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In silico analysis of gene content in tomato genomic regions mapped to the *Ty-2* resistance gene

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ABSTRACT. Tomato yellow leaf curl virus is one of the main diseases affecting tomato production worldwide. Previous studies have shown that *Ty-2* is an important resistance gene located between molecular markers C2_At2g28250 (82.3 cM) and T0302 (89.0 cM), and exhibits strong resistance to tomato yellow leaf curl virus in Asia. In this study, *Ty-2* candidate genes were subjected to bioinformatic analysis for the sequenced tomato genome. We identified 69 genes between molecular markers C2_At2g28250 and T0302, 22 of which were disease-related resistant genes, including nucleotide binding site-leucine-rich repeat disease resistance genes, protease genes (protein kinase, kinase receptor, and protein isomerase), cytochromes, and transcription factors. Expressed sequence tag analysis revealed that 77.3% (17/22) of candidate disease-resistance genes were expressed, involving 143 expressed sequence tags. Based on full-length cDNA sequence analysis, 7 candidate genes were found, 4 of which were involved in tomato responses to pathogens. Microarray ex-

pression analysis also showed that most candidate genes were involved in the tomato responses to multiple pathogens, including fungi, viruses, and bacteria. RNA-seq expression analysis revealed that all candidate genes participated in tomato growth and development.

Key words: Disease resistance; Expressed sequence tag; Tomato; *Ty-2;* Tomato yellow leaf curl virus

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) belongs to the family Solanaceae and is an important vegetable crop worldwide. Tomato yellow leaf curl virus (TYLCV) has severely affected the growth of tomato crops and world tomato production in recent years, and has been reported on a global scale (Accotto et al., 2000, 2003; Delatte et al., 2005; Wu et al., 2006; Lefeuvre et al., 2010; Melzer et al., 2010). Currently, the disease is primarily managed by spraying pesticides to control the virus vector whitefly. However, previous studies have shown that overuse of insecticides can increase the drug resistance of whitefly populations (Palumbo et al., 2001).

Breeding disease-resistant varieties is the most economic and efficient method for controlling TYLCV in tomato. However, no TYLCV-resistant gene resources exist in cultivated tomato cultivars, and wild tomato species such as *Solanum pimpinellifolium*, *S. peruvianum*, *S. chilense*, *S. habrochaites*, and *S. cheesmaniae* exhibit a high degree of disease resistance (Picó et al., 1996; Ji et al., 2007b; Scott, 2007; Vidavski, 2007). A total of 6 tomato TYLCV genes have been identified and named as *Ty-1*, *Ty-2*, *Ty-3*, *Ty-4*, *Ty-5*, and *Ty-5* (Hanson et al., 2000, 2006; Garcia et al., 2007b; Ji et al., 2007a, 2009b; Hutton et al., 2012). These disease-resistant genes were derived from different wild-type tomatoes. The *Ty-2* gene has been shown to exhibit clear resistance to TYLCV in India, Taiwan, Japan, and other Asian regions (Hanson et al., 2000), and has been widely used for TYLCV-resistance breeding in tomato. The *Ty-2* gene was first identified and located on the long arm of chromosome 11 between the molecular markers TG36 (84 cM) and TG393 (103 cM) (Hanson et al., 2000). Further studies localized this gene to between molecular markers TG36 (84 cM) and TG26 (92 cM) (Hanson et al., 2006), and finally between C2_At2g28250 (82.3 cM) and T0302 (89.0 cM) (Ji et al., 2009a). Studies also reported that the marker TG105A was tightly linked to *Ty-2*, but may also be related to the *I2* gene, which can cause false-positive results (Garcia et al., 2007a). Currently, though molecular markers for the *Ty-2* gene have been developed (Hanson et al., 2006; Garcia et al., 2007b; Ji et al., 2007a, 2009b; Hutton et al., 2012), further analysis on *Ty-2* candidate genes has not been reported. In 2011, whole-genome sequencing of the tomato was completed (Tomato Genome Consortium, 2012), providing an opportunity for the screening of *Ty-2* candidate genes using a bioinformatic approach.

