INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
17–0893/2018/20–3–481–485
DOI: 10.17957/IJAB/15.0502
http://www.fspublishers.org

Full Length Article

Stability of Antifungal Protein Chitinase (*AFPCHI*) Gene in the T_1 Generations of Transgenic Melon Plants

Ismail Bezirganoglu
Department of Molecular Biology and Genetics, Erzurum Technical University, Erzurum, Turkey
*For correspondence: i.bezirganoglu@hotmail.com; ismail.bezirganoglu@erzurum.edu.tr

Abstract

Melon is an important target for crop development by biotechnological tools. We describe the efficient and reproducible production of stable integration and expression transgenic melon plants after plant genetic transformation derived from mature seeds. To determine stability of the antifungal protein-chitinase gene in the T_1 generation of transgenic melon plants, transformed by co-cultivation with *Agrobacterium* mediated genetic transformation; the T_1, seeds of transgenic melon plants were germinated in Murashige and Skoog (MS) medium with kanamycin added at 150 mg/L. T_1 plants were tested by molecular analysis including expression analysis. Genetic stability of the transgenic plants was assessed by RT-PCR and western blot analysis. The results showed that all transgenic plants were phenotypically normal, fully fertile and no irregular responses in growth and other traits were observed. This suggests that both the antifungal protein-chitinase genes are present and expressed in the T_1 generation. © 2018 Friends Science Publishers

Keywords: Genetic transformation; Chitinase; Transgenic melon plants; Transgene stability and expression

Introduction

Cucumis melo L. (Melon) is an important vegetable species and it is an attractive fruit that is widely grown in many areas (Almodovar *et al*., 2017). Melon is sensitive to a number of fungal disease, pest and environmental stress requiring disease-resistant cultivars, extensively chemical pesticides uses and field rotation (Wu *et al*., 2010). Improvement of resistant cultivars and methods of chemical have been effectively used for plant protection against various fungal pathogens. However, in terms of fungal pathogen the desired level of germplasm is lacking in the cultivated melon and its wild relatives. Traditional breeding has been instrumental in improving fungal resistance cultivars in various crops. Recently, numerous studies, have been report to develop new cultivars and increase to important agronomic traits including flavor, sweetness of melon species but this process requires a lot of time and effort using long-established hybridization techniques (Taheri and Tarighi, 2010; Bezirganoglu *et al*., 2013). Genetic transformation is one of the most used techniques to obtain for disease resistant cultivars in different crop species. Genetic transfer provides an enormous scope for widening is known as genetic engineering. Genetic engineering has found wide application of the various crop species through stable transfer of different traits from different organisms (Bezirganoglu *et al*., 2013). *Rhizoctonia solani*, which lives in the soil and is the most widely distributed to fields worldwide. This soil fungus nearly destroyed crop production and causes large losses in crop yield most geographical areas. The symptom indication of this disease is a falling of the lower leaves (Ntui *et al*., 2010). Applications of chemical are ineffective in disease control. Soil rotation has provided excellent control in many areas but is mostly very expensive. However, the improvement and use of resistant cultivars have nearly eliminated the concern over this disease (Paul *et al*., 2011). Study of the gene inheritance and expression is of great importance in gene transformation programs and in fact, the outcome of research relies on it. Chitinases play an important role studies showed that the antifungal activities of chitinases increased in many plants infected by fungal pathogens (Metraux and Boller, 1986; Schlumbaum *et al*., 1986; Punja and Zhang, 1993). Expression of genes coding for PR proteins improves disease resistance in transgenic plants (Cornelissen and Melchers, 1993). The *Agrobacterium tumefaciens* system has been used to transfer disease-related genes into plants. These genes also include chitinase, glucanase, and antifungal protein. Plant chitinases are defense proteins widely distributed all over the plant kingdom. The antifungal property of chitinase enzyme provides good protection against fungal disease (Chen *et al*., 2009). The transformation of foreign chitinase gene in plants to increase levels of chitinase is a possible way to improve plant resistance against pathogens (Nitzche, 1983). Transgenic plants overexpressing are expected to have increased resistance against fungal pathogen. *In vitro* antifungal assays of tobacco class I chitinase and β-1, 3-

To cite this paper: Bezirganoglu, I., 2018. Stability of antifungal protein-chitinase (*AFPCHI*) gene in the T_1 generations of transgenic melon plants. *Int. J. Agric. Biol*., 20: 481–485

glucanase, used singly or combined, showed that the two enzymes acted synergistically (Sela-Buurlage *et al*., 1993).

