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Review paper

Functional genomics of tomato for the study of plant immunity

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Abstract

Tomato (*Solanum lycopersicum*), along with many other economically valuable species, belongs to the Solanaceae family. Understanding how plants in this family defend themselves against pathogens offers the opportunity of improving yield and quality of their edible products. The use of functional genomics has contributed to this purpose through both traditional and recently developed techniques that allow determination of changes in transcript abundance during pathogen attack. Such changes can implicate the affected gene as participating in plant defense. Testing the involvement of these candidate genes in defense has relied largely on posttranscriptional gene silencing, particularly virus-induced gene silencing. We discuss how functional genomics has played a key role in our current understanding of the defense response in tomato and related species and what are the challenges and opportunities for the future.

Key words: plant immunity; Pseudomonas syringae; Solanaceae; transcriptomics; virus-induced gene silencing

Introduction

Plants have evolved defense mechanisms to survive in an environment populated with phytopathogens. Current models include a first layer of defense against microbes that is activated by the recognition of highly conserved molecular features (microbe-associated molecular patterns, MAMPs). Examples of MAMPs include bacterial flagellin and fungal chitin. Plants perceive the presence of MAMPs in the apoplastic space through membrane-associated receptors or pattern-recognition receptors (PRRs) and activate a defense response termed pattern-triggered immunity (PTI) [1]. Additional membrane-associated receptors are involved in the recognition of damage-associated molecular patterns, such as endogenous peptides [2] and oligogalacturonides [3] that are released upon damage or pathogen recognition. The detection of some proteins secreted by fungi, such as AVR9 from *Cladosporium fulvum*, also relies on PRRs [4]. Insect chewing is associated with the release of herbivore-associated molecular patterns that trigger plant defense responses, different from those that result from mechanical damage [5].

Certain pathogens deliver effectors that are able to suppress PTI activation signaling [6]. An additional level of defense termed effector-triggered immunity (ETI) is activated when plants detect, through intracellular receptors (R proteins), the activity of pathogen effectors [7]. A localized programmed cell death or hypersensitive response (HR) is usually associated with ETI activation, which leads to the suppression of pathogen growth [8].

The Solanaceae family includes many economically important species such as tomato (*Solanum lycopersicum*), potato, eggplant, pepper, petunia and tobacco. Because of tomato's relevance as fresh and processed produce, there is great interest in using genomic resources to improve this crop [9]. Tomato is susceptible to a wide range of pathogens including bacteria, fungi, oomycetes, viruses and nematodes [10] and has become one of the model species within this family for the study of plant defense mechanisms [11]. A high degree of gene sequence similarity among the Solanaceae [12] raises the possibility of transferring knowledge generated using tomato to other species and vice versa. Wild relatives of tomato also provide a source of

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valuable traits, which can be introgressed into cultivated tomato. In this sense, natural resistance to pathogens has proven useful in the identification of novel immune-related genes [13–19]. A high-quality genome sequence for tomato became available in 2012 [20], further enhancing the use of this species as a model to study plant defensive mechanisms.

In this review, we focus on the use of functional genomics as a means to study tomato plant immunity. In particular, we discuss how different experimental approaches have been used to enhance our knowledge in this field.

Mutagenesis approach

A thorough overview of mutagenesis in tomato has been presented elsewhere [21]. This approach requires a subsequent screening method that relies on identifying individuals with the phenotype of interest. The *Pto* gene, which encodes a serine/threonine kinase, confers tomato resistance to *Pseudomonas syringae* pv. *tomato* (*Pst*) strains carrying AvrPto and/or AvrPtoB effectors [22]. Pto-mediated resistance requires Prf, an NBS-LRR (nucleotide-binding site/leucine rich repeat) protein identified through mutagenesis with diepoxibutane and fast neutron irradiation, followed by map-based cloning [23, 24].

