Differential gene expression, induced by salicylic acid and *Fusarium oxysporum* f. sp. *lycopersici* infection, in tomato

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Abstract – The objective of this work was to determine the transcript profile of tomato plants (*Lycopersicon esculentum* Mill.), during *Fusarium oxysporum* f. sp. *lycopersici* infection and after foliar application of salicylic acid. The suppression subtractive hybridization (SSH) technique was used to generate a cDNA library enriched for transcripts differentially expressed. A total of 307 clones was identified in two subtractive libraries, which allowed the isolation of several defense-related genes that play roles in different mechanisms of plant resistance to phytopathogens. Genes with unknown roles were also isolated from the two libraries, which indicates the possibility of identifying new genes not yet reported in studies of stress/defense response. The SSH technique is effective for identification of resistance genes activated by salicylic acid and *F. oxysporum* f. sp. *lycopersici* infection. Not only the application of this technique enables a cost effective isolation of differentially expressed sequences, but also it allows the identification of novel sequences in tomato from a relative small number of sequences.

Index terms: *Lycopersicon esculentum*, cDNA libraries, complementary DNA, gene expression, salicylic acids, suppression subtractive hybridization.

Expressão diferencial de genes induzida por ácido salicílico e por *Fusarium oxysporum* f. sp. *lycopersici*, em tomateiro

Resumo – O objetivo deste trabalho foi determinar o perfil de transcritos em plantas de tomate (*Lycopersicon esculentum* Mill.), durante a infecção com *Fusarium oxysporum* f. sp. *lycopersici* e após a aplicação foliar de ácido salicílico. A técnica de hibridização subtrativa por supressão (SSH) foi utilizada para gerar uma biblioteca de cDNA enriquecida por transcritos diferencialmente expressos. Foram identificados 307 clones, em duas bibliotecas subtrativas, que permitiram o isolamento de diversos genes de defesa com função em diferentes processos relacionados à resistência vegetal contra patógenos. Também foram isolados, nas duas bibliotecas, genes com função desconhecida, o que indica a possibilidade de identificação de novos genes que ainda não tenham sido relatados em estudos anteriores de resposta a estresses e defesa, em plantas. A técnica SSH é eficiente em identificar genes de resistência, ativados pelo ácido salicílico e pela infecção com *Fusarium oxysporum* f. sp. *lycopersici*. A aplicação dessa técnica não apenas possibilita isolar seqüências diferencialmente expressas, a baixo custo, como também permite a identificação de novas seqüências, em tomate, a partir de um número relativamente pequeno de seqüências.

Termos para indexação: *Lycopersicon esculentum*, bibliotecas de cDNA, DNA complementar, expressão gênica, ácido salicílico, hibridização subtrativa por supressão.

Introduction

Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *lycopersici*, is one of the main diseases of tomato (*Lycopersicon esculentum* Mill.). Three physiological races (1, 2 and 3) have been identified. Resistant cultivars are the best wilt control strategy, since the pathogen remains in the soil for some decades and chemical control is ineffective (Reis et al., 2004).

Plant resistance to diseases is associated with a number of defense responses, activated by the host after contact with the pathogens. Resistance is often expressed as a hypersensitive reaction, which results in localized cell death at the pathogen penetration site. Other resistance responses may include structural alterations, accumulation of reactive oxygen species, synthesis of secondary metabolites and production

of a wide variety of defense molecules, such as antimicrobial proteins (Park et al., 2003; Shah, 2003; Ros et al., 2004). Defense responses are induced in pathogenesis-related situations, observed after the application of chemical substances, which simulate the effect of pathogen infection (e.g. salicylic acid – SA), which triggers the production of PR proteins (pathogenesis related proteins) (Van Loon, 1985).

The identification of host genes, involved in defense responses, is important to understand plant resistance mechanisms against phytopathogens (Thatcher et al., 2005; Desender et al., 2007). Suppression subtractive hybridization (SSH) is a promising technique for the isolation of genes expressed in plants subjected to biotic and abiotic stress, because it increases the relative abundance of some cDNA species (Diatchenko et al., 1996; Soares, 1997). The advantages of this technique include the detection of low-abundant and differentially expressed transcripts through suppression of the abundant ones, and the capacity of isolating genes with no previous knowledge of their sequence or identity (Diatchenko et al., 1999; Moody, 2001).

