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Analysis of the tomato leaf transcriptome during successive hemibiotrophic stages of a compatible interaction with the oomycete pathogen *Phytophthora infestans*

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SUMMARY

The infection of plants by hemibiotrophic pathogens involves a complex and highly regulated transition from an initial biotrophic, asymptomatic stage to a later necrotrophic state, characterized by cell death. Little is known about how this transition is regulated, and there are conflicting views regarding the significance of the plant hormones jasmonic acid (JA) and salicylic acid (SA) in the different phases of infection. To provide a broad view of the hemibiotrophic infection process from the plant perspective, we surveyed the transcriptome of tomato (*Solanum lycopersicum*) during a compatible interaction with the hemibiotrophic oomycete *Phytophthora infestans* during three infection stages: biotrophic, the transition from biotrophy to necrotrophy, and the necrotrophic phase. Nearly 10 000 genes corresponding to proteins in approximately 400 biochemical pathways showed differential transcript abundance during the three infection stages, revealing a major reorganization of plant metabolism, including major changes in source–sink relations, as well as secondary metabolites. In addition, more than 100 putative resistance genes and pattern recognition receptor genes were induced, and both JA and SA levels and associated signalling pathways showed dynamic changes during the infection time course. The biotrophic phase was characterized by the induction of many defence systems, which were either insufficient, evaded or suppressed by the pathogen.

Keywords: hemibiotrophic interaction, *Solanum lycopersicum*, transcriptome.

INTRODUCTION

Plants have evolved an array of mechanisms to detect and respond to a wide range of potential pathogens (Cohn *et al*., 2001). One of these is known as pathogen-associated molecular pattern-triggered immunity (PTI), and involves the recognition of structurally conserved molecules, termed microbial- or pathogen-associated molecular patterns (MAMPS or PAMPS, respectively), derived from oomycetes, bacteria and fungi, using transmembrane pattern recognition receptors (PRRs) (Jones and Dangl, 2006). PRRs comprise a class of leucine-rich repeat (LRR)-receptor kinases (RKs), examples of which include β-glucan-binding protein (GBP) (oomycete perception), Flagellin-sensing 2 (FLS2), Xa21 and the elongation factor-Tu (EF-Tu) receptor (EFR) (bacterial perception), and ethylene-inducing xylanase (EIX1/2) and LysM RK (fungal perception) (Boller and Felix, 2009). Some PRRs are present in diverse plant families (e.g. FLS2), whereas others are apparently limited to specific plant taxa. An example of the latter is EFR, which has only been found in the Brassicaceae family, although LRR-RK homologues of EFR are present in the poplar and rice genomes (Boller and Felix, 2009). Indeed, the discovery that heterologous expression of an EFR from *Arabidopsis thaliana* in tomato (*Solanum lycopersicum*) and *N. benthamiana* confers broad-spectrum resistance to bacteria (Lacombe *et al*., 2010) suggests that downstream elements of PRR resistance are conserved in these members of the Solanaceae family.

In response to these PTI defence systems, pathogens have evolved so-called effectors, secreted proteins and other molecules that suppress plant basal defence strategies and facilitate infection (Schneider and Collmer, 2010). However, as a countermeasure, plants can, in turn, deploy intracellular defence mechanisms that either directly or indirectly recognize pathogen effectors (McHale *et al*., 2006). Molecular recognition of pathogen effector proteins is typically mediated by host proteins with nucleotide-binding sites (NBSs) and LRRs that are encoded by resistance (*R*) genes (Jones and Dangl, 2006). The resulting resistance is referred to as effector-triggered immunity (ETI), and typically results in a rapid induction of defence responses that often triggers a localized programmed cell death, known as the hypersensitive response (HR) (Jones and Dangl, 2006). Following HR,

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the accumulation of salicylic acid (SA) stimulates systemic acquired resistance (SAR), whereby uninfected parts of the plant develop resistance to infection by some pathogens (Martin, 1999; Yang *et al*., 1997).