Based on the whole-genome sequence of tomato, *Ty-2* candidate genes were identified *in silico* in this study. Furthermore, the expression patterns of these genes were analyzed using expressed sequence tags (ESTs), full-length cDNA, microarray, and RNA-Seq. The results showed that *Ty-2* disease-resistant genes were restricted to within 22 candidate genes, providing a foundation for the further development of molecular markers for fine mapping and function analysis. In addition, these candidate genes were found to not only be involved in the growth and development of tomato, but they also participated in tomato responses to pathogens.

MATERIAL AND METHODS

Identification of *Ty-2* candidate genes

According to a previous study, the *Ty-2* disease resistance gene is located between molecular markers C2_At2g28250 (82.3 cM) and T0302 (89.0 cM) (Ji et al., 2009a). In our study, based on the whole tomato genomic sequencing, the positions of these 2 molecular markers in the tomato genome database were 51305217 and 51879030, respectively. The *Ty-2* molecular marker gene number, position, and nucleotide sequence were retrieved using the Solanaceae genome website (http://solgenomics.net).

EST-based expression analysis of candidate genes in the *Ty-2* regions

Three EST databases (SOLHA, SOLLC, and SOLPN) were downloaded from the Solanaceae EST database website (http://biosrv.cab.unina.it/solestdb/). The local database was set up using the BioEdit software (Ibis Biosciences, Carlsbad, CA, USA). The *Ty-2* candidate genes were compared to search for the corresponding candidate genes. The sequence similarity score was greater than 150 and the E value was less than -30 (Anderson and Brass, 1998).

cDNA-based full-length expression analysis

The full-length cDNAs of the *Ty-2* candidate genes in tomato were searched using Blast on the tomato full-length cDNAs website (http://www.pgb.kazusa.or.jp/kaftom/) with the selected E value of -30.

Microarray- and RNA-Seq-based expression analysis of *Ty-2* candidate genes

The microarray analysis platform included the tomato TOM2 database. Tomato *Ty-2* candidate gene array probes were retrieved using Blast (http://ted.bti.cornell.edu/cgi-bin/TFGD/array/blast.cgi). For genes with multiple corresponding probes, the gene probe with the highest E-value was selected. The RNA-Seq analysis platform was the tomato tissue expression dataset DOO4. The software MeV was used for clustering analysis of expression data to conduct.

RESULTS

Identification of *Ty-2* candidate genes in tomato

Previous studies showed that *Ty-2* disease-resistant genes were located between molecular markers C2_At2g28250 (82.3 cM) and T0302 (89.0 cM) (Ji et al., 2009a). Based on the tomato genome sequence, the physical distance between these 2 molecular markers was 573,813 bp. Sixty-nine genes were annotated in this region according to the analysis of the tomato genome sequence, 22 of which were related to resistance to TYLCV disease based on previous transcriptomics studies (Chen et al., 2013) (Table 1). These disease-resistance genes were divided into 4 classes, including nucleotide binding site-leucine-rich repeat (NBS-LRR), protease genes (protein kinase, kinase receptor, and protein isomerase), cytochromes, and transcription factors. Among the 22 candidate genes, 5 belonged to the NBS-LRR family,

3 were receptor-like protein kinase family members, 3 were transcription factors, 2 were protein isomerases, 2 were involved in oxidation reduction in organisms, 1 encoded structural proteins, 1 encoded allergic pathogenic proteins, 2 were ubiquitin ligases, and 1 was related to cytochromes.

Table 1. *Ty-2* candidate genes.