The objective of this work was to determine the transcript profile in tomato plants (*Lycopersicon esculentum* Mill.), during *Fusarium oxysporum* f. sp. *lycopersici* infection, and after the foliar application of the signaling molecules of salicylic acid.

Materials and Methods

Tomato seeds of the cultivar BHR, resistant to *F. oxysporum* f. sp. *lycopersici*, were germinated aseptically on MS culture medium (0.8% w/v agar), with the pH adjusted to 5.8±0.2, photoperiod of 16 hours of light and 8 hours of dark, and temperature at 25±2ºC. Plants were kept in these conditions for three weeks after germination.

Three-week old plants were, then, sprayed with 5 mM sodium salicylate (Sigma, USA) dissolved in autoclaved distilled water. Untreated control plants were sprayed with autoclaved distilled water. Leaves of ten plants were harvested 24 hours after the treatment, mixed and used for total RNA extraction. Mahalingam et al. (2003) identified differentially expressed genes in *Arabidopsis* using leaf tissue 24 hours after exposure to salicylic acid to build their SSH library. However, gene expression alterations, in response to soil pathogens, are usually studied in root tissues, after a pathogen germination and penetration period (Benhamou et al., 1990; Divon et al., 2005; Olivain et al., 2006). In order to capture a wide spectrum of differentially expressed genes, leaf and root tissues were collected after the treatment and pooled before RNA extraction.

For inoculation, *Fusarium oxysporum* f. sp. *lycopersici* (race 2) conidiospores were collected from potato dextrose agar (PDA) plates by flooding a 2-week old culture with a solution of 0.01% (v/v) Tween 20 and rubbing the surface. The spore suspension was filtered through glass wool to remove mycelial fragments. The spore concentration was determined using a hemocytometer. The plants were infected through the root cut method (Santos, 1997) for 10 min. Control plants were treated with autoclaved distilled water. After inoculation, the plants were transferred to new tubes containing MS liquid medium, and the roots were harvested 72 hours after, mixed and used for total RNA extraction.

For total RNA extraction, the leaves from SA treated plants and roots from *Fusarium* infected plants were used with Trizol (100 mg mL^{-1}). RNA yield was 50 µg per 100 mg of plant tissue. Complementary DNA was obtained through SMART PCR cDNA Synthesis Kit.

Suppression subtractive hybridization was performed with the PCR-select cDNA subtraction kit. Treated and control samples were processed simultaneously to reduce false positives. Complementary DNA, prepared from the treated samples, was used as "tester", and that from the control sample as "driver", for the forward subtraction carried out to isolate fragments corresponding to genes whose expression level was increased following the treatments. The PCR-based enrichment of differentially expressed sequences depends on the number of tester molecules with adaptors ligated to their ends. When the fraction of tester cDNA with the adaptors was less than 25%, the ligations were repeated. It was designed a plant-specific translation elongation factor 1α (EF-1α) primer to test the ligation efficiency, as recommended by the manufacturer.

A EF-1α gene fragment of approximately 300 bp was amplified with: EF-1α R: 5'GACAATCAAGCACTGGAGCA3' and EF-1α F: 5'GATGCTACCACCCCCAAGTA3', using the adaptor-ligated cDNA as template. Samples from the EF-1α gene fragment amplifications, using the subtracted and unsubtracted cDNA pools, were analyzed after 15, 20, 25 and 30 cycles of PCR

(Pokalsky et al., 1989). Primers used to amplify regions without an *Rsa*I site were designed for one stress-induced gene, the pathogen-inducible PR4 gene: *Chi3* F: 5’GTTTCCAGGTTTTGGTACTGCTGGT3’ and *Chi3* R: 5’CCACAATACCTCCTGTAAAATCCA A3’; these were used to test the subtraction efficiency of the corresponding libraries before cloning.

Cloned PCR products, in the vector T/A PCR2.1 TOPO were, then, transformed with DH5α competent cells to produce all the subtracted libraries (forward SSH), according to manufacturer’s instructions. Cell colonies were transferred to LB liquid medium containing 100 μg mL^{-1} kanamycin, and were cultured for 16 hours. Plasmid DNA was extracted by the alkaline lysis mini-preparation method (Sambrook & Russell, 2001). Recombinant plasmids were identified, by restriction analysis, and subjected to DNA sequencing.