Part of the complex response of plants to herbivore insects involves the transcriptional activation of genes encoding protease inhibitors (PIs) that reduce the activity of digestive enzymes present in the herbivore gut [25]. An 18-amino acid peptide isolated from wounded tomato leaves, named systemin, was found to induce gene expression of PIs [26]. Later studies showed that systemin originates through proteolytic cleavage of prosystemin (Solyc05g051750), a larger precursor protein [27], and that prosystemin overexpression under the CaMV 35S promoter (35S::*PS*) results in plants with a higher basal level of PIs in the absence of insect challenge [28]. Systemin is the molecular link between herbivore wounding and the signaling cascade, which leads to increased levels of the phytohormone jasmonic acid (JA) and the consequent transcriptional activation of defense genes [29]. Howe *et al.* [30] used a mutagenesis approach with ethyl methanesulfonate (EMS) on 35S::*PS* tomato plants to identify components of the systemin-dependent response. Non-mutagenized overexpressing lines possessed several-fold higher polyphenol oxidase activity compared with wild-type plants [31]. This characteristic was used to perform a rapid screening to identify *spr* (*suppressor of prosystemin-mediated responses*) mutants. The use of map-based cloning allowed the identification of *Spr2* or *LeFad7* (Solyc06g051400) that encodes a chloroplastic fatty acid desaturase that participates in JA synthesis and whose mutation resulted in increased susceptibility to tobacco hornworm larvae [32]. Recently, also with the use of map-based cloning, *Spr8* or *TomLoxD* (Solyc03g122340) was found to encode for a chloroplast-localized lipoxygenase that is involved in JA biosynthesis [33]. In this case, overexpression of *TomLoxD* led to increased immunity against cotton bollworm larvae and *Botrytis cinerea*.

Mutagenesis was also successfully used to study the interaction between tomato and *C. fulvum*. This pathogen secretes avirulence (Avr) proteins that trigger an HR-based immune response that arrests fungal growth in resistant tomato plants expressing the corresponding Cf resistance protein [4]. The tomato *Cf-9* gene, which was identified using a transposon tagging approach [34], encodes a membrane-anchored protein with LRRs that confers resistance to *C. fulvum* strains expressing the corresponding Avr9 protein [35]. *Cf-2* from resistant *Solanum pimpinellifolium* has been bred into commercial tomato [15]. Using such resistant plants, Dixon *et al.* [36] performed mutagenesis with EMS or diepoxybutane and tested for compromised resistance to *C. fulvum*. The screening strategy involved the use of a *C. fulvum* race 4 β-glucuronidase (GUS) whose growth can be followed both by macroscopic abundance of mycelium and a GUS activity assay. This study led, by using positional cloning, to the identification of *Rcr3* (required for *C. fulvum* resistance 3; Solyc02g076980) that encodes a secreted cysteine protease required for Cf-2-dependent response [36, 37].

A system for studying the interaction of tomato and the oomycete *Phytophthora infestans* that can be combined with mutagenesis was recently developed by Jia *et al.* [38]. The potato gene *R3a* confers resistance to *P. infestans* strains expressing the corresponding avirulence gene, Avr3a [39, 40]. Co-expression in tomato of this pair of genes results in HR development, suggesting a conserved signaling cascade. In the future, mutant screenings using this overexpressing line could help in the identification of novel Avr3a/R3a signaling components.

Mutagenesis has been used in combination with TILLING (targeting induced local lesions in genomes) [41, 42] as a means of identifying individuals that carry mutations in a specific gene [43, 44]. Piron *et al.* [45] generated a tomato-based TILLING platform to identify individuals with mutations in the eukaryotic initiation factor 4E [46, 47] associated with resistance to members of the *Potyvirus* genus.

In recent years, precise genome-editing strategies have been developed [48]. Even though these have not been used yet to study tomato immunity, they present a promising approach. Zn-finger nucleases [49] and TALE (transcription activator-like effectors) nucleases [50, 51] can be modified to target specific genomic regions to generate double-stranded cleavage sites that when repaired by plant cell machinery result in a broad range of mutations. The use of CRISPR (clustered regularly interspaced short palindromic repeats) approach has emerged as a particularly powerful genome-editing tool. This method relies on RNA-guided endonucleases (such as Cas9, for CRISPR-associated nuclease 9) that can direct a double-stranded DNA break at a specific target sequence with an associated higher mutagenesis rate, compared with Zn-finger or TALE approaches [48, 52].

Differential transcript abundance approach

A popular approach for functional genomics of plant immunity is to investigate differences in transcript abundance when comparing different treatment conditions. This approach usually results in the identification of a set of candidate genes and requires further investigation of their involvement in plant defense. In this section, we discuss examples that range from pioneering to the most recent techniques, focusing on those studies that led to the identification and characterization of novel genes involved in immunity. Even though they can be informative, we will not address examples of general descriptive transcriptomic studies.

