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Trifluralin herbicide-induced resistance of melon to fusarium wilt involves expression of stress- and defence-related genes

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SUMMARY

To identify genes involved in trifluralin herbicide-induced resistance of melon to *Fusarium oxysporum* f. sp. *melonis*, suppression subtractive hybridization (SSH) and cDNA-amplified fragment-length polymorphism (cDNA-AFLP) were used. A total of 123 clones—60 of which have never been isolated from melon—were isolated, sequenced and annotated. A significant proportion (35%) of the total 123 clones exhibited similarity to genes that have been formerly described as stress- or defence-related. Thirty-two selected clones were subjected to a detailed expression analysis, one-third of which were found to be up-regulated in response to trifluralin treatment and/or fusarium inoculation. The putative roles of seven of these clones in stress are discussed. Furthermore, the expression of four stress-related and up-regulated genes was enhanced when the plants were subjected to salinity stress, suggesting that trifluralin induces a general stress response which protects the plant against fusarium wilt.

INTRODUCTION

Plant resistance to pathogens appears to be activated via a complex set of stress or defence responses. In contrast to monogenic resistance, which appears to result from specific pathogen recognition and a host response against it (Flor, 1971), there are general defence mechanisms that are induced in response to various diseases and stresses (reviewed by Bostock, 2005; da Rocha and Hammerschmidt, 2005). Besides the familiar mechanisms of systemic acquired resistance (SAR) and induced systemic resistance (ISR) (Gozzo, 2003; Kloepper *et al.*, 2004; Vallad and Goodman, 2004), various chemicals have been found to induce plant resistance to pathogens (Andreu *et al.*, 2006; Oostendorp *et al.*, 2001). Herbicides as well as plant growth regulators may alter plant susceptibility to soil-borne diseases, such that they become more resistant to infection by pathogens (Cohen *et al.*, 1987, 1992, 1996; Graham, 2005; Katan and Eshel, 1973). Generally, resistance conferred by exposing plants to sublethal concentrations of herbicide is not attributed to a direct toxicity effect of the herbicide on the pathogens but to the activation of a defence mechanism in the plant (Grinstein *et al.*, 1976, 1984; Katan and Eshel, 1973). Dinitroanilines are herbicides that are widely applied in many agricultural crops; they act by disrupting spindle-apparatus formation in the mitotic sequence, causing plant-growth retardation (Hess, 1989). Among the dinitroanilines, trifluralin has been the most extensively studied. Low concentrations of trifluralin have been reported to reduce disease incidence caused by different pathogens in various crops (Altman and Campbell, 1977; Cohen *et al.*, 1986, 1992, 1996; Eshel and Katan, 1972; Grinstein *et al.*, 1976, 1984; Katan and Eshel, 1973). Pre-treatment of tomato, eggplant, melon and watermelon plants with dinitroaniline herbicides markedly increased their resistance to vascular wilt caused by *Fusarium* and *Verticillium* species (Grinstein *et al.*, 1976, 1984).

The soil-borne pathogen *Fusarium oxysporum* f. sp. *melonis* (FOM) specifically attacks melon plants, causing severe wilt disease. Monogenic resistance genes to various races of this pathogen have been described in melon (Martyn and Gordon, 1996) and recently a gene conferring resistance to races 0 and 1 was cloned (Joobeur *et al.*, 2004). Cohen *et al.* (1986) reported that the dinitroaniline herbicides trifluralin and dinitramine are the most effective at inducing resistance to FOM and suggested that reduction of wilt symptoms is associated with a reduction in ethylene production. In addition, higher glutathione levels following dinitroaniline treatment have been suggested to confer protection against FOM (Bolter *et al.*, 1993).

Gene-expression profiles associated with biotic or abiotic stresses have been extensively studied (e.g. Cheong *et al.*, 2002; Dowd *et al.*, 2004; Kazan *et al.*, 2001; Wan *et al.*, 2002). In addition,

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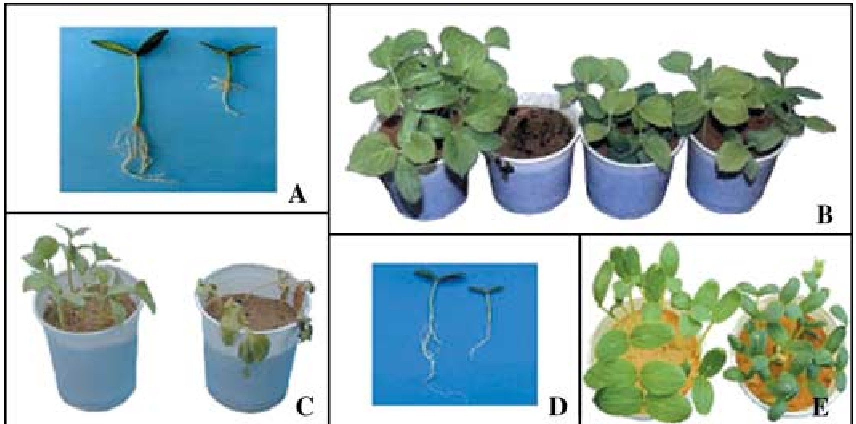

Fig. 1 The effect of trifluralin, NaCl and fusarium (FOM) on melon growth. (A) The stunting effect of trifluralin on melon seedlings: an untreated seedling (left) and a seedling treated with 1 μg/g trifluralin 1 week post-germination (right). (B) Herbicide-induced resistance, 3 weeks post-germination and 2 weeks post-FOM inoculation. Left to right: untreated seedlings, FOM-inoculated seedlings, 1 μg/g trifluralin-treated seedlings and seedlings treated with a combination of 1 μg/g trifluralin and FOM. (C) Wilt caused by FOM, 2 weeks post-germination and 1 week post-FOM inoculation: untreated seedlings (left) and FOM-inoculated seedlings (right). (D,E) The stunting effect of salinity on melon seedlings 4 days post-emergence: (D) an untreated seedling (left) and a seedling irrigated with 100 mM NaCl (right); (E) untreated seedlings (left), and greenish seedlings which were irrigated with 100 mM NaCl (right).