In this report, we studied the genetic stability and inheritance pattern of the AFPCHI gene in the T1 generations of the melon plants by expression analysis.

Materials and Methods

Progeny Analysis

Transgenic seeds of the T_1 generation of *AFPCHI*-transgenic and T_0 transgenic melon were obtained in from the self-crossed of primary-transformants. The non-transgenic melon *Cucumis melo* L. Silver light, a parental plant of *AFPCHI* was used as control. Seeds of T_1 plants were surface-disinfected and were germinated on 150 µg/mL kanamycin containing MS medium. After 10 days of incubation, Observations were taken by counting the germinated seeds and recording the ratios between germinated and non-germinated seeds (Fig. 1).

RT-PCR

To analyze the level of gene expression, total RNA samples were prepared from leaves of three transgenic lines after inoculated with *R. solani.* Total RNA was isolated from leaf using promega RNA kit. After measuring RNA quality, RT-PCR analysis was carried out using 100 ng total RNA employing *afp/Chi* primers cDNA was performed in 5 µL volume consisting of 1 µL 0.1 mM DTT, 1 µL 10 mM dNTPs, 2 µL 0.1 mM 5X first strand buffer, 1 µL reverse transcriptase. The cycling conditions for the RT-PCR reaction was as follows: 94°C 5 min, 58°C 30 s, 72°C 50 s, for 30 cycles. PCR primers used for amplification of AFPCHI gene expression were described in genomic PCR section. Individual PCR products were separated via electrophoresis on 1.2% (w/v) agarose gel with EtBr.

Western Blot Analysis

Melon leaf tissues (0.2 g) were ground in a mortar using liquid nitrogen and the tissue powder was transferred to a eppendorf tube protein extraction buffer was then added to the eppendorf tube and mixed. After centrifuge for 10 min at 12.000 rpm at 4°C the supernatant was transferred to an eppendorf tube. Protein quantity of sample extracts was determined using bovine serum albumin (BSA) as a standard, according to the method of Bradford (1976). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was carried out in15% polyacrilamide gel (Laemmli, 1970). A medium range protein marker was used as a molecular mass standard. After electrophoresis proteins were transferred to a PVDF membrane and immunostained. The primary anti-CHI antibody wash was done with the anti rabbit IgG antibody, was detected on nitroblue tetrazolium and 5-bromo, 4-chloro, 3 indoly phosphate (BCIP/NBT).

Antifungal Assay

Rhizoctania solani was placed on the potato dextrose agar (PDA) medium. The center of plate was inoculated with a small plug of actively growing *R. solani* and incubated at room temperature. After one week Erlenmeyer flask (250 mL) containing 100 g potato was inoculated with one piece of *R. solani* (Fig. 2).

Results

Progeny Analysis

After *AFPCHI* transgenic melon was self-crossed, kanamycin studies have proved that the *NPTII* gene was linked closely with *AFPCHI* gene in T-DNA of transgenic melon plants. T_1 transgenic plants were selected based on regeneration under kanamycin-containing MS plates, and kanamycin resistance was observed 80–90% among transgenic melon plants. There were morphological differences between control and transgenic plants (Fig. 3). It was found that this relationship still stayed. The result display that the *AFPCHI* gene was closely inherited and co-expressed with the selectable gene *NPTII*. To identify the presence of *AFPCHI* gene in the *NPTII* positive plants of the progenies of *AFPCHI* transgenic melon plants. Expression analysis was conducted with specific DNA primers and CHI antibody.

Antifungal Assay

T_1 three transgenic plants (B4, D1, M1), control were carried out to determine the resistance against *R solani.* Plants from control (WT) and offspring of transgenic melon B4, D1, and M1 were infected with pathogen. After 1 week, control plants displayed high disease symptoms than transgenic plants. After 2 week, a marked difference between B4, D1 and M1 and control plants was observed. Finaly one month later, control plants showed necrosis and wilt in greenhouse (Fig. 4).

Expression of the Transgenic Plants

Three T_1 transgenic melon plants B4, D1 and M1 with different resistant to *R. solani* were selected to determinate the *AFP* and *CHI* gene expression levels by RT-PCR analysis. Our results revealed large amounts of variation in gene expression levels among individual transgenic lines and no accumulation of *AFPCHI* were observed transgenic plants. After infection, the transcripts of AFP (D1) were accumulated in a higher level; however, the transcript of B4, M1 revealed no expression change (Fig. 5).