Differential complementary DNA libraries

The use of complementary DNA (cDNA) libraries to identify differentially expressed genes relies on the differential hybridization or subtraction between cDNAs derived from the tissues under study. This technique was used for the identification of genes encoding components of tomato immunity against 'Tomato yellow leaf curl virus' (TYLCV) [53–55]. Comparing cDNAs from a susceptible and a resistant tomato line

challenged with TYLCV, 70 candidate genes with higher expression levels in the resistant plants were identified. Further analysis of this set of genes using virus-induced gene silencing (VIGS, see section below) in the resistant line background, led to the identification of a permease-like protein (Solyc03g114030), a hexose transporter (LeHT1; Solyc02g079220) and a lipocain-like protein (SlVRSLip; Solyc10g0744580), as having a key role in the resistance to TYLCV.

This approach has also been used to study the interaction between tomato and the parasitic plant *Cuscuta reflexa*. Albert *et al*. [56] identified 15 genes upregulated in infected (12 h post-inoculation, hpi) plants. A gene encoding an arabinogalactan protein (attAGP; Solyc08g078020) was further studied owing to its predicted extracellular localization. Silencing *attAGP* using VIGS and interference RNA resulted in a looser attachment of *C. reflexa* to tomato stems.

Selective fragment amplification

DNA-AFLP

The cDNA amplified fragment length polymorphism (cDNA-AFLP) method is based on the analysis of PCR amplification products using several combinations of primers. The polyacrylamide gel-resolved pattern of amplification products allows the identification of fragments with different abundance when comparing two conditions or treatments [57]. Further isolation and sequencing of these differential fragments lead to establishing the identity of candidate genes. This is a laborious technique that nevertheless proved to be successful for studying the interaction of tomato and *C. fulvum*.

Using a cDNA-AFLP approach, a tobacco cell line expressing Cf-9 was used for the identification of novel components of Avr9/Cf-9-mediated defense response [58]. Such *Avr9/Cf9 rapidly elicited* (ACRE) genes were identified by treating the suspension cells with a fluid containing Avr9. Taking advantage of the rapid and synchronous response of these culture cells [59], of the 30 000 fragments inspected, 290 were found with differential abundance 30 min after treatment. This study was the basis for the identification of a protein kinase (ACIK1, for Avr9/Cf-9-induced kinase; Solyc06g062920) and a U-box protein with armadillo repeat domains (ACRE276; Solyc02g072080) that participate in Avr9/Cf-9-associated immunity [60, 61]. Experiments using tomato VIGS-silenced plants for these genes indicated that they are required for Avr9/Cf-9-mediated HR and resistance.

A similar approach was used to study Avr4/Cf-4-mediated immunity [62] using tomato plants expressing both Avr4 and Cf-4 [63]. Co-expression of these proteins leads to systemic HR symptoms when grown at room temperature, but when seedlings are incubated at high temperature (33°C) these symptoms are suppressed. Thus, tissue was collected at different time-points after transferring the plants from high temperature to room temperature [62]. From 343 *ART* (for *Avr4-responsive tomato*) genes identified, 192 were selected for further analysis using VIGS in *Nicotiana benthamiana*. A *CC-NB-LRR* (*NRC1*, for *NB-LRR protein required for HR-associated cell death 1*; Solyc06g062920) candidate whose silencing affected Avr4/Cf-4-mediated HR development, was further studied using VIGS in tomato plants [64].

GeneCalling

This technique also relies on the differences in cDNA amplification products, but does not require laborious fragment cloning (necessary for cDNA-AFLP) to identity the corresponding gene [65, 66]. Only fragments derived from unknown genes (not present in databases) need to be cloned for identification. GeneCalling was used to study the transcript changes that occur in the interaction of tomato and *Pst*. Mysore *et al*. [67] studied the transcript abundance changes that follow AvrPto-mediated recognition, comparing the response in Rio Grande (RG) plants lacking Pto (RG-PtoS), lacking Prf (RG-prf3) and fully resistant RG-PtoR plants. These latter plants had that largest transcript-level changes and accounted for nearly 90% (395 of 432) of all the differential genes identified. The data generated in this work led to the identification of several proteins that participate in the tomato–Pst interaction [68–70], using VIGS as a first approach to study their involvement in immunity.

DNA microarrays

This approach is based on the hybridization of a probe derived from RNA isolated from the tissue under study and DNA spotted on a glass slide [71]. Three microarrays have been generated for tomato: TOM1 cDNA array, TOM2 oligo array and an Affimetrix genome array [72].