The efficiency of subtraction was evaluated by PCR amplification of one of several differentially expressed genes, and of a housekeeping gene – the one for translation elongation factor 1α (EF-1α). To test enrichment for differentially expressed genes, the PR4 gene (*Chi3*) was amplified for the biotic stress.

Nucleotide sequences of each insert were determined in an automatic DNA sequencer (ABI PRISM 377), with the Big Dye Terminator kit. Conventional M13-forward and M13-reverse primers were used to determine DNA sequences. Obtained sequences were compared with the ones in the databanks of: the National Center for Biotechnology Information, NCBI (http://www.ncbi.nlm.nih.gov/blast), using the program BlastX (Altschul et al., 1997); the Institute for Genomic Research, TIGR (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/Blast/index.cgi); and the DNA Data Bank of Japan, DDBJ (http://www.ddbj.nig.ac.jp/search/blast). Sequences were aligned with the software ClustalW (http://www.ebi.ac.uk) (Thompson et al., 1994).

Results and Discussion

Two cDNA libraries were constructed through SSH technique, one with genes induced by salicylic acid (SA) e another with genes induced by *F. oxysporum* f. sp. *lycopersici* (FO) infection, as shown in Table 1.

If subtraction is efficient, transcripts of housekeeping genes should be reduced, while those of differentially expressed genes should be substantially enriched in the population of cDNA fragments used for library construction. Figure 1 shows that the EF-1α fragment is barely detectable, even after 30 cycles of amplification in the subtracted sample, while it is clearly detectable in the unsubtracted sample after 20 cycles. To test enrichment for differentially expressed genes, the PR4 gene (*Chi3*), for the biotic stress, was tested and showed strong amplification in the subtracted samples, after 20 cycles of PCR whereas, in the unsubtracted samples, the PCR product was seen only after 5 additional cycles (Figure 1). Subtraction was efficient, because the expression of EF-1α transcripts was reduced, while the differentially expressed PR4 gene was substantially enriched in the cDNA fragment population used in the SSH library construction. On the basis of the number of PCR cycles required for equal amplification of the corresponding PCR products, from the subtracted and unsubtracted cDNA samples, it was estimated that the subtracted libraries were 64-fold enriched for differentially expressed genes. One of the main advantages of SSH is that it normalizes the cDNA abundance, so that cDNAs encoded by genes that are expressed infrequently, but nonetheless differentially, can be identified readily (Diatchenko et al., 1996; Kürkcüoglu et al., 2006).

After SSH, 307 cDNA clones were obtained, being 143 clones from the SA library and 164 from the FO library (Table 1). Inserted length ranged from 300 to 1,100 bp, in the two libraries. Clone similarity was considered at E-values lower than 10^{-5} in the different data banks analyzed, being, thus, significant

Table 1. Treatments used to obtain suppression subtractive hybridization (SSH) cDNA libraries.

Treatment	Control	Time (hours)	Library identification	Total clones	Number of clones per category
5 mM salicylic acid	H_2O	24	Tomato – SA	143	46 unknown roles 34 defense/stress related 24 general metabolism 39 no similarity
F. oxysporum f.sp. *lycopersici* (10^7 mL^{-1})	H_2O	72	Tomato – FO	164	49 unknown roles 42 defense/stress related 44 general metabolism 29 no similarity

(Fernández et al., 2003). In the SA library, 39 clones encoded proteins with no similarity, 46 were similar to putative proteins with unknown roles, 34 clones represented genes involved in defense mechanisms and 24 clones were associated to cell maintenance and plant development. From the 164 clones identified in the FO library, 29 showed no similarity with data bank sequences, 49 were analogous to hypothetical proteins, 42 corresponded to genes involved in defense mechanisms, and 44 were related to cell maintenance and plant development. Results are summarized in Table 1.

The identification of host genes involved in defense responses is important to unravel mechanisms of plant resistance against phytopathogens. Suppression subtractive hybridization combines suppression PCR with subtraction and normalization steps in a single reaction, increasing, therefore, the possibility of identifying low expressed genes (Diatchenko et al., 1999; Moody, 2001). Genes with unknown roles were identified in SA and FO libraries, which indicates the possibility of identifying new genes that have not yet been reported in previous studies of stress/defense response. Similar results were observed in SSH libraries from *Arabidopsis*, potato and tomato, in biotic stress conditions (Mahalingam et al., 2003; Ros et al., 2004; Ouyang et al., 2007).