Gene Name	Physical position (bp)	Size (bp)	Exon No.	Predicted protein function
Solyc11g069590.1.1	51305442-51307901	2460	6	Receptor-like protein kinase
Solyc11g069620.1.1	51346017-51349831	3815	2	CC-NBS-LRR, resistance protein
Solyc11g069630.1.1	51353722-51351654	2069	2	Receptor-like protein kinase
Solyc11g069660.1.1	51374081-51371454	2628	1	NBS-LRR, resistance protein
Solyc11g069670.1.1	51376047-51375871	177	1	Disease resistance protein R3a-like protein
Solyc11g069690.1.1	51383609-51389361	5753	4	Protein disulfide isomerase
Solyc11g069770.1.1	51482195-51486514	4320	2	Transcription factor MADS-box protein
Solyc11g069800.1.1	51514060-51512528	1533	1	Cytochrome P450
Solyc11g069860.1.1	51566625-51571040	4416	4	Glutaredoxin
Solyc11g069920.1.1	51610237-51610882	646	2	NBS, resistance protein fragment
Solyc11g069930.1.1	51612577-51616365	3789	2	Disease resistance protein R3a-like protein
Solyc11g069940.1.1	51622746-51623174	429	1	Glutaredoxin
Solyc11g069960.1.1	51646669-51643713	2957	3	Receptor like kinase, RLK
Solyc11g069970.1.1	51650118-51649333	786	1	Harpin-induced 1
Solyc11g069990.1.1	51664081-51661763	2319	1	NBS-LRR, resistance protein
Solyc11g070000.1.1	51664969-51664166	804	1	NBS, resistance protein fragment
Solyc11g070040.1.1	51683590-51685026	1437	1	Pentatricopeptide repeat-containing protein
Solyc11g070070.1.1	51703065-51701395	1671	2	Zinc finger CCCH domain-containing protein 39
Solyc11g071190.1.1	51800380-51801390	1011	2	C3HC4 type zinc-finger domain-containing protein
Solyc11g071230.1.1	51854001-51851248	2754	3	Galactosylgalactosylxylosyl protein 3-beta-glucuronosyl transferase
Solyc11g071240.1.1	51857580-51859206	1627	2	U-box domain-containing protein
Solyc11g071260.1.1	51882669-51878146	4524	5	Ubiquitin-conjugating enzyme E2

According to the gene clusters and tandem repeats criteria used in previous studies (Meyers et al., 2003; Yang et al., 2008; Huang et al., 2012), further analysis revealed that the 5 NBS-LRR family genes in the *Ty-2* disease-resistant genes region belonged to 2 gene clusters. Solyc11g069620.1.1 and Solyc11g069660.1.1 belonged to the same gene cluster, and exhibited tandem repeat modes; the other 3 NBS-LRR family genes (Solyc11g069920.1.1, Solyc11g069990.1.1, and Solyc11g070000.1.1) belonged to another cluster.

EST expression analysis

To determine the potential function of *Ty-2* candidate genes, the expression of the 22 candidate genes was analyzed using the tomato EST database (http://biosrv.cab.unina.it/solestdb/). The results showed that 17 candidate genes were supported by 143 ESTs (Table 2). Each gene harbored various numbers of ESTs, ranging from 1-25. These ESTs were mostly from cultivated tomato (*S. lycopersicum*), but some were also identified in wild-type tomatoes, in which the similarity between the *Ty-2* candidate gene Solyc11g069590.1.1 and EST (AW618332) from *S. pennellii* was as high as 99%.

According to previously reported criteria, 3 different categories of genes were identified based on the number of EST matches to a gene. If the number of EST matches to a gene were < 7, it was considered minimally expressed; if matches were between 7 and 200, it was considered relatively highly expressed; and if matches exceeded 200, it was considered highly expressed (Mahalingam et al., 2003; Dubey and Chandel, 2010). Among the 17 candidate genes, 9 showed relatively high expression levels, including Solyc11g069590.1.1,

Solyc11g069620.1.1, Solyc11g069660.1.1, Solyc11g069800.1.1, Solyc11g069860.1.1, Solyc11g069940.1.1, Solyc11g069990.1.1, Solyc11g070000.1.1, and Solyc11g071260.1.1. Of these 9, the EST matches to Solyc11g069800.1.1 reached a maximum of 24. The remaining 8 genes were minimally expressed, with EST matches of < 7, respectively. No highly expressed genes were observed.

Table 2. EST-based expression analysis of *Ty-2* candidates.