Table 1 Summary of 16 tissue samplings.*

Treatments	Tissue
Untreated (control)	[a]Root + stem
	[b]Cotyledon
FOM	[c]Root + stem
	[d]Cotyledon
Trifluralin/Salinity	[e]Root + stem
	[f]Cotyledon
Trifluralin/Salinity + FOM	[g]Root + stem
	[h]Cotyledon

*Samples a–h were collected on days 3 and 6 following FOM inoculation (16 samples).

cross-talk between defence- and stress-signalling, as well as additional plant responses, has been established (Glombitza *et al.*, 2004; Taylor and McAinsh, 2004). Various differential-screening molecular methods have been applied to compare multiple gene expression during plant defence and stress responses. One of these methods is cDNA-amplified fragment-length polymorphism (cDNA-AFLP) (Bachem *et al.*, 1996; Vos *et al.*, 1995), which has been employed for differentially expressed gene discovery. Another method is suppression subtractive hybridization (SSH), which was specifically designed to compare gene expression in different tissues or at different developmental stages (Diatchenko *et al.*, 1996). Combining the SSH method with a cDNA macroarray provides an efficient study system for the rapid identification of differentially expressed genes (Shi *et al.*, 2005; Zheng *et al.*, 2004).

Even though the physiological and morphological aspects of resistance to FOM disease induced by dinitroaniline have been extensively studied, the genetic components and the molecular mechanistic basis of this resistance have yet to be determined. Here we utilized SSH and cDNA-AFLP for the identification of genes whose expression is associated with resistance to FOM race 2 following trifluralin treatment, and we present our analysis of the expression of these genes following the application of an additional abiotic stress.

RESULTS

Effect of the herbicide trifluralin on seedling development and incidence of fusarium wilt

Melon seedlings were germinated in sandy soil treated with a series of trifluralin concentrations (1, 2, 5 and 10 μg/g) and in untreated soil as a control. A concentration of 1 μg/g trifluralin conferred resistance to FOM, retarded plant growth but did not affect plant viability. This concentration was therefore selected for subsequent trials (Fig. 1A,B). FOM inoculation of trifluralin-treated seedlings did not cause wilting symptoms for at least 1 month post-inoculation, whereas FOM inoculation of control seedlings not treated with the herbicide resulted in first wilt symptoms 7 days post-inoculation (Fig. 1C). For the molecular analysis of differential gene expression, the days 3 and 6 post-inoculation were chosen, day 3 being the mid-point between inoculation and the onset of wilt symptoms and day 6 being 1 day prior to appearance of the first wilt symptoms (Table 1).

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Owing to the delicate nature of the melon seedlings, once wilting symptoms develop, sampling of live tissue is almost impossible.

Effect of irrigation with saline water on melon seedling development and disease incidence

To test the hypothesis that FOM resistance induced by trifluralin is the result of a general response mechanism, we attempted to induce resistance using another abiotic stress, salinity. We predicted that genes up-regulated by trifluralin will also be up-regulated by salinity. A preliminary study comparing NaCl solutions at concentrations of 0, 50, 100 and 150 mM was conducted in order to assess the effect of salinity on the seedlings. Irrigation with 100 mM NaCl resulted in retardation of seedling development, but the plants remained viable. The cotyledons of the treated seedlings were slightly smaller (similar in size to those that had been pretreated with trifluralin) and greener than those of the untreated seedlings (Fig. 1D,E). This concentration was therefore chosen for subsequent analyses. When irrigated with a 100 mM NaCl solution and inoculated with FOM, the seedlings exhibited a 3-day delay in wilt-symptom appearance, in contrast to the stable, long-term resistance induced by trifluralin.

The isolation of differentially expressed genes by combined SSH and macroarray analyses

Four SSH libraries were constructed from the trifluralin-FOM trials in order to isolate genes that are specifically up-regulated following trifluralin treatment combined with FOM inoculation (Table 2). Subtractions were performed reciprocally between seedlings with or without trifluralin and FOM treatments. The seedlings were collected on day 6 post-inoculation. The subtractions were performed separately for the root + stem RNA extracts and for the cotyledon RNA extracts. Arbitrary clones (576) from the four libraries were printed in arrays, in duplicate, on nylon membranes (Fig. 2). The macroarrays were then differentially screened using the four non-cloned subtracted cDNA mixtures as probes (tester and driver populations, see Table 2). A total of 470 clones, with clear, above-background signals, in duplicate, were considered to be positive (Table 3). Of these, 384 were positive following hybridization with the tester cDNA-subtracted population but not with the reciprocal one (driver), and were therefore considered to be differentially expressed (Fig. 2, Table 3). Among the differentially expressed clones of the trifluralin and FOM-treated libraries, 187 out of 206 (91%) were derived from the

Table 2 List of the SSH libraries constructed.

Library designation*	Tester (cDNA synthesized from)	Driver (cDNA synthesized from)
1. Treated root + stem	Trifluralin-treated + FOM-inoculated root + stem	Untreated root + stem
2. Untreated root + stem	Untreated root + stem	Trifluralin-treated + FOM-inoculated root + stem
3. Treated cotyledon	Trifluralin-treated + FOM-inoculated cotyledon	Untreated cotyledon
4. Untreated cotyledon	Untreated cotyledon	Trifluralin-treated + FOM-inoculated cotyledon

*RNA was extracted from tissues sampled on day 6.

A1 B1 A2 B2

Fig. 2 Macroarray hybridizations. Randomly selected clones (576) from the four SSH libraries were spotted in duplicate on two nylon membranes and hybridized with the four cDNA probes described in Table 2: (A1) treated root + stem, (A2) untreated root + stem; (B1) treated cotyledon, (B2) untreated cotyledon. The squares mark clones that were up-regulated in the trifluralin-treated FOM-inoculated subtracted libraries. The circles mark clones that were up-regulated in the untreated subtracted libraries.

Table 3 Differentially expressed clones detected using macroarray membranes.

SSH libraries	No. of clones on macroarrays	cDNA probe*	Origin of positive clones	Positive clones (no.)	Positive clones (%)
Treated root + stem	257	Treated root + stem	Treated root + stem	187	91
			Untreated root + stem	0	0
			Treated and untreated root + stem	8	4
			Others:		
			Treated or untreated cotyledon	11	5
				206	
Untreated root + stem	91	Untreated root + stem	Untreated root + stem	39	59
			Treated root + stem	0	0
			Untreated and treated root + stem	2	3
			Others:		
			Treated or untreated cotyledon	23	38
				64	
Treated cotyledon	192	Treated cotyledon	Treated cotyledon	140	91
			Untreated cotyledon	0	0
			Treated and untreated cotyledon	1	1
			Others:		
			Treated or untreated root + stem	13	8
				154	
Untreated cotyledon	36	Untreated cotyledon	Untreated cotyledon	18	39
			Treated cotyledon	0	0
			Untreated and treated cotyledon	8	17
			Others:		
			Treated or untreated root + stem	20	44
				46	
	576			470	

*cDNA probes were the subtracted cDNA populations generated by subtraction of the driver from the tester populations.

root + stem tissues and 140 out of 154 (91%) from the cotyledon (Table 3).