Mi-1.2, which confers resistance to root-knot nematodes (RKNs), potato aphids and sweet potato whitefly, provides a good example of the use of functional genomics to investigate plant immunity. Tomato *Mi-1.2* (Solyc06g008450) was first introgressed from resistant *Solanum peruvianum* into cultivated tomato in the 1940s and later identified by using a map-based cloning approach [17]. *Mi-1.2* encodes a resistance (R) protein, containing coiled-coil nucleotide-binding leucine-rich rich repeat domains. Bhattarai *et al*. [73, 74] used the TOM1 microarray, which contains 12 860 ESTs, to study transcriptomic changes occurring during compatible and incompatible interaction between tomato and RKNs. With particular emphasis on transcription factors that were induced only in the incompatible interaction at 24 h after challenge, two WRKY genes (*SlWRKY72a* and *SlWRKY72b*) were identified using VIGS. Follow-up experiments suggested that these candidates are additionally involved in tomato defense against aphids and *Pst*.

Giant cells (GCs) are vascular root cells that differentiate as a consequence of the interaction with RKN [75]. Using laser capture microdissection combined with microarray analysis, Portillo *et al*. [76] were able to study transcriptome changes occurring specifically in GCs. Transcript abundance comparisons with uninfected vascular cells, allowed the identification of a cell wall peroxidase (TPX1; Solyc07g052510) downregulated in the GCs, that had previously been associated with lignin biosynthesis [77]. Overexpression of *TPX1* in susceptible tomato plants impaired nematode infection.

Next-generation sequencing

Next-generation sequencing (NGS) techniques, which produce massive quantities of sequencing data, have greatly accelerated research in the biological sciences [78]. In particular, RNA-Seq methods allow deep sequencing of the transcriptome under study [79]. Chen *et al*. [80] used an RNA-Seq approach to compare the response of two tomato lines (susceptible and resistant) with TYLCV. Identification of differentially expressed genes was done by comparing transcript abundance between a pool of treated (3, 5 and 7 days postinoculation) and untreated tissues. Special emphasis on genes whose expression increased in the resistant line while being decreased or unaffected in the susceptible one, allowed the identification of a gene encoding an

NBS-LRR protein (Solyc05g009760), whose silencing in the resistant background led to increased TYLCV accumulation.

Tomato terpenes, specialized metabolites that are found in high concentrations in glandular trichomes, participate in plant defense [81, 82]. Using NGS, Spyropoulou *et al*. [83] generated a trichome-specific transcript database. Mining transcription factors that are expressed in these structures, SlMYC1 (KF430611) and SlWRKY73 (Solyc03g113120) were identified and shown to bind and activate terpene synthase promoters.

The interaction of *Pst* with tomato was recently studied using an NGS-based transcriptomic approach [84, 85]. Genes were identified whose expression was increased upon treatment with flagellin-derived flgII-28 MAMP [86, 87], but decreased by the activity of DC3000 effectors AvrPto and AvrPtoB, and termed *FIRE* genes (after flagellin increased, repressed by effectors). With a particular emphasis on genes encoding protein kinases, a subset of *FIRE* genes was used in a VIGS-based screen. This strategy led to the identification of a gene encoding a wall-associated kinase SlWAK1 (Solyc09g014720), whose silencing compromised plant immunity, leading to increased symptoms and pathogen growth [84].

More recently, using a series of DC3000 mutants and different tomato lines with intact or impaired AvrPto and AvrPtoB-mediated ETI response, a set of genes specifically associated to this signaling pathway was identified [85]. Silencing one of these ETI-specific candidates, *SlEpk1* (for *ETI-specific kinase 1*), reduced cell death symptoms associated to AvrPto/AvrPtoB and HopQ1-1 recognition pathways.

The availability of NGS techniques opens up the possibility of not only characterizing tomato transcriptomic response to different pathogen challenges, but also studying other relevant features such as the abundance of alternative splicing variants, microRNA populations and DNA methylation status, which could lead to the discovery of unexplored immunity-associated processes. Using a whole-genome bisulfide sequencing approach [88, 89], the DNA methylation state was recently shown to be a dynamic process during tomato fruit ripening [90]. Yu *et al*. [91] studied DNA methylation during *Pst* infection in Arabidopsis and found that this epigenetic modification is part of the immune response of plants. The use of a similar approach may shed light into tomato's defensive mechanisms.

Recently, Ouyang *et al*. [92] used NGS to identify microRNAs (slmiR482f and slmiR5300) that were downregulated in a tomato line resistant to *Fusarium oxysporum* as compared with the levels found in a susceptible line. Using *in silico* prediction of genes that are targeted by these microRNAs with psRNATarget algorithm [93], four genes with full or partial NBS-containing domains were identified (Table 1). Further experiments using VIGS in the resistant background implicated these genes in the resistance to *F. oxysporum*.