Figures 2 and 3 show that a higher number of genes involved in defense mechanisms were found in the FO library. The group of unknown genes represented 32% of the SA library and 29% of the FO library. One of the most interesting characteristics of SSH was achieved with the description of relatively rare cDNA fragments, obtained by simultaneous normalization and subtraction steps. The normalization step equalizes the abundance of mRNA within the target population, and the subtraction step excludes sequences that are common to the two populations being compared. However, the SSH methodology allows the detection of low copy transcripts, and it allows the identification of unknown genes (Diatchenko et al., 1996, 1999; Moody, 2001).

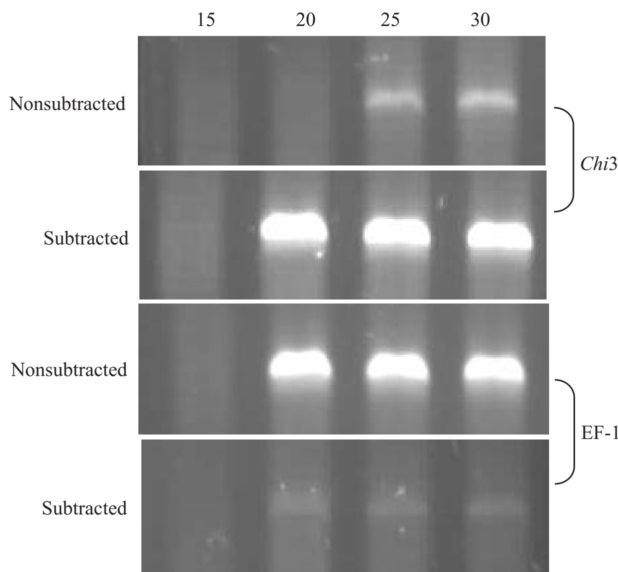

Figure 1. Subtraction efficiency analysis using PCR. Tester cDNA was prepared from total RNA from plants infected with *F. oxysporum* f. sp. *lycopersici*, and driver cDNA was prepared from water treated controls. Subtracted and nonsubtracted cDNA pools were amplified with the primer that amplifies *Chi3* induced by pathogen infection or amplifies the gene EF-1α expressed constitutively. Numbers above are aliquots taken after 15, 20, 25 and 30 PCR cycles. Amplified products were analyzed in 1.5% agarose gel.

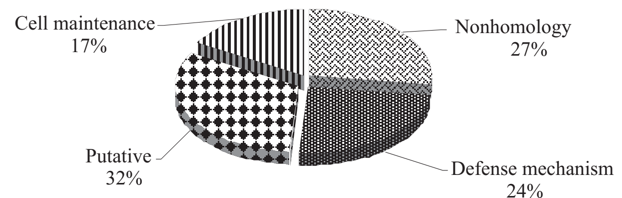

Figure 2. Genes activated in the tomato cultivar BHR treated with salicylic acid (SA). Genes were isolated by suppression subtractive hybridization, from tomato mRNAs, 24 hours after SA treatment.

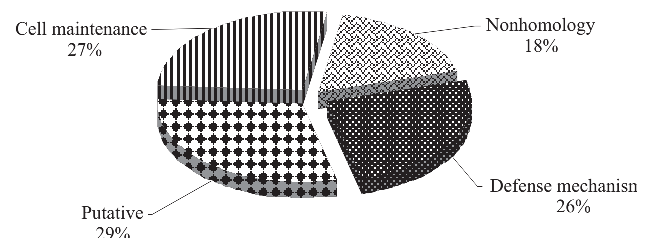

Figure 3. Genes expressed in the tomato cultivar BHR after *F. oxysporum* f. sp. *lycopersici* inoculation. Genes were isolated by suppression subtractive hybridization from tomato mRNAs 72 hours after the inoculation of 107 spores mL^{-1}.

The amount of genes involved in each process related to plant resistance, activated in both SSH libraries, are shown in Figure 4. A higher number of genes related to signal perception and transduction, encoding antimicrobial proteins related to oxidative stress and cell death, was observed in the FO library. However, the number of genes related to the synthesis and transport of secondary metabolism compounds and controlled protein breakdown was higher in the SA library. Figure 4 also shows a higher amount of genes related to cell maintenance in the FO library. Genes with cell maintenance and plant development roles were identified in both libraries. Results indicate that there is activation of primary metabolism pathways, together with the induction of several signaling pathways, for the expression of defense-related genes (Hammond-Kosack & Jones, 1996; Birch et al., 1999; Mahalingam et al., 2003).