Expression profiles of selected genes isolated from the SSH libraries

Altogether, 94 clones from the trifluralin and FOM-treated subtracted libraries (Table 2, rows 1 and 3) were sequenced. The sequenced clones were annotated using the predicted peptide sequence (BLASTX). Similarity was considered significant if the expectation value was less than 1e-5 for all but four clones whose similarity was accepted with a higher expectation value. Based on closest homologies, 56 clones were annotated, 21 of which were unigenes, and approximately half of these showed significant sequence similarity to genes that had been previously described as stress- or defence-related (Table 4). Twenty-six clones demonstrated sequence similarity to genes coding for proteins of unknown function or putative proteins, and 12 showed no significant homology to sequences available in the databases. Eighteen clones (unigenes) were chosen for further analysis by Northern blot (Table 4). Six clones were selected based on an expression pattern detected by macroarray and 12 were selected arbitrarily. Four clones exhibited the most distinct trifluralin- and/or FOM-related expression pattern (Table 4: DR974753, DR974840, DR974832, DR974838) and were therefore selected for further characterization as described below. Among the 18 clones tested by Northern blot, seven were root + stem-specific and two were cotyledon-specific (Table 4).

Characterization of the four selected clones

Detailed Northern analysis of the four selected clones demonstrated up-regulation for two of them following FOM inoculation (Fig. 3A,B), while the other two were up-regulated following either trifluralin and/or FOM treatments (Fig. 3C,D). The expression pattern of two of these clones was further verified by real-time PCR analysis, and was similar to the patterns obtained by Northern blot (Fig. 3A-2 and A-3 vs. Fig. 4A). Differences were observed between the expression profiles of the day 3 and day 6 samples (Fig. 3A-1/A-2 and C-1/C-2). In addition, the expression profiles of all four clones were examined following salinity stress (Figs 3 and 4).

Table 4 Similarity information and Northern blot analyses of clones isolated by SSH and cDNA-AFLP.

Accession no.*	Accession number and best similarity	Identities (aa)/ probability/ clone length (bp)	Stress/ defence related refs.†	Northern analyses‡ a b c d e f g h
1. DR974753	AAC06243; Osmotic stress-induced zinc-finger protein	19/47/1.7/171	1	
2. DR974754	AAP34361; Fibre protein Fb14	33/38/1e-15/116		
3. DR974756	XP_481233; Stromal cell-derived factor 2-like protein precursor	57/72/2e-21/215		
4. DR974760	NP_029567; VLN1 (VILLIN 1) actin binding	29/44/3e-08/136		
5. DR974764	No significant similarity found			
6. DR974774	CAD33533; Probable major latex protein	25/45/6e-07/184	2,3	
7. DR974780	CAJ91132; Ketol-acid reductoisomerase	46/91/7e-10/275	4,5	
8. DR974786	P27692; Transcription elongation factor SPT5	15/27/8.6/139		
9. DR974790	NP_566156.2; YrdC family protein	11/15/7.9/132		
10. DR974832	AAB91466; ADP-glucose pyrophosphorylase small subunit	76/87/9e-37/264	6,7	
11. DR974834	AAQ08194; Eukaryotic translation initiation factor 5A	124/135/3e-68/422		
12. DR974838	NP_188029; Glycolate oxidase	27/41/1e-09/134	8,9	
13. DR974839	NP_181996; Protein kinase CK2 regulator	37/51/7e-12/340	10,11	
14. DR974840	CAB43344; 1-Deoxy-D-xylulose-5-phosphate reductoisomerase	49/52/1e-20/158	12,13	
15. DR974794	CAA85388; Acyl-(acyl carrier protein) thioesterase	43/59/2e-18/181		
16. DR974750	ABE84419; CTP synthase-like protein	74/81/3e-24/267		
17. DR974796	CAE02649; Myospryn protein	22/46/6.7/269		
18. DR974820	ABC59515; Photosystem II 22-kDa protein precursor	181/200/3e-84/641	14	
19. DR974849	BAB86927; Glucosyltransferase-9	27/55/0.002/405	15,16,17,18	
20. DR974856	BAA96453; F1-ATPase	29/30/5e-08/382		
21. DR974857	NP_180811; Hydrolase	75/87/2e-32/359	19	
22. DR974867	BAD36524; Putative UDP-glucuronic acid	48/54/2e-20/212		
23. DR974868	CAB61749; Putative water-channel protein	53/56/4e-23/221	20,21	
24. DR974842	CAI11456; Putative glycosyltransferase	95/100/7e53/386	22	
25. DR974843	ABE88499; Hypothetical protein	32/46/2e-13/316		
26. DR974844	XP_466086; Putative multiple stress-responsive zinc-finger	29/37/8e-09/321	23	
27. DR974845	NP_566320; Protein binding/zinc ion binding	73/87/1e-39/367		
28. DR974847	BAA21651; Proteasome subunit alpha type 3	36/40/2e-13/120	24,25	
29. DR974858	BAB64345; EIN3-like protein	87/88/2e-45/328	26,27	

Table 4 *continued.*

Accession no.*	Accession number and best similarity	Identities (aa)/ probability/ clone length (bp)	Stress/ defence related refs.†	Northern analyses‡ a b c d e f g h
30. DR974869	BAD30034; Gibberellin 20-oxidase2	46/71/2e-21/217		
31. DR974860	AAO42190; Palmitoyl protein thioesterase family protein	26/36/7e-08/184		
32. DR974862	AAM61531; Putative SKP1-like protein	22/35/0.27/163		
Ribosomal RNA				

*Accession number: clones 1–18 isolated by SSH and clones 19–32 isolated by cDNA-AFLP.

†References: 1, J.C. Kim *et al.* (1997) Unpublished results; 2, Osmark *et al.* (1998); 3, Stromvik *et al.* (1999); 4, Pillmoor *et al.* (1991); 5, Schloss and Aulabaugh (1990); 6, Ahmadi and Baker (2001); 7, Lafta and Lorenzen (1995); 8, Fedina *et al.* (1994); 9, Xu *et al.* (2001); 10, Gerber *et al.* (2000); 11, Eisenreich *et al.* (2001); 12, Espunya *et al.* (1999); 13, Hans *et al.* (2004); 14, Rorat *et al.* (2001); 15, Fernandez *et al.*, 2004); 16, Fraissinet-Tachet *et al.* (1998); 17, Horvath and Chua (1996); 18, Taguchi *et al.* (2001); 19, Benedetti *et al.* (1998); 20, Luu and Maurel (2005); 21, Suga *et al.* (2002); 22, Poppenberger *et al.* (2003); 23, T. Sasaki, T. Matsumoto and K. Yamamoto (2002), Unpublished results; 24, Becker *et al.* (2000); 25, Ingvardsen and Veierskov (2001); 26, Cohen *et al.* (1986); 27, Waki *et al.* (2001).

