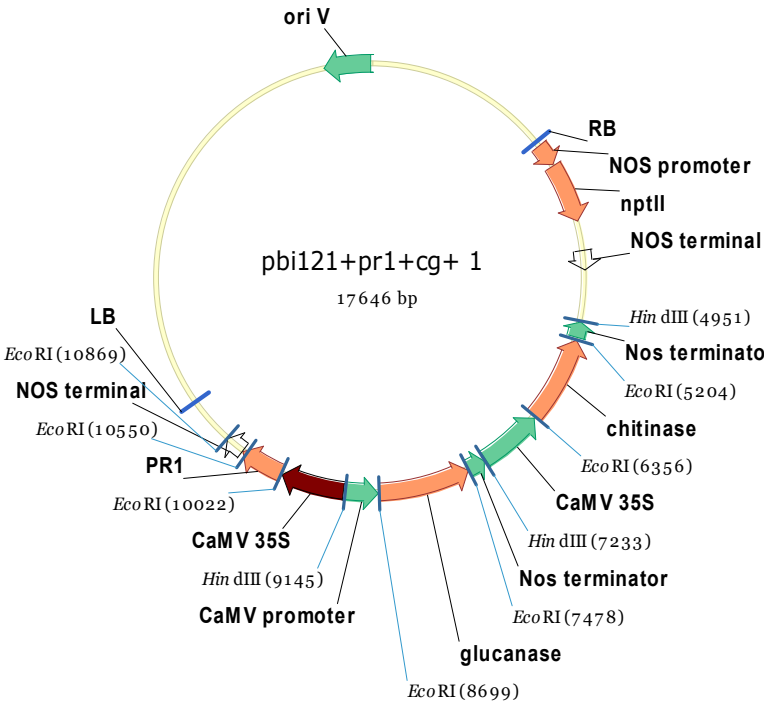

Figure 1. The schematic presentation of binary vector pBI121 harboring PRs gene and *nptII* selective marker in T-DNA region.