RenSeq, for resistance gene enrichment and sequencing [94], is another example of the use of NGS. Using this approach, Andolfo *et al*. [95] identified novel genes encoding NB-LRRs that were not predicted in the current tomato genome. Custom probes based on NB-LRR sequences from Solanaceous species were used to hybridize with tomato DNA fragments, which were sequenced using NGS. This strategy, which allowed the identification of 105 novel NB-LRR sequences, could be useful for identifying members of other gene families.

The use of other species in tomato functional genomics

As mentioned previously, sequence similarity among *Solanaceae* species opens the possibility of use and transference of information between species. Koening *et al*. [96] conducted a transcriptomic-based analysis to study patterns of selection in domesticated and wild tomato species. The search of genes with a high non-synonymous to synonymous (dN/dS) substitution ratio led to the identification of 51 genes with evidence of positive selection. Most of these have not been characterized in tomato and include at least two immune-related genes such as the ortholog of the *Arabidopsis ARGONAUTE 2* (Solyc02g069260) [97] and *immunity to fusarium wilt-2C4* (Solyc08g007640) [98]. Further study of this set of genes may contribute to the identification of novel components of the tomato immune response.

Another example of the use of wild tomato species is the work related to the identification of the tomato receptor for the flgII-28 flagellin epitope. This epitope has been recently identified and is mainly perceived by a subgroup of solanaceous species [86, 87]. Natural variation in flgII-28 perception among heirloom tomato varieties had been previously reported [99]. A screen based on the production of reactive oxygen species as a consequence of flgII-28 perception, identified 'responsive' and 'non-responsive' accessions [100]. Crosses between accessions with contrasting responsiveness, followed by genomic DNA sequencing using NGS led to the identification of a region in chromosome 4 with high frequency of single-nucleotide polymorphism that contained nine predicted LRR-receptor-like kinases (LRR-RLKs). Further experiments allowed the identification of *FLS3* (for FLAGELLIN-SENSITIVE 3; Solyc04g009640) while conclusively demonstrating that it encoded the flgII-28 receptor [100].

The use of information generated using species outside the Solanaceous, such as *Arabidopsis*, has also proven to be valuable in contributing to understanding tomato immunity [101]. The Arabidopsis *C repeat/dehydration-responsive element binding factor 1* (*AtCBF1*) gene was overexpressed in tomato in an attempt to improve stress tolerance [102, 103]. In addition to increased resistance to chilling, water-deficit and oxidative stress, overexpressing lines showed tolerance to bacterial wilt (*Ralstonia solanacearum*). Subtractive hybridization and use of a DNA array allowed the identification of several pathogenesis-related (PR) encoding genes differentially expressed in the overexpressing lines [104]. Testing transcription factors that would mediate the transcriptional activation pathway between AtCBF1 and PRs, SlRAV1 (for related to ABI3/VP1, Solyc05g009790) and SlERF5 (for ethylene-responsive factor, Solyc03g093560) were identified as putative signaling components. Further experiments using knockdown and overexpression lines confirmed their involvement in tomato defense against *R. solanacearum*.

Gene silencing in tomato functional genomics

Assessment of gene function in tomato immunity has largely involved post-transcriptional gene silencing (PTGS) and particularly VIGS (Table 1). The use of differential expression techniques results in the identification of a set of candidate genes that may be involved in plant immunity. Such downstream analysis has usually relied on PTGS. In addition, PTGS has been used in high-throughput screens of random genes to identify those that participate in plant immunity [105–108]. These techniques depend on the plant silencing machinery targeting an endogenous gene. Efficient silencing can be achieved by overexpression of an intron-spliced hairpin RNA (hpRNA) construct [109]. Alternatively, in the VIGS approach, silencing is achieved by overexpressing a fragment with high degree of homology to