‡Northern blot analyses: lanes a–h represent the different treatments: (a) untreated root + stem; (b) untreated cotyledon; (c) FOM-inoculated root + stem; (d) FOM-inoculated cotyledon; (e) trifluralin-treated root + stem; (f) trifluralin-treated cotyledon; (g) trifluralin + FOM-treated root + stem; (h) trifluralin + FOM-treated cotyledon. Northern blots were performed using day 6 samples except for clones 29–32, 24–25, 27–28, 32 which refer to the day 3 samples.

The first clone characterized (DR974753) exhibited similarity to a zinc-finger DNA-binding protein from *Petunia hybrida*. On day 3 post-inoculation, its expression pattern was slightly enhanced in the root + stem samples of all treatments (FOM, trifluralin and their combination) (Fig. 3A-1). However, on day 6 post-inoculation, enhanced expression levels were observed in both samples (root + stem and cotyledon) of the FOM-inoculated seedlings (Figs 3A-2 and 4A). In the salinity trials, the expression of this gene was up-regulated mainly following FOM inoculation (Figs 3A-3 and 4A). This enhanced expression was observed in the root + stem samples only, similar to day 3 of the trifluralin treatment. It should be noted that in the salinity trials, the probe hybridized to three bands with similar intensities, whereas in the trifluralin trials, one of the hybridizing signals was more intense than the others.

The second clone (DR974840) shared sequence similarity with a 1-deoxy-D-xylulose 5-phosphate reductoisomerase previously described in *Arabidopsis thaliana* (Fig. 3B). On day 6 following the trifluralin treatment, higher expression levels of this gene were detected in all the cotyledon samples, with the highest level detected in seedlings inoculated with FOM (Fig. 3B-1). This gene was highly abundant in the SSH cotyledon library (it was cloned and sequenced ten times). In the salinity trials, enhanced expression was observed mainly following inoculation with FOM or irrigation with saline water (Fig. 3B-2).

The expression pattern of a clone with similarity to ADP-glucose-pyrophosphorylase from *Cucumis melo* (DR974832) showed that on day 3 post-inoculation, it was highly expressed mainly in the cotyledon of the combined trifluralin and FOM treatment (Fig. 3C-1). The transcript abundance of this gene was also increased in the samples from the other treatments, albeit to a lesser extent. On day 6, however, the highest expression level was detected in the cotyledon tissue treated with trifluralin without FOM inoculation (Fig. 3C-2). Similarly, in the salinity trials, enhanced expression was detected, mainly following the saline water irrigation treatment (Fig. 3C-3).

The fourth clone (DR974838) was similar to glycolate oxidase from *A. thaliana* and was up-regulated mainly in the root + stem tissues treated with trifluralin or with a combination of FOM and trifluralin. A lower level of expression was also detected in the cotyledon samples (Fig. 3D-1). The expression pattern of this gene in the salinity trials, as determined by real-time PCR, resembled that observed in extracts from the herbicide-treated plants: elevated expression levels were observed mainly in the root + stem samples of the saline-treated samples, with or without FOM inoculation (Fig. 4B).

Expression profile of genes isolated by cDNA-AFLP analysis

An additional technique, cDNA-AFLP, was applied for the identification of genes that were differentially expressed following the above-described treatments (Table 1). A screen of 18 primer combinations (*MSE*I and *Eco*RI, Table 5) was carried out on the 16 samples representing four treatments, two tissues and two sampling days (see Tissue sampling section of the Experimental procedures and Table 1). Twenty-nine fragments that exhibited differential expression profiles between the trifluralin- and FOM-

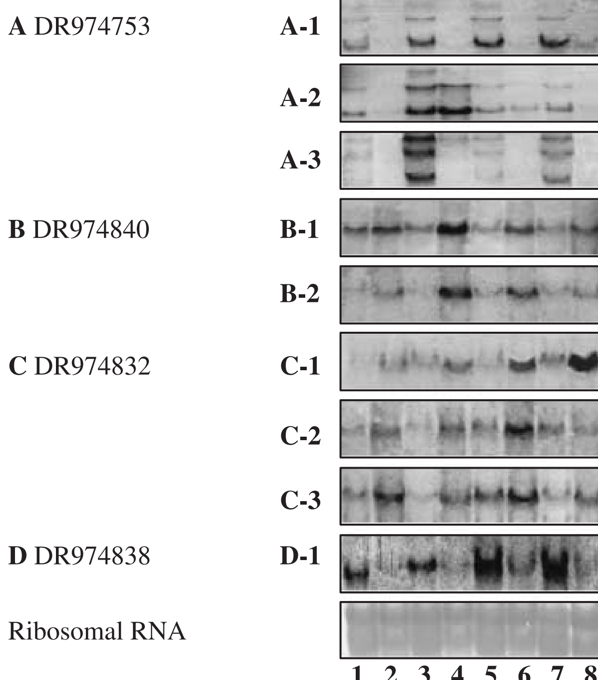


Fig. 3 Northern blot analysis of four clones isolated by SSH. Clone A—isolated from the treated root + stem library. Clones B to D—isolated from the treated cotelydon library. (A-1,2,3) Clone DR974753 analysed in day 3 and day 6 trifluralin-treated samples and in day 6 salinity-treated sample, respectively. (B-1,2) DR974840 analysed in day 6 samples from the trifluralin and salinity trials, respectively. (C-1,2,3) DR974832 analysed in day 3 and day 6 trifluralin-treated samples and in the day 6 salinity-treated sample, respectively. (D-1) DR974838 analysed in the day 6 trifluralin-treated sample. Lanes 1–8 represent the different treatments: (1) untreated root + stem; (2) untreated cotyledon; (3) FOM-inoculated root + stem; (4) FOM-inoculated cotyledon; (5) trifluralin (or salinity)-treated root + stem; (6) trifluralin (or salinity)-treated cotyledon; (7) trifluralin (or salinity) + FOM-treated root + stem; (8) trifluralin (or salinity) + FOM-treated cotyledon.