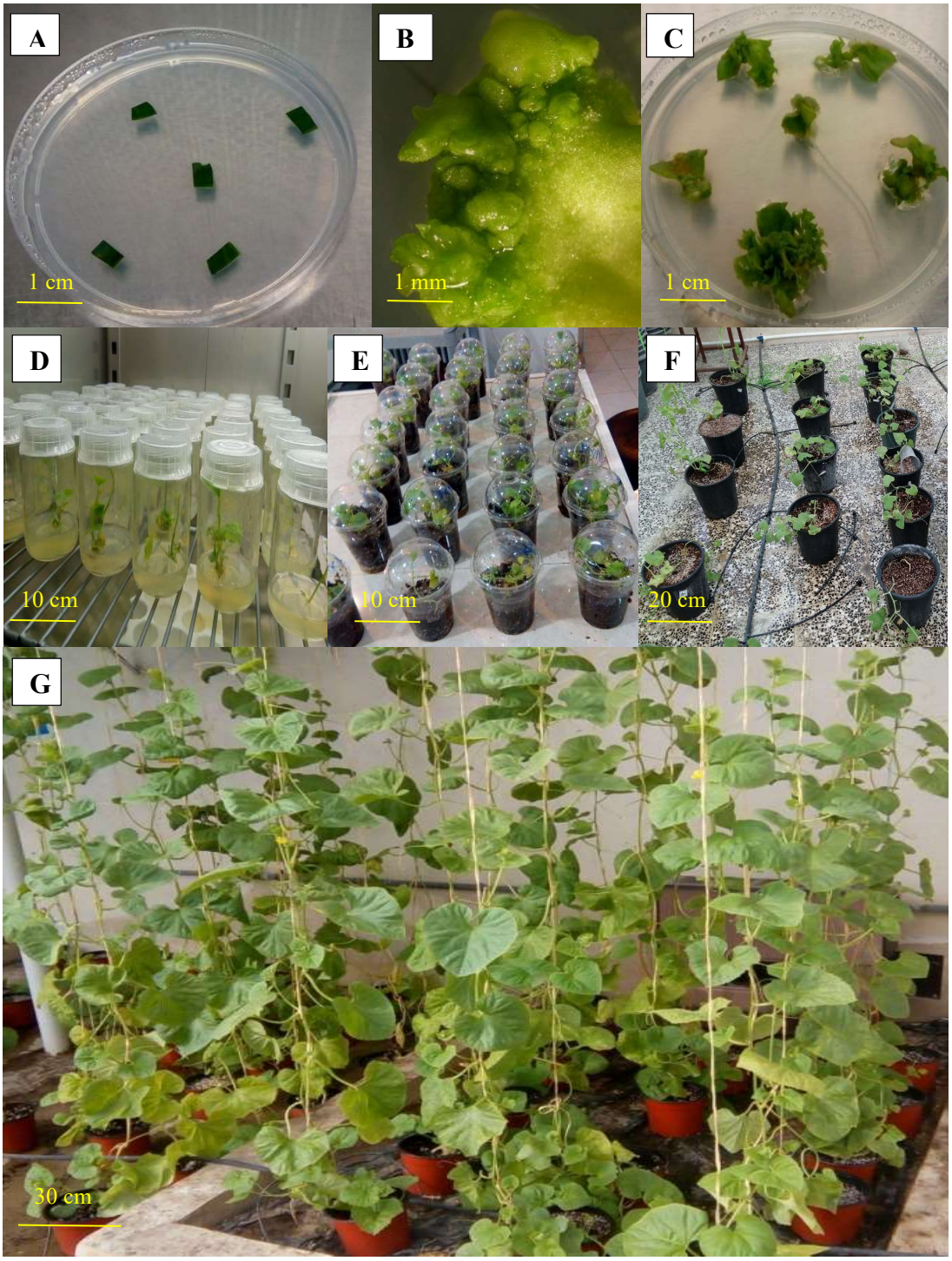

Figure 2. Stages of preparation of explants from 4-day cotyledons, regeneration, rooting and transfer of regenerated melons to jars. A: Cultivation of inoculated explants in regenerative medium, B: Regeneration, C: Shoot formation, D: Transfer of regenerated plants to jars to form roots and complete plants, E: Transfer of tissue culture plants to closed glass containing perlite cocopeat (1: 1), F: Transfer ofthe plants to the greenhouse, G: Adult plants.

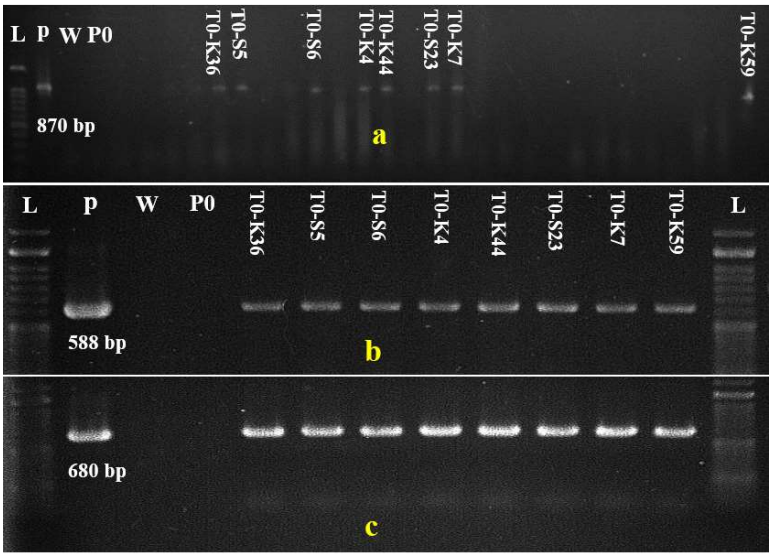

Figure 3. PCR analysis of validated transgenic plants. A) Chitinase (expected size 870 bp); B) PR-1 gene (expected size 588 bp); C) Glucanase (expected size 680 bp). L: DNA marker (1100 bp); T0-K 4, 7, 36, 44, 59 and T0- S5, 6, 23: transformed plants; W: water; P: plasmid (positive control); P0: non-transformed plant (negative control).