Table 1: Tomato immune-related genes identified through functional genomics

Gene name	*ID*	*Classification*	*Proposed function/process*	*Methodology/strategy*	*Criteria for candidate gene selection*	*Time-point*[a]	*Follow-up or screening experiments' strategy*[b]	*Pathogen type*	*Reference*
SlWRKY72	Solyc03g113120 Solyc06g070990	Transcription factor	Transcriptional activation downstream of *Mi-1* resistance gene	Microarray TOM1	Transcriptional induction in incompatible interaction	24 hpt	VIGS	Nematode	[73, 74]
SlWAK1	Solyc09g014720	Cell wall associated kinase	PTI against *Pst*	RNA-Seq (HiSeq2000)	Transcriptional induction by flgII-28 and suppressed by AvrPto and/or AvrPtoB at 6 hpt	6 hpt	VIGS	Bacterium	[84]
SlEpk1	Solyc12g009340	GmPK6/AtMRK1-like protein kinase	AvrPto and HopQ1-1 mediated ETI activation	RNA-Seq (HiSeq2000)	Specific ETI (AvrPto and/or AvrPtoB) transcriptional induction at 6 hpt	6 hpt	VIGS	Bacterium	[85]
SlFLS3	Solyc04g009640	RLK	Receptor of flgII-28 epitope from flagellin	Crosses of flgII-28 responsive and non-responsive accessions, followed by DNA-Seq (HiSeq2500)	RLK within a region enriched for SNPs in chromosome 4	NA	Transient overexpression in non-responsive species	Bacterium	[100]
NA	Solyc05g009760	NBS-LRR	Resistance to TYLCV	RNA-Seq (HiSeq2000)	NBS-LRR induced in resistant cultivar	3, 5, 7 dpt pool	VIGS	Virus	[80]
SlMYC1 *SlWRKY73*	KF430611[c] Solyc03g113120	MYC TF WRKY TF	Terpene biosynthesis regulation	RNA-Seq (Genome Analyzer II)	Transcritionally active TFs in trichomes	24 hpt	Transactivation of terpene synthase promoters	Insect	[83]
Permease 1-like	Solyc03g114030	Permease 1-like protein	Resistance to TYLCV	Screening of differentially expressed cDNA library	Higher expression levels in resistant cultivar	1, 3, 5, 7 dpt pool	VIGS	Virus	[53]
LeHT1	Solyc02g079220	Hexose transporter	Resistance to TYLCV	Screening of differentially expressed cDNA library	Higher expression levels in resistant cultivar	1, 3, 5, 7 dpt pool	VIGS	Virus	[53, 54]
SlVRSLip	Solyc10g074580	Lipocain-like protein	Resistance to TYLCV	Screening of differentially expressed cDNA library	Higher expression levels in resistant cultivar	1, 3, 5, 7 dpt pool	VIGS	Virus	[53, 55]
Spr2 (LeFAD7)	Solyc06g051400	Fatty acid desaturase	JA biosynthesis, resistance to tobacco hornworm larvae	Mutagenesis in 35S::*prosys* background followed by map-based cloning	Mutation reverted 35S::*prosys* phenotype	NA	Mutant's susceptibility to tobacco hornworm larvae	Insect	[30, 32]
Spr8 (TomLoxD)	Solyc03g122340	Lipoxygenase	JA biosynthesis, resistance to cotton bollworm larvae and *Botrytis cinerea*	Mutagenesis in 35S::*prosys* background followed by map-based cloning	Mutation reverted 35S::*prosys* phenotype	NA	Overexpression of candidate gene	Insect/fungus	[30, 33]
SlGRAS6	Solyc04g064550	GRAS transcription factor	Resistance to *Pst*	GeneCalling, EST search in publicly available databases and microarray analysis	Induction of GRAS gene family members by *Pst* and *Xcv*	2, 4, 8 hpt	VIGS	Bacterium	[67, 70]
ACIK1	Solyc06g062920	RLCK	Resistance to *Cladosporium fulvum* (Cf4/Avr4 and Cf9/Avr9 signalling pathway)	AFLP-based identification of genes differentially expressed upon Avr9 perception	Rapid transcription activation upon Avr9 perception	30 mpt	VIGS	Fungus	[58, 60]
Rcr3	Solyc02g076980	Cystein protease	Resistance to *Cladosporium fulvum* (Cf2/Avr2)	Mutagenesis in resistant Cf-2 background, followed by positional cloning	Gene envolved in Cf-2 pathway	NA	Complementation by expression under native promoter in *rcr3* mutant line	Fungus	[36, 37]

(Continued)