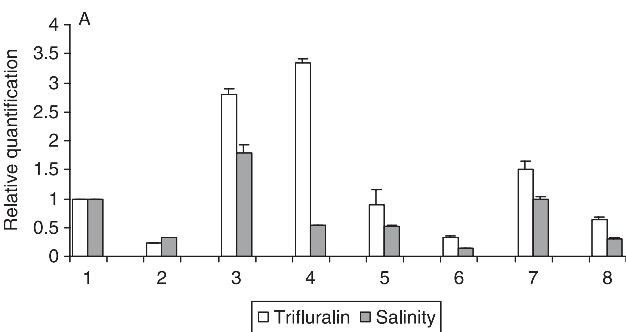
Table 5 Primer combinations used in the cDNA-AFLP analysis.

*Mse*I *Eco*RI	M-CTG	M-CTC	M-CTA	M-CAT	M-CAC	M-CAA
E-AAC		x	x	x		
E-AAG				x	x	
E-ACC	x	x	x	x		
E-ACG	x	x	x			
E-ACT						x
E-AGA						x
E-AGC		x				x
E-AGT		x				
E-ATC				x		

treated seedlings and their untreated counterparts were cloned and sequenced. Twenty of these clones were annotated (the *E*-value was higher than 1e-5 for three of the 20 clones). Of the annotated clones, 18 were unigenes; eight of them (DR974849/-57/-68/-42/-44/-47/-58/-63) had been previously related to stress or defence responses (Table 4). Nine sequences did not show significant similarity to genes available in the databases.

Fourteen of the 29 clones were analysed by Northern blot using day 3 and day 6 samples (Table 4). Genes with enhanced expression levels following the various treatments in the root + stem (Table 4, DR974856/-42), cotyledon (Table 4, DR974844/-60) or both (Table 4, DR974849/-57/-47) were identified. Genes with specific expression in either root + stem (Table 4, DR974843) or cotyledon (Table 4, DR974845/-62) that were not affected by the various treatments were also observed (Table 4, DR974867/-68/-58/-69).

Three of the clones were noticeably differentially expressed: DR974857 and DR974860 in response to FOM inoculation and DR974844 in response to trifluralin and FOM inoculation. These clones showed sequence similarity to hydrolase, palmitol thioesterase and zinc-finger protein, respectively.



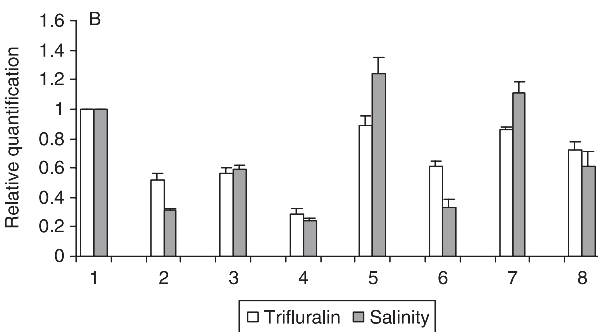


Fig. 4 Quantitative real-time PCR analysis of two clones isolated by SSH. (A) DR974753 and (B) DR974838 analysed in the day 6 samples of the trifluralin and salinity trials. Expression levels of the two clones were normalized with respect to the internal control cyclophilin and are plotted relative to the expression from the untreated root sample. Data bars represent the mean of at least four replicates ± SE. Lanes 1–8 represent the different treatments: (1) untreated root + stem; (2) untreated cotyledon; (3) FOM-inoculated root + stem; (4) FOM-inoculated cotyledon; (5) trifluralin (or salinity)-treated root + stem; (6) trifluralin (or salinity)-treated cotyledon; (7) trifluralin (or salinity) + FOM-treated root + stem; (8) trifluralin (or salinity) + FOM-treated cotyledon.

Sequence accession numbers

The new clones isolated by SSH and cDNA-AFLP have been deposited in the EMBL Nucleotide Sequence Database under accession numbers DR974747 to DR974869.

DISCUSSION

Plant survival depends, in many instances, on the ability to respond to biotic and abiotic stresses (Dombrowski, 2003; Genoud and Metraux, 1999; Rizhsky *et al*., 2002). In this study, plants pretreated with a low concentration of the herbicide trifluralin (generating an abiotic stress) exhibited induced resistance to the biotic stress imposed by FOM. The screening for genes involved in this herbicide-induced resistance system on the basis of their altered expression patterns was described. We were able to identify genes exhibiting increased expression in the presence of trifluralin and determined that they were also up-regulated by another abiotic stress—salinity. As a first step in determining the molecular basis of the induced resistance response in melon plants, SSH and cDNA-AFLP techniques were used to identify the potential genetic elements involved, on the basis of differential gene expression. SSH is a powerful technique that has been successfully applied in the identification of differentially expressed plant genes (Beyer *et al*., 2001; Fernandez *et al*., 2004; Gepstein *et al*., 2003; Xiong *et al*., 2001). The main advantage of SSH is that it allows the detection of low-abundance, differentially expressed cDNAs, in contrast to other differential screening methods. The cDNA-AFLP approach is another notable method which has been previously used for the isolation of differentially expressed genes (Breyne *et al*., 2003; Kemp *et al*., 2005; Santaella *et al*., 2004). Combining the methods seemed to be a rational means of increasing the number of differentially expressed genes identified, as well as of cross-validating some of the obtained results (e.g. the zinc-finger genes isolated by both techniques; discussed below).

A total of 123 clones were isolated by the SSH and cDNA-AFLP techniques and sequenced. A significant proportion of these genes (50%) had not been previously isolated from melon, as determined by comparison to the GenBank and Melon EST databases (http://melon.bti.cornell.edu). Based on sequence similarities, about 35% of the annotated clones exhibited homology to defence- or stress-response genes. The rest were classified as belonging to different functional categories, such as metabolism and regulation. The fact that a large proportion of the annotated clones were correlated with stress or defence responses confirms the efficiency of these approaches for the isolation of uniquely expressed genes. Using similar approaches, a large number of relevant differentially expressed genes have been detected in other studies (Fernandez *et al*., 2004; Gepstein *et al*., 2003; Van der Biezen *et al*., 2000; Xiong *et al*., 2001). About 35% of the total 123 clones coded for undefined genes. The relatively large number of undefined clones can be attributed to the short average lengths of the clones obtained by these techniques, due to the cDNA-restriction step in both methods (Diatchenko *et al*., 1996; Vos *et al*., 1995).