Induced resistance pathways are regulated by the phytohormones SA, jasmonic acid (JA) and ethylene (ET), which trigger substantial changes in plant host gene expression (Glazebrook, 2005). A current model of host resistance posits that the defence mechanisms deployed by plants depend on the type of pathogenicity (Glazebrook, 2005). According to this model, plants respond to biotrophic pathogens, which require living host cells for survival, by activating the SA signalling pathway, whereas responses to necrotrophic pathogens, which kill host cells, are mediated by the JA and ET signalling pathways (Glazebrook, 2005). Hemibiotrophic pathogens employ both infection strategies, suppressing or avoiding plant defences during early stages of infection, thereby prolonging the biotrophic (asymptomatic) stage, and then undergoing a physiological change, becoming necrotrophs and rapidly killing the adjacent host tissues (Baxter *et al*., 2010). Accordingly, when plants encounter hemibiotrophic pathogens, the SA pathway is induced at the early stages of the interaction, and the JA and ET pathways are activated at later stages of infection (Glazebrook, 2005).

Phytophthora infestans is a hemibiotrophic pathogen that causes late blight, one of the most devastating diseases of both potato (*Solanum tuberosum*) and tomato (Fry, 2008). Reflecting its agricultural importance, many studies have sought to identify the mechanisms by which potato and tomato respond to *P. infestans* and the factors that are associated with the various stages of its infection (Fry, 2008; Kamoun and Smart, 2005; Rietman *et al*., 2012). For example, differences have been noted in the duration of the biotrophic phase of *P. infestans* when comparing infection between highly specialized tomato isolates, which have a longer biotrophic phase in tomato than in potato (Berg, 1926; Cai *et al*., 2013; Smart *et al*., 2003; Vega-Sanchez *et al*., 2000). Specifically, although necrosis is seen at the early stages of pathogenesis in both partially compatible and highly compatible *P. infestans*–potato interactions, the interaction between tomato and *P. infestans* can vary considerably, with a more prolonged biotrophic phase in a highly compatible than in a partially compatible interaction (Smart *et al*., 2003). In addition, Cooke *et al*. (2012) demonstrated differences in the biotrophic phase that correlated with pathogen aggressiveness among *P. infestans* genotypes against potato.

Despite such insights into the pathology and physiology of the interactions, there is still little understanding of the numerous underlying molecular responses of tomato and potato to this hemibiotrophic pathogen, particularly when contrasting the three different pathogenicity stages (biotrophic, transition and necrotrophic). Examples are the poorly resolved roles of SA and JA in the responses of potato and tomato to *P. infestans*. Although it has been reported that SA-deficient transgenic potato lines are more susceptible to *P. infestans* (Halim *et al*., 2007), it has been suggested that this is not the case for JA-deficient potato (Halim *et al*., 2009). In addition, JA production is not induced in either susceptible or partially resistant potato cultivars after *P. infestans* inoculation (Gobel *et al*., 2002; Weber *et al*., 1999). However, JA is induced in an incompatible interaction between *Pseudomonas syringae* pv. *maculicola* and potato, leading to the hypothesis that JA-mediated defence responses occur only in non-host pathogen interactions (Gobel *et al*., 2002). Moreover, a case of partial resistance of tomato to *P. infestans* has been shown to be independent of SA, JA and ET (Smart *et al*., 2003), although Cohen *et al*. (1993) found that the induction of JA enhanced the resistance of tomato and potato to *P. infestans*, and Thaler *et al*. (2004) demonstrated that JA-deficient tomatoes were more susceptible. Such studies underline the complexity of the interactions and indicate that much remains to be learnt about the key molecular determinants and associated signalling pathways that influence the outcome of pathogen challenge and progression of infection.

To gain a better understanding of the molecular mechanisms that underlie the defence response of tomato against *P. infestans*, we evaluated the transcriptome of tomato leaves during a compatible interaction with the tomato-specialized isolate *P. infestans* (*US11*) using a 454-based RNA-Seq strategy. We describe major changes in the transcriptome of the host plant during the biotrophic, transition to necrotrophic and necrotrophic phases of infection, and present these data in the context of altered plant metabolism, physiology and hormone signalling.