After infection, the transcripts of *CHI* (D1, B4 and M1) were accumulated in a higher level; the transcript of three revealed no significant change between each other (Fig. 5).

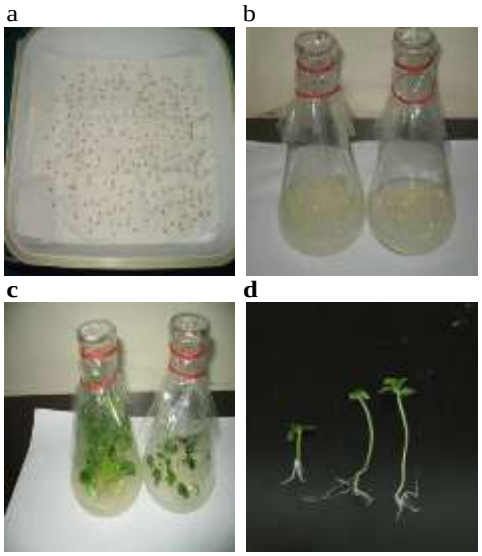

Fig. 1: (a) T_1 transgenic melon seeds, (b) T_1 transgenic melon seeds were placed on MS medium with containing 150 mg/ml kanamycin, (c) Compared with control and T_1 transgenic melon seeds, shoots-rooted on the kanamycin medium after 15 days, (d) T_1 transgenic melon plants

Fig. 2: (a) *Rhizoctonia solani* was placed on the potato dextrose agar (PDA) medium, the center of plate was inoculated with a small plug actively growing *Rhizoctonia solani* and incubated at room temperature, (b) After one week, transfer one piece PDA culture with *Rhizoctonia solani* into 100 g potato medium, (c) 1 kg steril soil was inoculated with *Rhizoctonia solani*

Western Blot analysis showed the expression of *CHI* in T_1 plants, M1 showing that low level of expression was obtained conversely B4 and D1 plants, which display higher level of transgene expression. As observed for plants of T_1 confirmed same result showing that higher expression level of *CHI* gene is directly related to disease resistant symptoms (Fig. 6).

Discussion

Improvement of genetically engineered to fungal pathogen is an important and economically sustainable method that might provide and application of crop improvement. In melon, as in many plant species, conventional breeding is alternative approach to develop crop improvement. However, efficient production of transgenic plants through *Agrobacterium*-mediated transformation is limited in melon

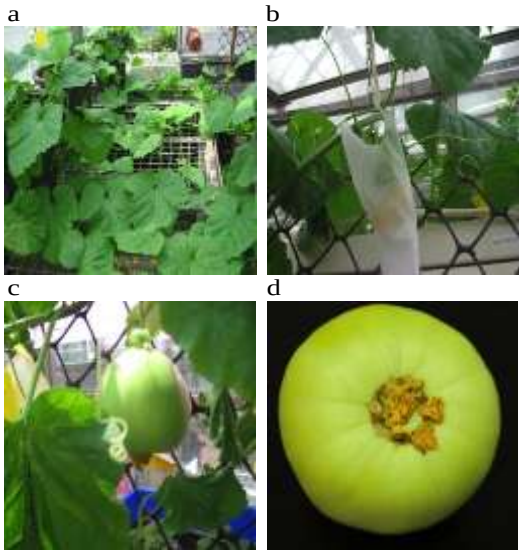

Fig. 3: (a) Transgenic melon plants 30 days in greenhouse, (b) Transgenic melon plants self crossed in greenhouse, (c) Immature transgenic melon fruit, (d) Transgenic melon fruit is harvested within 2 months