Most of the clones with high similarity to genes related to stress or defence responses were subjected to Northern blot analysis, leading to the identification of genes that were up-regulated in response to trifluralin treatment combined with FOM inoculation. Of the 18 clones isolated by SSH five were clearly up-regulated while three were slightly up-regulated (Table 4, DR974753/-832/-838/-839/-840 and DR974754/-786/-796, respectively). Of the 14 clones isolated by cDNA-AFLP three showed enhanced expression (Table 4, DR974857/-44/-60) while three were only slightly up-regulated (Table 4, DR974856/-42/-43).

It should be noted that several of the genes verified by Northern blot analysis were also expressed in the untreated plants. A possible explanation is that pre-inoculation with FOM required seedling removal, washing and cutting (performed for all treatments, including the control). These manipulations might have imposed stress, potentially resulting in increased expression of stress-related genes in the untreated plants. Notably, several genes exhibited an expression profile that was unique to a specific tissue, such as the cotyledon or combined root + stem tissues.

Four of the genes isolated by SSH were exceptional in their expression patterns following the various treatments. To determine whether this was a unique response to the trifluralin treatment or a general response to abiotic stress, their expression profiles were also tested in response to salinity stress. The conditions chosen for the salinity trials (100 mM NaCl) were based on previous reports (Chartzoulakis, 1994; Sivritepe *et al*., 1999), as well as preliminary testing in this study.

Plant genes that exhibit altered expression during infection have been previously described for a number of phytopathogenic fungi and oomycetes (e.g. Beyer *et al*., 2001; Dowd *et al*., 2004; McGrath *et al*., 2005). Here, two genes exhibited enhanced expression following FOM inoculation. The first, DR974753, showed the closest similarity to a zinc-finger protein, previously demonstrated to be associated with osmotic stress (*Nicotiana tabacum*) and cold stress (*Datisca glomerat*) (J.C. Kim *et al.* (1997), Unpublished results; P.J. Reinhoud *et al.* (1998), Unpublished results). This gene was slightly up-regulated in all three treatments on day 3 post-inoculation, whereas 3 days later it was markedly up-regulated in the FOM-inoculated seedlings; less intensive expression was observed in the trifluralin-treated FOM-inoculated seedlings (Figs 3A-2 and 4A). We assume that the plant defence or stress response is progressive, increasing in accordance with FOM growth and development. Thus, stronger gene expression, first noticed in the root + stem sample on day 3, was also observed in the cotyledon sample on day 6, 1 day prior to the appearance of wilt symptoms. In the salinity experiments,

enhanced expression appeared in the root + stem tissue of the FOM-inoculated seedlings (Figs 3A-3 and 4A). Owing to possible temporal differences in the respective physiological effects of salinity and trifluralin, the sampling on day 6 in the salinity experiment may have preceded this gene's expression in the cotyledon.

Therefore, we concluded that the main expression of this gene is induced in response to the FOM fungus, whereas the exposure to herbicide or salinity stresses resulted in reduced gene expression. In addition, the presence of multiple hybridization signals in both the trifluralin and the salinity trials (Fig. 3A) may be indicative of the expression of additional structurally related genes in the tissue, or perhaps of the production of more than one transcript by this gene. Using the cDNA-AFLP technique, additional zinc-finger-like genes were isolated (DR974844/-51/-64). One of these (DR974844) was tested by Northern blot and showed a distinctive expression pattern following the trifluralin and the combined treatments (further discussed below and Table 4).

The second selected clone, DR974840, showed the closest similarity to 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) enzyme. This enzyme catalyses the transformation of 1-deoxy-D-xylulose 5-phosphate (DOXP) into 2-*C*-methyl-D-erythritol 4-phosphate (MEP) in the non-mevalonate DOXP/MEP pathway. Walter *et al*. (2000) and Hans *et al*. (2004) reported that root colonization of wheat, maize, rice and barley by the arbuscular mycorrhizal fungal symbiont *Glomus intraradices* involves strong induction of the transcript levels of the pivotal enzymes of the MEP pathway, 1-deoxy-D-xylulose 5-phosphate synthase (DXS) and DXR. Those authors were the first to demonstrate that the plastidic MEP pathway is active in plant roots and that a fungus can induce its expression. In our study, DXR was also expressed mostly following inoculation with the FOM fungus in both the herbicide and the salinity experiments (Fig. 3B-1 and B-2). However, its expression was greater in the cotyledon tissue than in the root + stem sample.

Two additional genes whose expression was up-regulated following trifluralin treatment were DR974832, which is similar to an ADP-glucose pyrophosphorylase (ADP-Gppase) small subunit, and DR974838, a putative glycolate oxidase. Previous studies have reported that the activity of ADP-Gppase is reduced in potato tubers exposed to heat stress (Lafta and Lorenzen, 1995), and in wheat grain under osmotic stress (Ahmadi and Baker, 2001). Here, ADP-Gppase was found to be up-regulated and not down-regulated on day 3 and expressed mainly in the combined trifluralin and FOM treatment (Fig. 3C-1). By contrast, higher expression levels were detected mostly in the cotyledon tissue of the trifluralin- or salinity-treated seedlings on day 6 (Fig. 3C-2 and C-3).

Glycolate oxidase is a key enzyme in the photo-respiratory process and is known to be involved in different abiotic stresses, including drought (Rizhsky *et al*., 2002), oxidative (Camp *et al*., 1998) and osmotic (Xu *et al*., 2001) stresses. In the peroxisome, glycolate reacts with O_2 to produce glyoxylate and H_2O_2, a reaction catalysed by the glycolate oxidase enzyme. Moreover, Taler *et al*. (2004) suggested that glycolate oxidase provides resistance to downy mildew in the wild melon line PI 124111F. This disease resistance was associated with the enhanced activity of two glyoxylate aminotransferases. Presumably, these aminotransferases used a large amount of glyoxylate, which accelerates the activity of the upstream glycolate oxidase, which in turn produces a large quantity of H_2O_2. In our case, enhanced expression of this gene was demonstrated in the trifluralin-treated (Fig. 3D-1) and salinity-treated (Fig. 4B) seedlings, with or without FOM inoculation. The effect of trifluralin in the real-time PCR experiment was not clear-cut. This may stem from the fact that the RNA used in the Northern blot and in the real-time PCR experiments originated from different biological experiments. Moreover, the control samples may have been affected by stress due to their handling, as already discussed.

Our data from the herbicide and salinity systems further support the assumption that the glycolate oxidase gene is strongly associated with stress responses.