RESULTS

Defining the time frame of the tomato–*P. infestans* interaction

We conducted a preliminary experiment to identify the appropriate time points at which to assay gene expression during the different stages of the transition from biotrophic to necrotrophic growth of *P. infestans* in tomato. In this preliminary experiment, we inoculated detached leaflets of the tomato cultivar M82 with the tomato-specialized isolate *P. infestans* of the *US11* clonal lineage. We observed tissues at 12-h intervals (from 12 to 144 h after inoculation), both macroscopically and microscopically after staining the tissue with trypan blue to visualize pathogen development inside the leaflets (see Zuluaga *et al*., 2015). In addition, we analysed the expression of three *P. infestans* genes used as markers for biotrophy (*IpiO* and *SNE1*) and necrotrophy (*PiNPP1.1*). From these analyses, we determined that, at 48 h after inoculation (hai), the interaction was biotrophic, and that, at 144 hai, the interaction was necrotrophic. The transition occurred during that interval, and we selected 96 hai as the time point representing the transition.

Table 1 Summary of the number of 454 reads and BLASTX hits to the Sol Genomics Network (SGN).

Sample	Total number of reads	High-quality reads	Length average (nt)	BLASTX hits to tomato genome (e-value ≤ 9e-7)
48_{mock}	171 569	168 217	229	151 137
48 hai	248 172	245 631	225	224 966
96 hai	154 842	151 863	209	128 706
144 hai	187 459	184 620	201	102 230

hai, hours after inoculation; nt, nucleotides; 48_{mock}, mock inoculation.

Mock-inoculated tomato leaves were collected at 48 hai and used as a negative control (see Zuluaga *et al*., 2015). cDNA libraries were constructed from 7-mm-diameter leaf discs obtained from the inoculation sites at 48, 96 and 144 hai and from 7-mm discs obtained from the mock-inoculated control, which were sequenced on a 454 GS FLX system to generate transcriptome profiles of both pathogen and host over the infection time course. A summary of the number and average length of tomato reads after sequence filtering to remove short-length (<30 bp) polyadenylation sequences and low-quality sequences, including those screened against the National Center for Biotechnology Information (NCBI) UniVec database, *Escherichia coli* genome, is shown in Table 1. Approximately 98%–99% of the sequences from each sample were determined to be of high quality. In both the water control (48_{mock}) and 48-hai samples, an estimated 90% of the sequences showed a match to existing tomato unigenes in the Sol Genomics Network (SGN) database. There was a slight increase (~2%) in the number of tomato RNA-Seq reads that could be matched to the tomato genome at 48 hai compared with the water control, and one-half of these (~1%) correspond to plant biotic stress-responding genes. At 144 hai, when the leaf tissue was necrotic, more than one-half of the reads (55%) originated from tomato (Table 1).

Tomato transcriptome analyses

The combined 454 sequencing of cDNAs derived from infected tomato leaves at the three different stages and from the mock-inoculated control generated a total of 93 978 unigenes, 67 973 of which mapped to the annotated tomato gene regions (Table S1, see Supporting Information) and 26 005 of which did not (Table S1). After data integration, we identified 20 177 annotated tomato genes that corresponded to the 454 reads (Table S1). For statistical purposes, we only analysed unigenes that had at least five reads in at least one time point, reducing the list to 16 106 genes, 14 648 of which were annotated genes and 1458 of which were unmapped unigenes (filtered file, Table S1). (Sequence data described in this article can be found in the GenBank Sequence Read Archive (SRA) under accession number SRP041501. In addition, the fasta files can be accessed at: http://solgenomics.net/download/data/secretom/Secretome_Phytophthora_tomato_interactions/Tomato_unigenes.fasta.). All the analyses described here were based on the data in this filtered file.