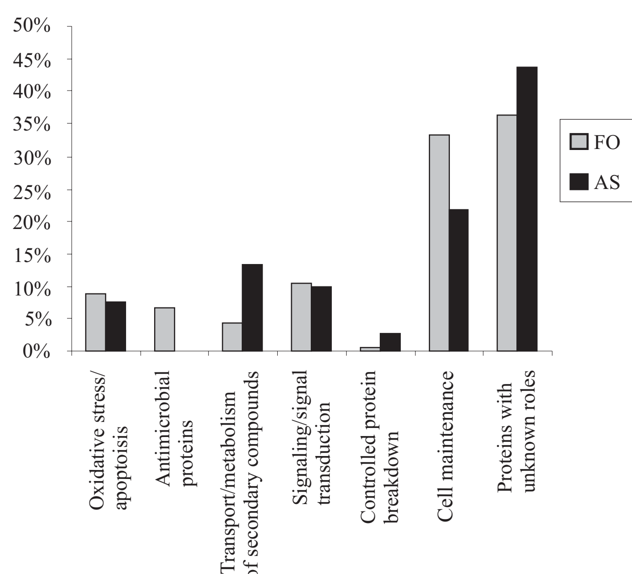

Figure 4. Comparison of Expressed Sequences Tags (ESTs) classified by the predicted role between two tomato cDNA libraries, induced by salicylic acid (SA) and *F. oxysporum* f. sp. *lycopersici* (FO).

Plant genes with known roles, involved in biotic and abiotic stress response and associated to cell maintenance and plant development, were identified in both SA and FO libraries. Some of these genes, as those encoding phenylalanine ammonia lyase, chitinase, late embryogenesis abundant protein, bZip transcription factor, zinc finger domain protein, and I-2 disease resistance protein with leucine-rich repeat, are related to defense mechanisms, while genes encoding cytochrome, ubiquitin, photosystem II apoprotein and ribosomal 60S protein are related to cell metabolism (Tables 2 and 3).

A gene that is exclusive to the plant-pathogen interaction type was identified in the FO library. It is a cDNA clone which showed highly significant similarity to an *Oryza sativa* gene, encoding a protein from the hypersensitive induced reaction (HIR) family. Members of this family are related to hypersensitivity reaction, involving cell death and resistance to pathogens (Nadimpalli et al., 2000). This gene was also induced in tomato plants with constitutive expression of the resistance gene *Pto* (35S::*Pto*) (Xiao et al., 2001).

Table 2. Identification of salicylic acid-induced genes in tomato (*Lycopersicon esculentum*) with similarity to known genes from other plant species.

Clone[(1)]	Gene description (acess no.)[(2)]	E-value[(3)]
AS - 010	Acil - CoA synthase [*Capsicum annuum*] (AAL29212)	1.4e-53
AS - 023	Late embryogenesis abundant protein [*Nicotiana tabacum*] (TC174494)	8.5e-62
AS - 044	Ubiquitin [*Solanum tuberosum*] (TC170024)	9.9e-55
AS - 053	Cytochrome b6 [*Arabidopsis thaliana*] (TC190079)	1.4e-21
AS - 061	Disease resistance protein PR-10 [*Capsicum annuum*] (Q2VT55)	9.1e-49
AS - 067	Chalcone synthase 2 [*Camellia sinensis*] (P48387)	2.0e-91
AS - 072	Manganese superoxide dismutase [*Lycopersicon esculentum*] (TC18915)	8.3e-34
AS - 080	Zinc finger domain protein [*Arabidopsis thaliana*] (TC172334)	4.7e-43
AS - 095	60S ribosomal protein [*Lycopersicon esculentum*] (TC172591)	4.5e-54
AS - 112	Caspase 1 [*Lycopersicon esculentum*] (TC188871)	1.8e-66
AS - 115	bZIP family transcription factor [*Solanum tuberosum*] (TC180148)	6.7e-86
AS - 127	Photosystem II apoprotein (47 kDa) [*Arabidopsis thaliana*] (TC182559)	1.9e-27
AS - 132	BHLH-like transcription factor [*Arabidopsis thaliana*] (Q8S3E0)	6.4e-46
AS - 142	Phenylalanine ammonia lyase [*Lycopersicon esculentum*] (TC172772)	1.2e-79
AS - 143	CIPK-like protein kinase [*Arabidopsis thaliana*] (NP566580)	9.0e-30

[(1)]Genes isolated by suppression subtractive hybridization, from salicylic acid treated tomato mRNAs. [(2)]Gene roles were identified with homologue sequences from NCBI, TIRG and DDBJ, using the program BlastX. [(3)]E-value was used to indicate the significance of the similarity for each gene.