Genes	EST database		
	SOLHA	SOLLC	SOLPN
Solyc11g069590.1.1	0	9	1
Solyc11g069620.1.1	0	20	0
Solyc11g069660.1.1	0	14	0
Solyc11g069670.1.1	0	5	0
Solyc11g069690.1.1	0	2	0
Solyc11g069800.1.1	1	24	0
Solyc11g069860.1.1	0	9	0
Solyc11g069930.1.1	0	4	0
Solyc11g069940.1.1	0	13	0
Solyc11g069960.1.1	0	3	0
Solyc11g069990.1.1	0	9	0
Solyc11g070000.1.1	0	7	0
Solyc11g070040.1.1	0	1	0
Solyc11g070070.1.1	0	1	0
Solyc11g071230.1.1	0	2	0
Solyc11g071240.1.1	0	1	0
Solyc11g071260.1.1	0	17	0

SOLHA: *Solanum habrochaites*; SOLLC: *S. lycopersicum* L; SOLPN: *S. pennellii*.

Full-length cDNA expression analysis

Full-length cDNA sequences of the 22 tomato *Ty-2* candidate genes were retrieved using the online Blast software from the tomato full-length cDNA database website (http://www.pgb.kazusa.or.jp/kaftom/). One or more full-length cDNAs were found for 7 of the 22 *Ty-2* candidate genes (Table 3). The functions of these full-length cDNAs included tomato growth and development and different pathogen stress treatment. For example, Solyc11g069590.1.1 participates in the growth and development of fruits, and Solyc11g069860.1.1 and Solyc11g071260.1.1 participate in the maturing process of fruit. In contrast, the remaining 4 genes were involved in the tomato responses to pathogens.

Table 3. cDNA-based full-length expression analysis.

Genes	cDNA	Description	Identity (%)	Alignment length	E value	Bit Score
Solyc11g069590.1.1	LEFL2012B21	Stage of fruit development	99	1464	0	2894
Solyc11g069800.1.1	LEFL1089CF06	Pathogen-treated (Leaf)	99	1533	0	3015
Solyc11g069860.1.1	FC11AD08	Maturing fruits	99	432	0	825
	LEFL2002BC12	Stage of fruit development	99	432	0	811
Solyc11g069940.1.1	LEFL1009CB09	Pathogen-treated (Leaf)	99	429	0	842
Solyc11g069960.1.1	LEFL1020AF06	Pathogen-treated (Leaf)	99	1830	0	3510
Solyc11g071230.1.1	LEFL1019AA04	Pathogen-treated (Leaf)	99	1335	0	2639
	LEFL1011BF06	Pathogen-treated (Leaf)	84	618	1.00E-120	432
Solyc11g071260.1.1	FC03AE04	Maturing fruits	99	474	0	924

Microarray expression analysis of *Ty-2* candidate genes

The expression patterns of the candidate genes were further analyzed using tomato TOM2 array data. Twelve candidate genes were found using corresponding probes. Under tomato spotted wilt virus infection, the expression levels of Solyc11g069990.1.1 and Solyc11g069660.1.1 in the root were high, while expression of the other genes was low. Except for 3 candidate genes (Solyc11g069590.1.1, Solyc11g069960.1.1, and Solyc11g069990.1.1), the expression levels of most genes in the leaves were high (Figure 1). Under the arbuscular mycorrhizal fungus *Glomus mosseae* infection, the expression levels of Solyc11g069990.1.1 and Solyc11g069800.1.1 in the roots were relatively high, while the expression of the other genes was low. In the leaves, 2 candidate genes (Solyc11g069590.1.1 and Solyc11g069770.1.1) were highly expressed, 4 genes (Solyc11g069620.1.1, Solyc11g069670.1.1, Solyc11g069930.1.1, and Solyc11g070000.1.1) were moderately expressed, and the expression levels of the remaining genes were low.