The significance of differential gene expression was determined using the R statistical package (Stekel, *et al*., 2000), and the resulting raw *P* values were adjusted for multiple testing using the false discovery rate (FDR) (Benjamini and Hochberg, 1995) (see Experimental procedures). A gene was considered to have a differential transcript abundance if the observed change in transcript abundance was >2.0 and FDR < 0.05 (Benjamini and Hochberg, 1995). Using these criteria, we designated 9608 genes (59.6% of the filtered file) as being differentially expressed (Table S2, see Supporting Information).

Comparison between hormone-associated transcript abundance and hormone concentrations during the *P. infestans*–tomato compatible interaction

To obtain support for the validity of the RNA-Seq results, we compared the patterns of transcript abundance associated with the defence signalling hormones JA and SA with the pattern of hormone concentrations using liquid chromatography-tandem mass spectrometry (LC-MS/MS). In comparison with the mock-inoculated control at 48 hai, there was a modest accumulation of transcripts associated with the JA pathway (Fig. 1A). JA-associated transcript abundance correlated with a three-fold increase in the amount of JA, as detected by LC-MS/MS (Fig. 1B). An even larger increase in SA-related transcripts was detected throughout the interaction when compared with the mock-inoculated samples (Fig. 1C), which was associated with a five-fold increase in the amount of the SA phytohormone in comparison with the mock-inoculated control (Fig. 1D). Gene transcript abundance was consistent with the concentrations of JA and SA over the time course of the experiment. Thus, the results of the RNA-Seq-based transcript abundance analysis were consistent with the accumulation of both JA and SA.

Transcriptome dynamics in tomato leaves infected with *P. infestans*: biotrophic phase

Numerous changes associated with a broad range of structural and regulatory pathways were observed in the tomato transcriptome at 48 hai compared with the water control (48mock). At this time point, the plants were symptomless [Fig. 2A; photographs are magnifications of those in the inset of

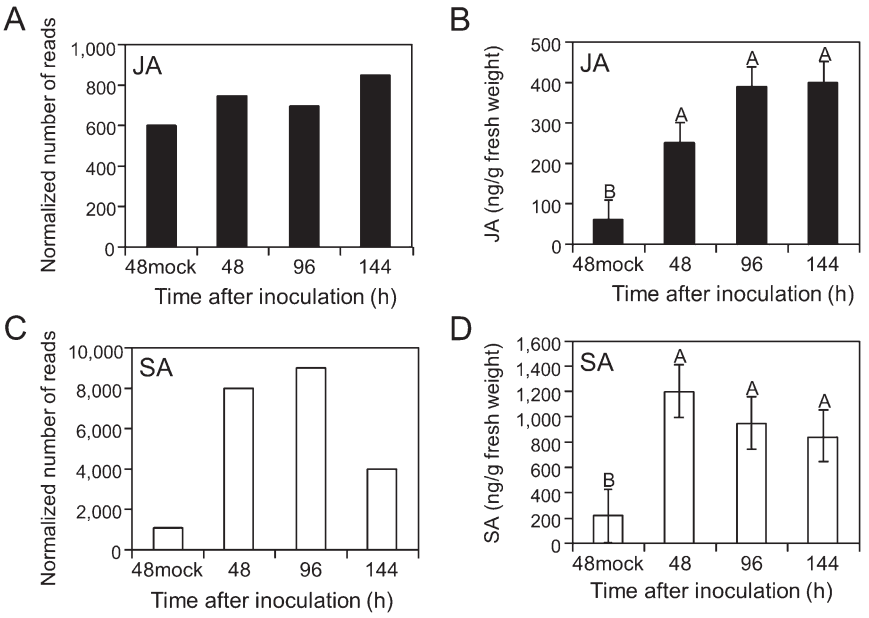

Fig. 1 Phytohormone-associated transcripts and concentration changes during pathogenicity. (A, C) Normalized number of reads for phytohormone-associated genes previously described to be involved in the synthesis of jasmonic acid (JA) and salicylic acid (SA). (B, D) JA and SA quantification by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis ($n = 3$). The data were transformed with $\log_{10}$ and Student's *t*-test was used to analyse the difference in phytohormone levels. Letters represent significant differences at $P < 0.05$.