Three clones isolated by cDNA-AFLP demonstrated enhanced expression patterns following FOM inoculation or trifluralin treatment. The two clones that showed significant up-regulation following FOM treatment, DR974860 and DR974857, may have been reflecting the melon FOM-susceptible response. DR974860 exhibited similarity to palmitol thioesterase while DR974857 exhibited similarity to hydrolase from Arabidopsis. This latter gene has been previously shown to be associated with stress or defence responses (Benedetti *et al*., 1998). The third clone, DR974844, showed similarity to a zinc-finger protein, previously associated with multiple stress response (T. Sasaki, T. Matsumoto and K. Yamamoto (2002), Unpublished results). This clone was up-regulated mainly in the cotyledon in response to the trifluralin and combined treatments. An additional zinc-finger protein (DR974753) was identified by the SSH method as already discussed. Overall, we did not identify identical clones simultaneously by both methods.

An ethylene-insensitive (*EIN*3) gene was among the genes cloned and tested by cDNA-AFLP. The EIN3-like protein is a transcription factor that participates in the signal-transduction pathway of ethylene (Chen *et al*., 2005; Potuschak *et al*., 2003). Ethylene regulates diverse plant processes, including the response to stress and pathogens. Susceptibility or resistance to pathogens following treatment with ethylene depends on specific host–pathogen interactions (Broekaert *et al*., 2006). In an early study of induced resistance in melon, an increase in ethylene production was detected in response to FOM inoculation; this increase was suppressed in plants treated by herbicides (Cohen *et al*., 1986). Here, the expression pattern of the *EIN*3-like clone was similar in all tested treatments, including the control. This

observation is in accordance with the current concept that *EIN*3 genes are not ethylene-induced; rather, they are regulated at a post-transcriptional level (Chen *et al.*, 2005; Mao *et al.*, 2006; Potuschak *et al.*, 2003).

In conclusion, this work provides the first report of an untargeted approach to analysing a transcriptional response during trifluralin-induced resistance against fusarium wilt in melon. The described response does not cover the complete melon transcriptome; however, based on our results, the enhanced expression of the seven genes we focused on in the induced-resistance system and salinity-stress trials emphasizes a strong link between defence responses and stress sensing at the transcriptional level. Future investigations of the functions of these genes will promote an understanding of the induced-resistance system.

EXPERIMENTAL PROCEDURES

Trifluralin pretreatment

Muskmelon plants (*Cucumis melo* L. cv. 'En Dor', Zeraim Gedera, Gedera, Israel) susceptible to *Fusarium oxysporum* Schlecht f. sp. *melonis* Synder and Hansen pathogen were used in all of the experiments. Seeds of this cultivar were sown in soil treated with trifluralin (Treflan; α,α,α-trifluoro-2,6-dinitro-N,N-dipropyl-p-toluidine, produced by Agan Chemicals, Ashdod, Israel) at a concentration of 1 μg/g active ingredient/soil, and in herbicide-free soil as a control. One day after emergence (about a week after germination), the seedlings were uprooted and washed thoroughly to remove adhering soil and traces of herbicide before inoculation with FOM fungus.

Plants irrigated with saline water

Melon seedlings were irrigated with saline water at a concentration of 100 mM NaCl for 4 days after emergence, and with tap water as a control. One week after germination, half of the control (tap water-irrigated) and the saline-water-treated seedlings were inoculated with FOM, re-transplanted in sandy soil and sampled as described below.

Fusarium inoculation

The FOM fungus originated from a wilting melon plant and was maintained on potato dextrose agar (Difco Laboratories, Detroit, MI) at 27 °C. The seedling roots were pruned slightly to allow better penetration of the pathogen conidia and then inoculated by dipping for 2 min in a 10^6 conidia/mL suspension of FOM race 2. The inoculated seedlings were transplanted into 250-cm^3 plastic pots containing herbicide-free sandy soil. In all experiments, seedlings were grown in a growth chamber at a temperature of 25 °C with 12-h lighting at a light intensity of 90 μE/ms.

Tissue sampling

Plant material for the herbicide and salinity experiments was sampled 3 and 6 days after inoculation from four treatment groups: (1) seedlings germinated in herbicide- or salinity-free soil without inoculation; (2) seedlings germinated in herbicide- or salinity-free soil with FOM inoculation; (3) seedlings germinated in trifluralin-treated soil or irrigated with saline water, without inoculation; and (4) seedlings germinated in trifluralin-treated soil or irrigated with saline water, with FOM inoculation (Table 1). Every plant was separated into root + stem tissues and cotyledon tissue and a total of 16 different samples (Table 1) from each experiment were immediately placed in liquid nitrogen and kept at –80 °C until processing. Four independent trifluralin experiments were performed, sampled and used in the various molecular analyses. Two experiments were performed and sampled for the salinity analyses.

Total-RNA extraction and mRNA isolation

Total RNA was extracted as described by La Claire and Herrin (1997) with slight modifications. Quantity and quality of total RNA were assessed by spectrophotometry and electrophoresis on 1% agarose gels. The PolyATract mRNA Isolation System (Promega Corp., Madison, WI) was used for mRNA isolation. Poly(A)$^+$ mRNA was purified from 1 mg of total RNA according to the manufacturer's recommendations.

cDNA-AFLP analysis

The first-strand cDNA synthesis was performed using 10 μL of reaction mix containing 200 U Superscript II reverse transcriptase enzyme (Invitrogen, Life Technologies, Carlsbad, CA), 2 μg poly(A)$^+$ RNA, 1 μL oligo (dT) primer (10 μM) and 10 mM dNTPs (10 mM each of dATP, dTTP, dGTP and dCTP). For the second-strand cDNA synthesis, an enzyme cocktail containing 6 U/μL DNA polymerase I (New England Biolabs Inc., Ontario, Canada), 0.25 U/μL RNase H (MBI Fermentas, Ontario, Canada) and 1.2 U/μL *Escherichia coli* DNA ligase (New England Biolabs) was used. The following reagents were added to the first-strand cDNA synthesis reaction: 16 μL 5× second-strand buffer (500 mM KCl, 50 mM ammonium sulfate, 25 mM $MgCl_2$, 0.75 mM β-NAD, 100 mM Tris-HCl pH 7.5, 0.25 mg/mL BSA), 1.6 μL dNTP mix, 4 μL of enzyme cocktail and ddH_2O to a final volume of 80 μL. The reaction mix was incubated for 2 h at 16 °C and 2 μL (6 U) of T4 DNA polymerase (New England Biolabs) was added for the next 30 min. Extraction with chloroform : isoamyl alcohol (24 : 1) was performed twice and precipitation was then done with ethanol. The final product was dissolved in 20 μL of ddH_2O and 2 μL were taken for quantitative and qualitative analyses. The cDNA-AFLP technique was performed using AFLP analysis System I and accompanying

instruction manual purchased from Gibco BRL® (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's recommendations. The selective PCR product (20 μL) mixed with 12 μL of loading buffer (98% formamide, 10 mM EDTA, 0.016% bromophenol blue and 0.016% xylene cyanol) was heated for 5 min at 95 °C and immediately cooled on ice. Each sample (4 μL) was loaded on a 6% sequencing polyacrylamide gel and electrophoresis was carried out in 1× TRIZMA® Base at 60 W for ~2.5 h. After electrophoresis, the gel was dried on a slab gel dryer model SE 1160 (Hoefer Scientific Instruments, San Francisco, CA) exposed to Kodak Biomax MR film (Kodak, Rochester, NY) and analysed on the basis of the intensity of the radioactive signal.