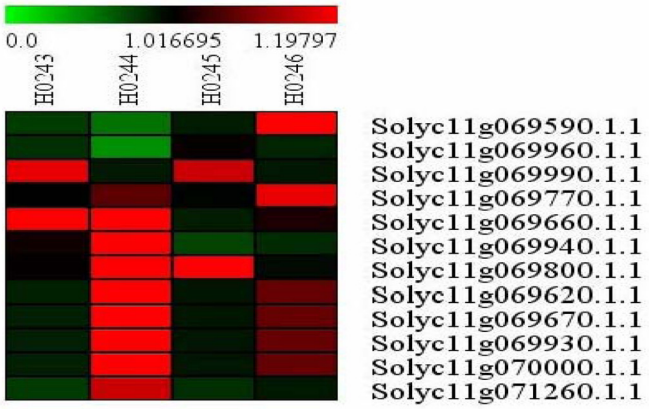

Figure 1. Expression profiles of tomato *Ty-2* candidates based on tomato microarray. Expression based on TOM2 Array. H0243: the Moneymaker root relative expression in plants systemically infected with tomato spotted wilt virus at 51 days; H0244: the Moneymaker leaves relative expression in plants systemically infected with tomato spotted wilt virus at 51 days. H0245: the Moneymaker root relative expression in plants colonized by the arbuscular mycorrhizal fungus *Glomus mosseae* at 51 days; H0246: the Moneymaker leaves relative expression in plants colonized by the arbuscular mycorrhizal fungus *Glomus mosseae* at 51 days.

Expression analysis of *Ty-2* candidate genes based on RNA-Seq

Based on the tomato RNA-Seq dataset, the expression patterns of the 22 *Ty-2* candidate genes in different stages of vegetative and reproductive development were determined (Figure 2). The results showed that the expression levels of Solyc11g069940.1.1, Solyc11g069930.1.1, Solyc11g069800.1.1, Solyc11g069860.1.1, Solyc11g069690.1.1, and Solyc11g071260.1.1 were high in all tested tissues; the expression level of Solyc11g069990.1.1 was moderate in all tested tissues; and the expression levels of Solyc11g071230.1.1, Solyc11g070070.1.1, Solyc11g071240.1.1, and Solyc11g069660.1.1 were moderate in most tissues tested. In addition, compared with those in the root, leaf, flower, and bud, the expression level of Solyc11g069900.1.1 was higher during fruit development. The expression levels of Solyc11g069770.1.1 were moderate in the root, bud, and flower, while in other tissues, expression levels were low. The expression levels of the remaining genes were low in all tissues tested.

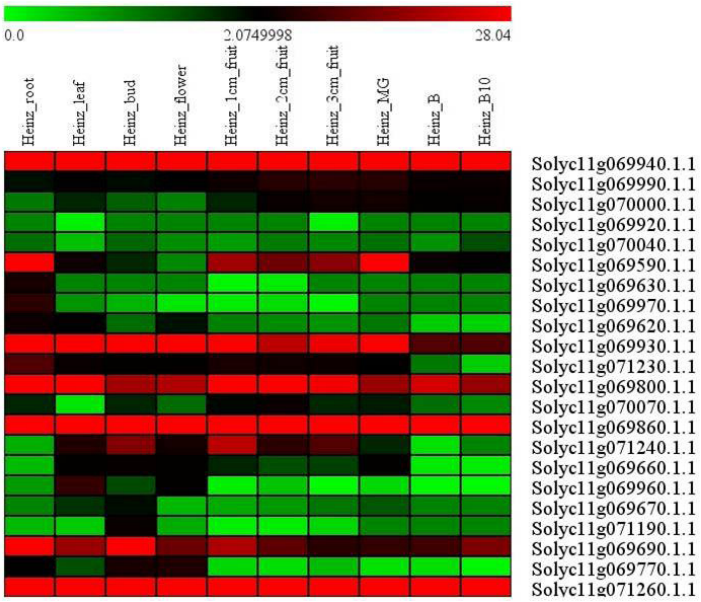

Figure 2. Expression profiles of tomato *Ty-2* candidate genes based on RNA-Seq data. The tissues from the left to right are roots, leaves, flower buds, fully opened flowers, and 1-cm, 2-cm, 3-cm, mature green, breaker, and breaker +10 fruits of tomato cultivar Heinz.