fig. 1 in Zuluaga *et al*. (2015) and depict the macroscopic symptoms on the leaflets at the inoculation site]. We determined that 4104 genes were differentially expressed, with 2079 being up-regulated and 2025 being down-regulated (for a complete list of up- and down-regulated genes, see Table S2). Approximately 12% of the genes induced more than two-fold at 48 hai were classified as 'unknown', 'predicted', 'hypothetical' or 'unnamed'. This suggests that many genes associated with highly specialized processes are expressed during the biotrophic phase of the interaction.

Tomato genes encoding proteins in 420 biochemical pathways responded to pathogen infection by 48 hai, including 55 pathways which, compared with the control, were only altered at this stage (Fig. 2B). Pathways that were either induced or repressed after pathogen inoculation included those involved in osmotic regulation (proline, glutathione, trehalose biosynthesis), cell structure (suberin biosynthesis) and phytohormone production [for a complete list of the differentially expressed pathways, see Table S3 (Supporting Information)]. These data indicated a major change in several areas of primary plant metabolism during biotrophy. The gene ontology (GO) classification of the differentially expressed pathways is shown in Fig. 3 and Table S4 (see Supporting Information). The most abundant categories of down-regulated genes included biosynthetic, metabolic and cellular processes (Fig. 3A), whereas transport, response to abiotic stimulus and response to stress were some of the most abundant categories for the up-regulated genes (Fig. 3B). Transcripts associated with photosynthesis were less abundant when compared with the mock-inoculated plants. The expression of photosynthesis-related genes, including ribulose bisphosphate carboxylase (RuBisCO), chlorophyll *a/b*-binding protein and photosystem *b* genes, showed a 50% reduction in transcript abundance (Fig. 4A and Table S4). This reduction was maintained for the duration of the interaction. In parallel, a reduction in the transcript accumulation of genes involved in glycolysis was noted (Fig. 4B). In contrast, there was an increase in transcripts related to fermentation (Fig. 4C) and mitochondrial metabolism, such as NADH dehydrogenase, proline oxidase, glycine cleavage, system H protein and mitochondrial electron transport complex III (Fig. 4D).

Some of the genes with the greatest change in transcript abundance at 48 hai have been suggested previously to be involved in the plant response to biotic stress (Table S2). These included genes encoding endo-β-1,3-glucanase [glycoside hydrolase (GH) family 17], chitinase (GH19), hevein-like protein and acidic thaumatin-like protein (Bowles, 1990). The increase in transcript abundance of some of these genes at 48 hai was over 200-fold when compared with the mock-inoculated plants (Table S2). The transcript abundance of some genes was substantially reduced during the biotrophic phase, including defensin (*PDF1.2*), chalcone synthase and genes involved in the production of reactive oxygen species (ROS) (Table S2).

Transcriptome dynamics in tomato leaves infected with *P. infestans*: transition phase

We used 96 hai to represent the transition phase and, at this time point, water soaking was evident in part of the tissue; 2287 genes were observed to be differentially expressed when comparing the transition stage (96 hai) with the biotrophic stage (48 hai, Fig. 2A), 1155 of which were up-regulated and 1132 were down-regulated. Genes in 349 pathways were affected