Table 3. Identification of tomato (*Lycopersicon esculentum*) genes induced by *F. oxysporum* f.sp. *lycopersici* with similarity to known genes from other plant species.

Clone[1]	Gene description (acess number)[2]	E-value[3]
FO - 007	ABC transporter protein [*Oryza sativa*] (BAC55994)	7.4e-50
FO - 023	Hypersensitivity-induced response protein [*Oryza sativa*] (AAK54610)	1.3e-44
FO - 027	Phospholipase [*Arabidopsis thaliana*] (AAL87258)	2.8e-41
FO - 041	Chitinase [*Solanum lycopersicum*] (BAC76900)	1.e-148
FO - 069	Cytochrome P450 [*Arabidopsis thaliana*] (AI776695)	1.1e-31
FO - 084	LeMir protein [*Lycopersicon esculentum*] (TC171566)	3.6e-45
FO - 089	Phenylalanine ammonia lyase [*Lycopersicon esculentum*] (TC172772)	3.1e-78
FO - 093	I-2 disease resistance protein with leucine-rich repeat [*Oryza sativa*] (BAB89710)	6.0e-35
FO - 098	Osmotin-like protein [*Solanum commersonii*] (P50702)	1.0e-32
FO - 099	Cysteine proteinase inhibitor [*Lycopersicon esculentum*] (TC172677)	1.2e-56
FO - 103	Xyloglucan-specific fungal endoglucanase inhibitor protein [*Lycopersicon esculentum*] (TC188664)	1.4e-63
FO - 115	Ubiquitin [*Arabidopsis thaliana*] (Q45W78)	2.3e-32
FO - 130	Serine/threonine protein kinase [*Nicotiana tabacum*] (Q1W0X1)	1.1e-25
FO - 145	CGI-144-like protein (pathogen-induced) [*Lycopersicon esculentum*] (CAC81814)	1.3e-61
FO - 152	Catalase [*Gossypium hirsutum*] (S10395)	2.2e-55
FO - 155	Cysteine proteinase [*Ipomea batatas*] (AAQ81938)	3.7e-19

(1)Genes isolated by suppression subtractive hybridization, from mRNAs of tomato plants infected with *F. oxysporum* f. sp. *lycopersici*. (2)Gene roles were identified with homologue sequences from NCBI, TIRG and DDBJ, using the program BlastX. (3)E-value was used to indicate the significance of the similarity for each gene.

The detection of a gene from the HIR family in tomato, which may be involved in the mediation of programmed cell death, was also made by Solomon et al. (1999).

Genes encoding antimicrobial proteins were only detected in the FO library. A chitinase-encoding gene, probably representing an acidic protein, likely belongs to a group of glycosyl hydrolases from family 19. In the interaction between a resistant potato cultivar and *Phytophthora infestans*, the expression of antimicrobial proteins related to defense mechanisms was observed at the formation of the first haustoria in the plant cell, from 48 to 72 hours after inoculation (Ros et al., 2004).

This is the first report of global gene expression in tomato induced by salicylic acid and *F. oxysporum* f. sp. *lycopersici* using the SSH technique. It was possible to isolate and identify novel sequences in tomato from a relative small number of analyzed sequences, when compared to major sequencing projects (Birch et al., 1999; Werne et al., 2001; Xiong et al., 2001; Mahalingam et al., 2003; Ros et al., 2004; Ouyang et al., 2007). The present work provides a valuable starting point for further elucidation of the roles played by these genes and gene products in pathogen resistance in tomato.

Conclusions

1. The technique of suppression subtractive hybridization (SSH) is effective for the identification of resistance genes activated by salicylic acid and *Fusarium oxysporum* f. sp. *lycopersici*.

2. Not only does the application of SSH technique enable a cost effective isolation of differentially expressed sequences, but also it allows the identification of novel sequences in tomato from a relative small number of sequences.

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