Table 1. Continued

Gene name	*ID*	*Classification*	*Proposed function/process*	*Methodology/strategy*	*Criteria for candidate gene selection*	*Time-point*[a]	*Follow-up or screening experiments' strategy*[b]	*Pathogen type*	*Reference*
APR134	Solyc10g074740	Calmodulin-like protein	Resistance to *Pst*	GeneCalling	Transcriptional induction in incompatible interaction	2, 4, 8 hpt	VIGS	Bacterium	[67, 69]
LePP2Ac1	Solyc05g006590	Phosphatase	De-sensitation of HR response due to effector protein recognition from *Pst* and *Cladosporium fulvum*	GeneCalling	Transcriptional induction in incompatible interaction	2, 4, 8 hpt	VIGS	Bacterium and fungus	[67, 68]
NRC1	Solyc01g090430	CC-NBS-LRR	Resistance to *Cladosporium fulvum* (Cf4/Avr4 signalling pathway)	AFLP-based identification of genes differentially expressed upon Avr4 perception	Transcriptional induction upon Cf4/Avr4 signalling pathway activation	30, 60, 90 mpt	VIGS	Fungus	[62, 64]
ACRE276	Solyc02g072080	E3 ubiquitin ligase	Resistance to *Cladosporium fulvum* (Cf9/Avr9 signalling pathway)	AFLP-based identification of genes differentially expressed upon Avr9 perception	Rapid transcription activation upon Avr9 perception	30 mpt	VIGS	Fungus	[58, 61]
aatAGP	Solyc08g078020	Arabinogalactan protein	*Cuscuta reflexa* adherence	Screening of differentially expressed cDNA libraries	Transcriptional induction upon infection	12 hpt	VIGS	Parasitic plant	[56]
TPX1	Solyc07g052510	Cell wall peroxidase	Resistance to RKN	LCM and microarray-based transcripome analysis of giant cells	Trancriptional suppression in giant cells	3, 7 dpt	Overexpression of candidate gene in susceptible line	Insect	[76]
Cf-9	U15936	membrane-anchored LRR	Resistance to *Cladosporium fulvum* (Cf9/Avr9 signalling pathway)	Transposon tagging	Tagging allowed survival of lines carring Avr9 and Cf-9	NA	Necrosis observation in variegating plant's leaves	Fungus	[34]
Prf	Solyc05g013280	NBS-LRR	Resistance to *Pst*	Mutagenesis in resistant line carrying the *Pto* locus, followed by map-based cloning	Mutation affected resistance to *Pst*	NA	Mutant phenotype complementation	Bacterium	[23, 24]
NA	Solyc08g075630 Solyc08g076000 Solyc05g008650 Solyc09g018220	Full or partial NBS-containing proteins	Resistance to *Fusarium oxysporum*	Differential microRNA abundance comparing challenged susceptible and resistant lines. Small-RNA sequencing (Illumina GSII)	Target of microRNA down-regulated in resistant cultivar	24 hpt	VIGS	Fungus	[92]

Examples are shown in chronological order, considering the year of publication.

AFLP, amplified fragment length polyomorphism; CC-NBS-LRR, coiled coil-nucleotide binding site-leucine rich repeat; EST, expressed sequence tag; ETI, effector triggered immunity; HR, hypersensitive response; JA, jasmonic acid; LCM, laser capture microdissection; (m, h or d)pt; minutes, hours or days post treatment; NA, not available; PTI, pattern-triggered immunity; *Pst*, *Pseudomonas syringe* pv. *tomato*; RLCK, receptor-like citoplasmatic kinase; RLK, receptor-like kinase; RKN, root-knot nematode; TF, transcription factor; TYLCV, tomato yellow leaf curl virus; SNP, single nucleotide polymorphism; VIGS, virus-induced gene silencing; *Xcv*, *Xantomonas campestris* pv. *vesicatoria*.

[a]Time-point used if candidate gene selection was based on differential expression.

[b]Initial strategy to test candidate gene's function.

[c]No gene model is currently available at SOL genomics database (http://solgenomics.net).

the selected gene, by using virus-derived vectors [110]. VIGS technique offers a great potential to study gene function not only in biotic and abiotic stresses, but also in other processes such as flower and fruit development [111]. Even though tomato is relatively amiable to VIGS, *N. benthamiana* shows a higher silencing efficiency [112, 113] and consequently has been widely used to investigate gene function. In addition, *N. benthamiana* genome has been made publicly available [114–116], further enhancing its use as a model for plant research.

The SOL Genomics Network (SGN) [117, 118] hosts a Web-based tool, the SGN VIGS tool, which helps in the selection of regions for VIGS construct design ([119]; http://vigs.solgenomics.net/). This tool allows the identification of regions with higher target/off-target ratios and also integrates the results with expression data to better predict potential silencing.

The current state, challenges and limitations of PTGS are described elsewhere [110, 120–122]. One of these limitations is that phenotypic characteristics may not be observable owing to functional redundancy of members from the same protein family, which are not efficiently targeted by the selected construct. A case of such redundancy was observed in Arabidopsis, in which progressive knocking-out of members of a calcium-dependent protein kinase family lead to a decrease in plant defense [123]. Cloning strategies for assembly of multimeric constructs, relying on type IIS restriction enzymes, have been recently developed for hpRNA-mediated silencing [124, 125]. We are currently working on a strategy amenable to VIGS, based on PRIVAS (programmable or random *in vivo* assembly shuttle) [126] that will allow concatenation of several fragments into a single construct. We believe this approach will help address gene functional redundancy.