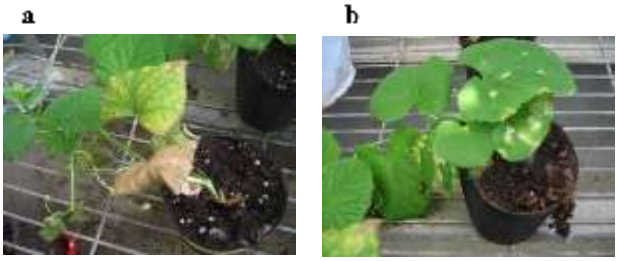

Fig. 4: Resistance of transgenic plants expressing AFPCHI gene against R solani a. Control melon plant of melon 1 month days after infection. b. D1 resistant transgenic plant represents 1 month after infection

due to the lacking number of established varieties as well as the difficulty in producing hybrids between cultivars (Rhimi *et al*., 2006). The use of micropropagation and genetic engineering is an important way of conserving the genetic variability of crop species and has become extensively popular for breeding purpose. There have been limited studies on *Agrobacterium tumefaciens*-mediated transformation and plant regeneration of several melon species via cotyledon, callus and leaf explants (Moreno *et al*., 1985; Kathal *et al*., 1988; Dirks and Van Buggenum, 1989). Consequently, successful plant regeneration in melon has been reported using cotyledon (Ntui *et al*., 2010) and leaf (Nora *et al*., 2001) explants, and *Agrobacterium*-mediated genetic transformation, in particular, is now possible to transform a wide range of plants including important horticulture crops, such as melon species. Antifungal proteins appear to be involved in either constitutive or induced resistance against pathogenic fungal

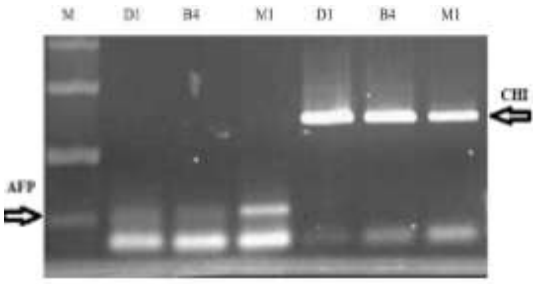

Fig. 5: RT-PCR analysis of T1 three transgenic plants. M. molecular marker; B4. M1. D1; first generation transgenic plants afp, Chi. Arrows show the amplified fragments of the introduced genes

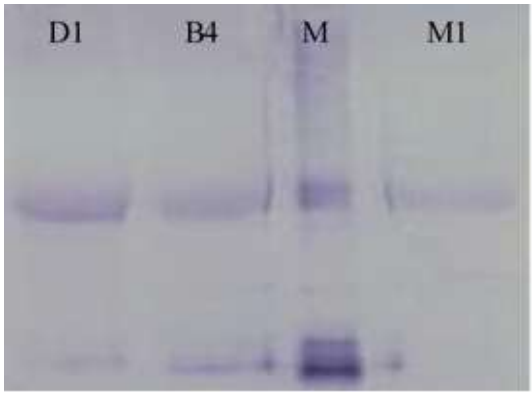

Fig. 6: Western blot analysis of T1 three transgenic plants. B4. M1. D1; first generation transgenic plants

attack. These proteins from different sources exhibited *in-vitro* antifungal activity by inhibiting hyphal and fungal growth as reported by many scientists (Rajasekaran *et al*., 2005; Hermanova *et al*., 2006). Antifungal proteins consist of pathogenic-related (PR) protein and ribosome inactivating proteins (RIPs). RIPs are plant enzymes with 28S rRNA *N*-glycosidase activity, which, depending on their specificity, can inactivate specific or foreign ribosomes, thereby shutting down protein synthesis. Pathogenic-related proteins represent a wide variety of proteins with different biochemical and enzymatic mechanisms. Genes encoding pathogenesis-related proteins, when over-expressed in crop plants, have been shown to enhance resistance to many fungal diseases (Jayraj *et al*., 2004; Bezirganoglu and Uysal, 2017). Many members of this protein group enhancing resistance of economically important plants are to express PR proteins or to improve and over-express their endogenous forms. Of these proteins, chitinase is considered as one important enzyme and prime candidate for further of plant defense against fungal disease. Most genes related to molecular defensive mechanisms are expressed by plants as responses against pathogen attacks. Graham *et al.* (1990) stated that chitinase is true pathogenesis-related (PR) protein and is the mostly studied defense protein in plants. Muzzarelli (1977) also explained that the hydrolysis of chitin is catalyzed by chitinase β-1,4 linkages of the N-acetyl-D-glucosamine (GlcNAc) linkage polymer chitin to oligomers and monomers. Techkarnjanaruk and Goodman (1999) further indicated that the hydrolysis of chitin polymers is also enabled via exo and endochitinase activity. The endochitinase, an enzyme that is usually referred to as chitinase, is responsible for the hydrolysis of oligomers and dimers from chitin. Dahiya *et al*. (2006) further showed that monomers which are products of degradation by chitobiase are formed from actions of endochitinase. Chitinases naturally occur in many organisms such as bacteria, nematodes, fungi, insects and higher plants (Clarke and Tracey, 1956). Plant and bacteria chitinases are the most significant in antifungal activity. For example, most of the plant chitinases is often contain lysozyme activity and function as endochitinases which produces multimers of N-acetlyglucosamine. Exochitinase displays progressive action starting at the non-reducing end of the polymer, and chitobiase hydrolyzes chitobiase to monomers of N-acetlyglucosamine.