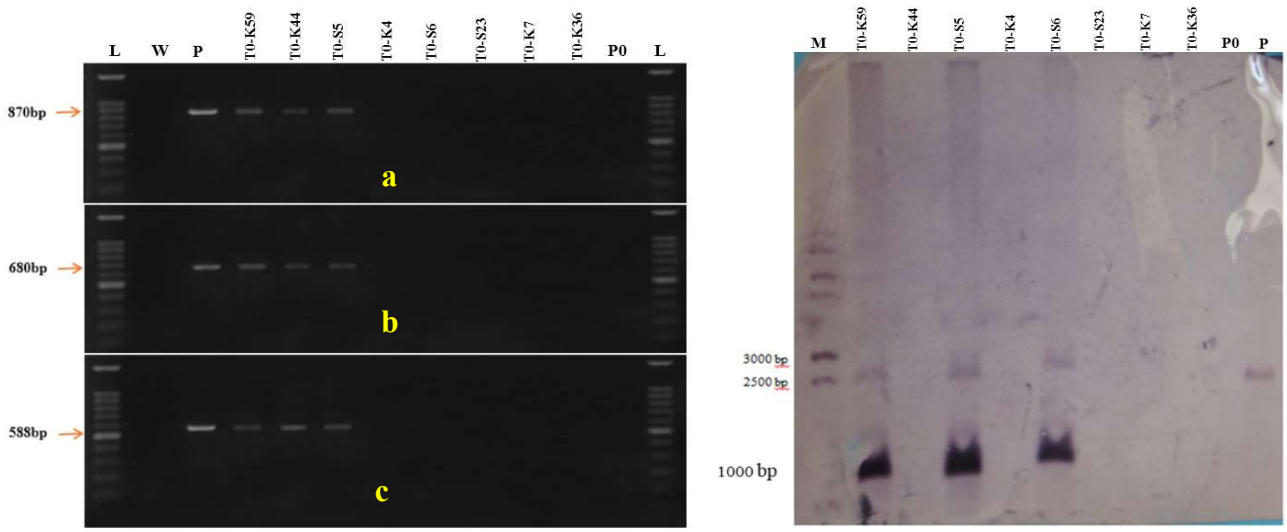

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Figure 4. A: RT-PCR analysis of 8 transgenic plant selected. a) Chitinase (expected size 870 bp); b) Glucanase (expected size 680 bp); c) PR-1 gene (expected size 588 bp). L: DNA marker (1100 bp marked); T0-K4, K7, K36, K44, K59 and T0- S5, S6, S23: PCR putative transgenic lines; W: water; P: plasmid (positive control); P0: non-transformed plant (negative control). B: Southern blotting analysis of DNA isolated from leaves of transformed melon lines. M, 1.0 kb plus DNA ladder (Gibco BRL); lane 2 to 9, *Eco*RI digested DNA from PCR putative transgenic lines: T0-K59, K44, S5, K4, S6, S23, K7, K36. lane N, *Eco*RI digested DNA from untransformed plant, lane P, *Eco*RI digested DNA from plasmid.

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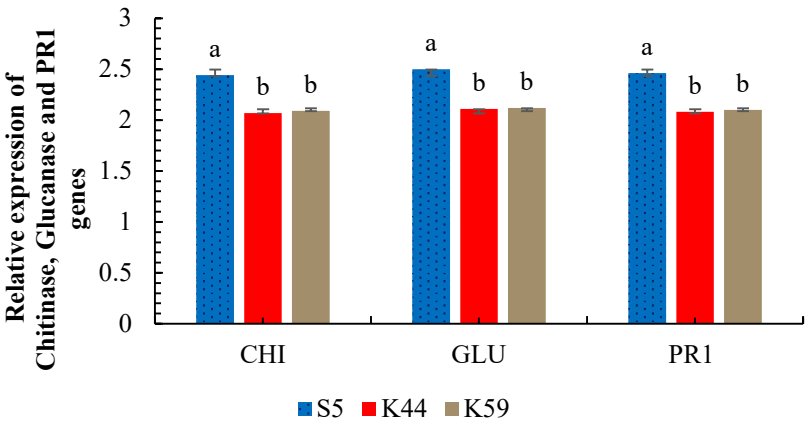

Figure 5. Relative expression of CHI, GLU and PR1 genes.

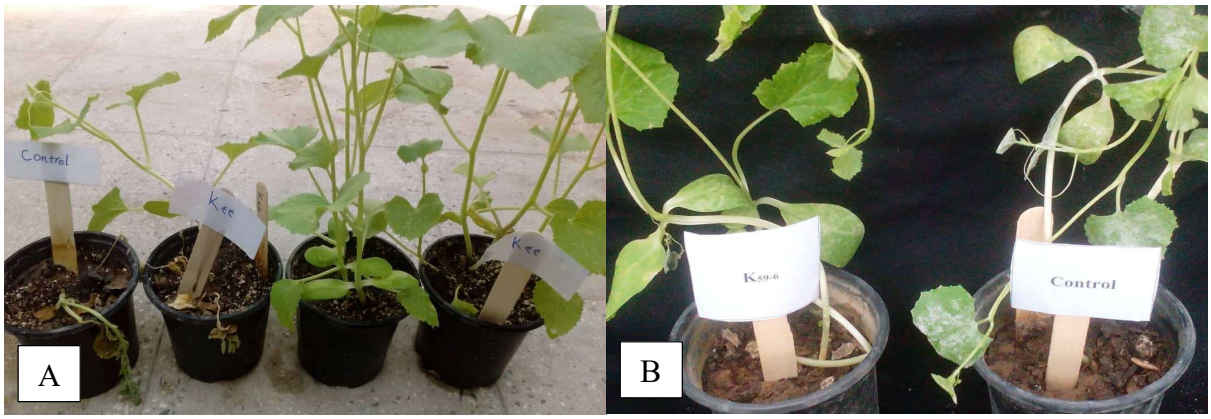

Figure 6. bioassay test. A: Resistance evaluation of transgenic T0 and T1 melon lines (K44) and non-transgenic (control) lines to FOM. B: Resistance evaluation of T1 transgenic melon line (K59-6) to powdery mildew fungus.