Tools and resources for functional genomics in tomato and *N. Benthamiana*

Many ambitious large-scale sequencing projects are currently underway that will positively impact functional genomics of tomato and related species. The 150 Tomato Genome ReSequencing project (http://www.tomatogenome.net/index.html), a common effort between the public and private sector, aims to explore tomato genetic variation. The SOL-100 sequencing project is planned to gain insight into variation among *Solanaceae* species (http://solgenomics.net/organism/sol100/view). Another project in progress, the Tomato Expression Atlas, will provide the community with tissue-specific expression data obtained using laser dissection [118].

Valuable Web resources are available that can assist with the research in this field: SOL Genomics (SGN, http://solgenomics.net) [118], Tomato Functional Genomics Database (TFGD, http://ted.bti.cornell.edu)[72], Tomato Genomics Resources Database (http://59.163.192.91/tomato2/index.html) [127], Kazusa Tomato Genomics Database (KaTomicsDB, http://www.kazusa.or.jp/tomato), TOMATOMICS (http://bioinf.mind.meiji.ac.jp/tomatomics), Plant Expression Database (PLEXdb, http://www.plexdb.org/index.php) [128], Tomato Genetics Resource Center (TGRC, http://tgrc.ucdavis.edu). In addition, TOMATOMA is a source for mutants of the dwarf cultivar Micro-Tom (http://tomatoma.nbrp.jp) [129], and TomExpress (http://gbf.toulouse.inra.fr/tomexpress/www/welcomeTomExpress.php) is a platform that allows the visualization of the RNA-seq data available for tomato.

The model species *N. benthamiana* has been used frequently, combined with VIGS, as a first step for dissecting the function of candidates genes identified in tomato. The community has benefited from the available draft genome sequence of this species [114–116] and will certainly take full advantage of this plant model with future improved genome versions.

Resources have been developed to identify and characterize R genes in plant species that include tomato and other Solanaceous species. The plant resistance gene database (PRGdb; http://prgdb.crg.eu/wiki/Main_Page) that centralizes information from 233 plant species [130, 131] has been used to study evolution, spatial and phylogenetic relationships of R genes [132]. Further putative R genes were identified by integrating the information of candidate R genes with known molecular markers associated to resistance phenotypes [133].

The availability of the tomato genome sequence also allowed the identification and analysis of WRKY transcription factors. Phylogeny-based comparison with WRKYs from other species allowed the identification of clusters that are species-specific, which may shed light on differential gene expression regulation [134].

Conclusion and future prospects

Research has historically focused on genes whose expression is increased during a specific response (Table 1). As a result, aspects of the plant response associated with decreased transcript abundance have been less explored. We are currently studying candidate genes whose expression is decreased on treatment with flgII-28 and is induced by the activities of bacterial effectors AvrPto and AvrPtoB [84]. Such genes may play a role in promoting susceptibility of the plant to pathogen attack.

Plant organs are a complex combination of tissues, but are often regarded as homogeneous material for experimental purposes in the tomato-pathogen research field. As a consequence, key differentially expressed genes may have been overlooked when analyzing such complex tissue mixtures. The advent of NGS and microdissection techniques [135] can help in overcoming this issue.

During the preparation of this review, we identified, when possible, the accession number of all the described genes (Table 1), in accordance with current tomato gene models. We encourage the community to use this unified nomenclature, for we believe it will help better coordinate research efforts.

Functional genomics research has benefited from numerous methodologies that were developed for studying differential gene expression. These technical advances, along with the availability of the tomato genome sequence, have allowed researchers to go from having <100 differentially expressed genes [53, 56] to several thousands [84, 85]. This deeper assessment of the tomato transcriptome presents the challenge of developing more efficient candidate-screening strategies and novel ways of data analysis to increase the chance of identifying genes with a role in plant immunity.

Key points

- Tomato, along with other members of the Solanaceae, constitutes a group of plants with great economical value and consequently it is important to understand the mechanisms underlying their immune responses.
- Current and future knowledge generated using tomato as a model has the potential of being transferred to other members of the Solanaceae.
- Differential gene expression approaches have been fruitful for the identification of novel components of the tomato immune system. Development of

next-generation sequencing techniques, along with the availability of high-quality genomes, has enhanced the research in this field.
- Virus-induced gene silencing has proven to be a powerful high-throughput method of assessing the function of genes in tomato and most importantly *Nicotiana benthamiana*, a related model plant species.

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