Our results showed strong evidence that the chitinase gene was stably integrated and expressed into the melon genome and transmitted to the next generation. Total RNA isolated from the three transgenic plants were used to analysis the change of gene expression level of *AFPCHI*. RT-PCR analysis indicated that the transgene in response of soil pathogen infection in all three transgenic lines. We used 35S, a constitutive promoter to induce transgene in response to pathogen infection. We have shown that the 35S promoter has strong systemic activity transgenic plants in response to pathogen infection. This results are similar to by Liu *et al.* (2015) displayed that MYB expression under CaMV35 promoter is involved in the resistance against *Fusarium oxysporum* and *Botrytis cinerea* in roots and leaves. As expected, the observed positive correlation between expression level and degree of disease resistance. RT-PCR analysis of a representative transgenic line (D1) revealed the highest level of AFPCHI gene expression after infection (Fig. 4(5)). In western blot analysis, a high accumulation of Chi was also observed from these lines. Comparison of the signal intensity, although a high accumulation of Chi was observed in the leaves after infection, however, Chi of resistant line (M1) was hardly observed after infection.

Conclusion

The findings of our study showed that the expression of antifungal protein-chitinase gene from transgenic melon plants can effectively improve resistance against to *R. solani* infection by inhibiting the growth and symptoms of lesion in transgenic plants. Our results are also benefit in improving the knowledge and resources available for effective management of soil pathogens can be used for resistance breeding of different melon cultivars using modern breeding techniques or molecular approach.

References

Almodovar, R.C.G., B. Gosalvez, M.A. Aranda and L. Burgos, 2017. Production of transgenic diploid *Cucumis melo* plants. *Plant Cell Tiss. Org. Cult*., 130: 323–333

Bezirganoglu, I. and P. Uysal, 2017. Impact of transgenic AFPCHI (*Cucumis melo* L. Silver Light) fungal resistance melon on soil microbial communities and enzyme activities. *J. Plant Biotechnol.*, 44: 156–163

Bezirganoglu, I., S.Y. Hwang, T.J. Fang and J.F. Shaw, 2013. Transgenic lines of melon (*Cucumis melo* L. var. makuwa cv. 'Silver Light') expressing antifungal protein and chitinase genes exhibit enhanced resistance to fungal pathogens. *Plant Cell Tiss. Org. Cult*., 112: 227–237

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*., 72: 248–254

Chen, S.C., A.R. Liu, F.H. Wang and G.J. Ahammed, 2009. Combined overexpression of chitinase and defensin genes in transgenic tomato enhances resistance to *Botrytis cinerea*. *Afr. J. Biotechnol*., 8: 5182–5188

Clarke, P.H. and M.V. Tracey, 1956. The occurrence of chitinase in some bacteria. *J. Gen. Microbiol*., 14: 188–196

Cornelissen, B.J.C. and L.S. Melchers, 1993. Strategies for control of fungal diseases with transgenic plants. *Plant Physiol*., 101: 709–712

Dahiya, N., R. Tewari and G.R. Hoondal, 2006. Biotechnological aspects of chitinolytic enzymes. A review. *Appl. Microbiol. Biotechnol*., 71: 773–782

Dirks, R. and M. Van Buggenum, 1989. *In vitro* plant regeneration from leaf and cotyledon explants of *Cucumis melo* L. *Plant Cell Rep*., 7: 626–627

Graham, T.L., J.E. Kim and M.Y. Graham, 1990. Role of constitutive isoflavone conjugates in the accumulation of glyceollin in soybean infected with Phytophthora megasperma. *Mol. Plant Microbiol. Interact.*, 3: 157–166

Hermanova, V., J. Barta and V. Curn, 2006. Antifungal plant proteins-classification, characterization and potential applications. *Chemicke Listy*., 100: 495–500