Isolation and cloning of cDNA fragments

cDNA bands which exhibited differential expression were excised from the gel, placed in 100 μL sterile water and heated for 2 h at 65 °C. After centrifugation, PCR amplification was performed with 3 μL of the supernatant and the selective reaction primer combinations used initially. The PCR cycles were as follows: initial denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 15 s and 56 °C for 30 s, and a final extension at 72 °C for 1 min. PCR products were separated on a 1.2% agarose gel, purified by GFX™ PCR DNA and Gel Band Purification kit (Amersham Biosciences Ltd, Little Chalfont, UK) and cloned into a pGEM-T EASY vector system (Promega Corp.), according to the manufacturer's instructions.

Constructing SSH libraries

Suppression subtractive hybridization (SSH) was performed using the PCR Select cDNA subtraction kit (Clontech Laboratories Inc., Palo Alto, CA) according to the manufacturer's instructions with slight modifications. cDNA synthesis was conducted according to the manufacturer's recommendations with 2 μg of driver and 2 μg of tester mRNA. Subtractions between the trifluralin- and FOM-treated vs. untreated seedlings in direct and reciprocal procedures were performed for each tissue (root + stem vs. cotyledon) separately (Table 2). A total of four SSH libraries were constructed, two from root + stem tissues and two from the cotyledon tissues, using day 6 samples. Secondary PCR products were cloned with the pGEM-T EASY Vector System kit. Positive clones were picked and transferred to 96-well plates, containing 100 μL Luria broth (LB) liquid medium. Altogether, 696 clones were stored at −80 °C.

Differential screening

A total of 576 clones from the subtraction libraries were printed on Genescreen plus hybridization transfer membranes (PerkinElmer Life Science Inc., Boston, MA). PCR amplification of the cDNA clones was performed using primers NPR1 and NPR2 (Clontech PCR-Select kit). A PCR product from each clone was spotted in a duplicate pattern on two replica array membranes (denatured filters: 0.5 N NaOH, 1.5 M NaCl), using the membrane cDNA array services at the Israeli National Knowledge Center for Gene Therapy of the Israeli Ministry of Science, the Goldyne Savad Institute of Gene Therapy, Hadassah University Hospital, Jerusalem, Israel. The filters were then neutralized in 1 M NaCl, 0.5 M Tris, pH 7.5, cross-linked by UV and dried. Each array membrane was divided into 96 squares, each containing six duplicated clones (Fig. 2). The subtracted cDNA from the SSH libraries was radioactively labelled with ^{33}P using a Rediprime TM II Prime Labeling System kit (Amersham Biosciences) and used as probes on the macroarray membranes (Fig. 2). Hybridizations were performed twice and the resulting radioactive signals were compared for redundancy. Clones that exhibited differential expression were subjected to sequencing.

Sequence analysis

DNA was sequenced (Macrogen Inc., Seoul, Korea) and similarity searches were performed with BLASTX programs at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Hits were considered significant if the expectation value was less than 1e-5.

Northern blot analysis

A series of Northern blots were run with total RNA extracted from the trifluralin-FOM and salinity-FOM experiments (Table 1). Total RNA samples (25 μg) were separated on formaldehyde-agarose gels and transferred to Hybond-N+ membranes (Amersham Biosciences) (Sambrook and Russell, 2001). DNA probes were labelled using the Rediprime TM II Prime Labeling System kit. Hybridization and washing were carried out as described by Church and Gilbert (1984). The radioactively labelled membranes were exposed to Kodak Biomax MS film and kept at −70 °C for several days, according to the intensity of the radioactive signal. Northern blot analyses (depicted in Fig. 3) were repeated at least twice for each clone. One-third of the Northern blots presented in Table 4 were repeated at least twice.

Quantitative real-time PCR analysis

First-strand cDNA was synthesized from 1 μg of total RNA by Reverse-iT™ 1st Strand Synthesis kit (Abgene®'s Inc., Epsom, UK) according to the manufacturer's instructions. A 2-μL aliquot of cDNA was used for each real-time PCR, performed with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster, CA). Amplifications were conducted using the qPCR™

Table 6 Primers used in quantitative real-time PCR.

Clone	Forward primer	Reverse primer
DR974753	CACTATGGCCTTACAAGCTCTCAA	ATCAAGGGGCGGAAGGATCG
DR974838	TATAATTGGAAGAAACGGACGGA	AATGGTTCTGTCCTACTCCTCGA
Cyclophilin	GATGGAGCTCTACGCCGATGTC	CCTCCCTGGCACATGAAATTAG

Mastermix kit for SYBR® green I dye (Eurogentec Inc., Seraing, Belgium) according to the manufacturer's instructions. Thermocycling was initiated by 15 min of incubation at 94 °C, followed by 40 cycles (90 °C, 15 s; 60 °C, 1 min) with a single fluorescent reading taken at the end of each cycle. Each reaction was completed with a melting-curve analysis to confirm the specificity of amplification. The housekeeping gene cyclophilin was used as a reference (Table 6). Real-time PCR for each primer combination was performed in duplicate at least twice. C_t values were determined by the ABI Prism 7000 SDS software and exported into MS Excel workbook (Microsoft Inc., Redmond, WA) for statistical analysis. Real-time efficiencies (*E*) were calculated from the slopes of standard curves for each gene [$E = 10^{(-1/\text{slope})}$]. The relative expression ratio (*R*) was calculated according to Pfaffl (2001) as shown in Eq. (1), while the control was the untreated root sample.

$$R = \frac{(E_{\text{target}})^{\Delta CP_{\text{target}}(\text{control}-\text{sample})}}{(E_{\text{ref}})^{\Delta CP_{\text{ref}}(\text{control}-\text{sample})}}$$

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