DISCUSSION

Currently, at least 6 TYLCV resistant genes (*Ty-1*, *Ty-2*, *Ty-3*, *Ty-4*, *Ty-5*, and *Ty-6*) have been identified in tomato, and molecular markers and gene mapping of these resistance genes were analyzed in previous studies (Hanson et al., 2006; Garcia et al., 2007b; Ji et al., 2007a, 2009b; Hutton et al., 2012). Of them, *Ty-1* and *Ty-3* resistance genes have been cloned and found to encode the DFDGD motif, which belongs to the RNA-dependent RNA γ polymerase family (Verlaan et al., 2013). However, cloning of the remaining TYLCV resistance genes has not been reported. In the present study, based on the mapping of *Ty-2* by previous researchers, candidate genes of *Ty-2* were analyzed using bioinformatics and tomato whole-genome sequencing information (Tomato Genome Consortium, 2012) to facilitate the identification and cloning of the *Ty-2* resistance gene.

Our results revealed abundant disease-resistant candidate genes in the chromosome regions encompassing the tomato TYLCV resistance locus *Ty-2* (Table 1). These disease-resistance genes accounted for 31.9% (22/69) of the total gene number in the *Ty-2* region, and for 0.92% (22/2385) of genes in tomato chromosome 11. Among the 22 candidate genes, 5 encoded NBS domain proteins and belonged to the NBS-LRR family of resistance genes. In all cloned disease-resistant genes in plants, most genes belonged to the NBS-LRR family (Liu et al., 2007). In tomato, most cloned resistance genes also belong to this family, including *Prf*, *I2*, *Mi-1*, *Mi-9*, *Sw-5/Mi*, and *Hero* (Salmeron et al., 1996; Ori et al., 1997; Brommonschenkel et al., 2000; Ernst et al., 2002; Tameling et al., 2002; Jablonska et al., 2007). The receptor-like protein kinase is also considered as a disease resistance gene family, and these genes have been shown to be involved in plant defense against pathogens (Kruijt et al., 2005; Hok et al., 2011).

Previous studies showed that the wheat rust resistance gene *Lr10* and rice blast resistance gene *Pi-d2* both encoded receptor-like protein kinases (Feuillet et al., 1997; Kouzai et al., 2013). In this study, the candidate genes Solyc11g069590.1.1 and Solyc11g069630.1.1 also encoded receptor-like protein kinase, indicating that the 2 candidate genes are associated with plant disease resistance. The pentatricopeptide repeat-containing protein is an RNA binding protein (Saha et al., 2007) that regulates mitochondrial and chloroplast genes through post-transcription modification of RNA. Expression analysis of the rice response to *Xanthomonas oryzae* showed that the gene encoding a pentatricopeptide repeat-containing protein was upregulated (Zhou et al., 2010), suggesting that these genes are induced by pathogenic bacteria.

The full-length cDNA library and the EST database are important resources for gene expression analysis (Adams et al., 1995). In this study, these databases were used to analyze candidate *Ty-2* genes. EST-based expression analysis revealed that 77.3% (17/22) of candidate disease-resistant genes contained EST in the regions of the *Ty-2* gene, indicating that most *Ty-2* candidate genes were expressed in tomato. In addition, 53% (9/17) of expressed candidate genes contained more than 7 ESTs, indicating their relatively high expression levels (Mahalingam et al., 2003; Dubey and Chandel, 2010).

Based on RNA-Seq datasets, the expression patterns of the 22 tomato *Ty-2* candidate genes in the roots, leaves, blossom buds, other tissues as well as the fruit development stages were examined (Figure 2). The results showed high expression levels of 6 genes (Solyc11g069940.1.1, Solyc11g069930.1.1, Solyc11g069800.1.1, Solyc11g069860.1.1, Solyc11g069690.1.1, and Solyc11g071260.1.1) in all tested tissues. These 6 genes encoded redox protein, cytochrome, plant disease resistance proteins, isomerase, and ubiquitin conjugating enzyme, respectively. For the remaining 16 genes, each was expressed in at least 1 tissue under normal growth conditions. These results showed that the genes were not only involved in tomato responses to environment stresses, but they also participated in tomato growth and development.

CONCLUSIONS

Using bioinformatic methods, 22 disease-resistance candidate genes of *Ty-2* were identified. The EST, cDNA, microarray, and RNA-Seq data of expression pattern results confirmed the involvement of these genes in tomato growth and development, as well as responses to multiple stresses. This study provides a foundation for the further development of molecular markers for the fine mapping and function analysis of *Ty-2* resistance genes.

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