Jayraj, J., A. Anand and S. Muthukrishnan, 2004. *Pathogenesis-related Proteins and their Roles in Resistance to Fungal Pathogen*, pp: 139–178. Food Products Press, New York, USA

Kathal, R., S.P. Bhatnagar and S.S. Bhojwani, 1988. Regeneration of plants from leaf explants of Cucumis melo cv. Pusa Sharbati. *Plant Cell Rep*., 7: 449–451

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227: 680–685

Liu, Z., Y. Luan, J. Li and Y. Yin, 2015. Expression of a tomato MYB gene in transgenic tobacco increases resistance to Fusarium oxysporum and Botrytis cinerea. *Eur. J. Plant Pathol.*, 144: 607–617

Metraux, J.P. and T. Boller, 1986. Local and systemic induction of chitinase in cucumber plant in response to viral, bacterial and fungal infections. *Physiol. Mol. Plant Pathol*., 28: 161–169

Muzzarelli, R., 1977. *Chitin*, pp: 1–309. Pergamon Press, New York, USA

Moreno, V., M. Garcia-Sogo, I. Granell, B. Garcia-Sogo and L.A. Roig, 1985. Plant regeneration from calli of melon (*Cucumis melo* L., cv. Amarillo Oro). *Plant Cell Tiss. Org. Cult*., 5: 139–146

Nitzche, W., 1983. Chitinase as a possible resistance factor for higher plants. *Theor. Genet*., 65: 171–172

Nora, F., J. Peters, L. Lucchetta, M. Schuch, L. Marini, J. Silva and C. Rombaldi, 2001. Melon regeneration and transformation using an apple ACC oxidaxe antisense gene. *Rev. Bras. Agrocienc*., 7: 201–204

Ntui, V.O., R.S. Khan, D.P. Chin, I. Nakamura and M. Mii, 2010. An efficient *Agrobacterium* tumefaciens-mediated genetic transformation of''Egusi'' melon (*Colocynthis citrullus* L.). *Plant Cell Tiss. Org. Cult*., 103: 15–22

Paul, C.Y., D.K. Becker, M.B. Dickman, R.M. Harding, H.K. Khanna and C.L. Dale, 2011. Apoptosis-related genes confer resistance to Fusarium wilt in transgenic 'Lady Finger' bananas. *Plant Biotechnol. J.,* 9: 1141–1148

Punja, Z.K. and Y.Y. Zhang, 1993. Plant chitinases and their roles in resistance to fungal diseases. *J. Nematol*., 25: 526–540

Rajasekaran, K., J.W. Cary, J.M. Jaynes and T.E. Cleveland, 2005. Disease resistance conferred by the expression of a gene encoding a synthetic peptide in transgenic cotton (*Gossypium hirsutum* L.) plants. *J. Plant Biotechnol*., 3: 545–554

Rhimi, A., N.B. Fadhel and M. Boussaid, 2006. Plant regeneration via somatic embryogenesis from *in vitro* tissue culture in two Tunisian Cucumis melo cultivars Maazoun and Beji. *Plant Cell Tiss. Org. Cult*., 84: 239–243

Schlumbaum, A., F. Mauch, U. Vogeli and T. Boller, 1986. Plant chitinases differ in antifungal activity. *Nature*, 324: 365–367

Sela-Buurlage, M.B., A.S. Ponstein, S.A. Bres-Vloemans, L.S. Melchers, P.M.J. Van den Elzen and B.J.C. Cornelissen, 1993. Only specific tobacco (*Nicotiana tabacum*) chitinases and β-1,3-glucanases exhibit antifungal activity. *Plant Physiol*., 101: 857–863

Taheri, P. and S. Tarighi, 2010. Riboflavin induces resistance in rice against Rhizoctonia solani via jasmonate-mediated priming of phenyl propanoid pathway. *J. Plant Physiol.*, 167: 201–208

Techkarnjanaruk, S. and A.E. Goodman, 1999. Multiple genes involved in chitin degradation from the marine bacteria *Pseudoalteromonas* sp. Strain S91. *Microbiology*, 145: 925–934

Wu, H.W., T.A. Yu, J.A.J. Raja, S.J. Christopher, S.L. Wang and S.D. Yeh, 2010. Double-virus resistance of transgenic oriental melon conferred by untranslatable chimeric construct carrying partial coat protein genes of two viruses. *Plant Dis.*, 94: 1341–1347

(Received 10 August 2017; Accepted 26 September 2017)