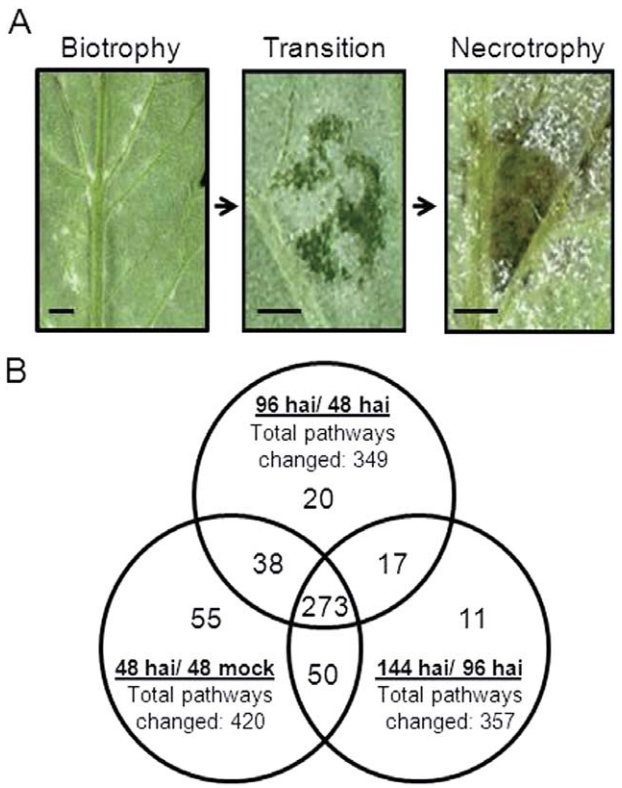

Fig. 2 Hemibiotrophic interaction between *Phytophthora infestans* and tomato with associated changes in the patterns of tomato gene expression. (A) Macroscopic visualization of the biotrophic [48 h after inoculation (hai)], transitional (96 hai) and necrotrophic (144 hai) stages of interaction of *P. infestans* with tomato (scale bar, 3 mm); the photographs are a magnification of the leaflets shown in Zuluaga *et al*. (2015). (B) Venn diagram showing the number of tomato genes with significant changes in transcript abundance, defined as at least a two-fold change in expression and a false discovery rate (FDR) ≤ 0.05, at 48, 96 and 144 hai.

(Fig. 2B; for a complete list of genes that were differentially expressed, see Table S2). The GO classification of the differentially expressed metabolic pathways is shown in Fig. 3A,B (see Table S4 for a complete list of GO annotations). During the transitional stage, the numbers of down-regulated or up-regulated genes were less than during the biotrophic or necrotrophic stages. Thus, this seems to be a stationary phase, where genes that were up-regulated after pathogen perception are either suppressed by the pathogen effectors or down-regulated by the plant as part of the defence response. Differences in 20 pathways were detected only at 96 hai, including those leading to the production of secondary metabolites, 2,3-dihydroxybenzoate biosynthesis, fatty acid biosynthesis, glutathione biosynthesis, purine biosynthesis, triacylglycerol degradation, urea degradation and xylose degradation. Differences associated with other pathways were also observed at 144 hai (Table S3). For example, transcription factors, bZIP, MYB and WRKY, were differentially increased at 96 hai and the differential increase continued until 144 hai (Jakoby *et al*., 2002; Yanhui *et al*., 2006; Zhang and Wang, 2005). The genes that showed the highest transcript abundance at 96 hai included endo-β-1,3-glucanase (GH-17), lipoxygenase, chitinase (GH-19) and *PR1* (Table S2). After an initial down-regulation of the defensin (*PDF1.2*) gene during the biotrophic stage, an increase in the accumulation of this gene was observed at this transition stage (Table S2). In addition to the primary metabolism genes, others that showed a major reduction in transcript abundance at this stage included those associated with ROS production, calcium sensing receptors and hypothetical proteins (for a complete list, see Table S2).

Transcriptome dynamics in tomato leaves infected with *P. infestans*: necrotrophic phase

Finally, during the necrotrophic phase (Fig. 2A), genes encoding proteins in 357 pathways were differentially expressed compared with the transition phase. Of these, differential expression of 11 pathways was first identified at this final time point (Fig. 2B; Table S3). At this time point, 3217 genes were differentially expressed when compared with the transitional stage. Of these, 1688 were down-regulated and 1529 were up-regulated. The GO classification of the differentially expressed pathways during the necrotrophic stage is shown in Fig. 3A (down-regulated) and 3B (up-regulated). Genes related to transcription and translation were down-regulated compared with the transition phase. In addition, pathways involved in the structural integrity of a complex within or outside the cell were down-regulated (Fig. 3A). In contrast, genes involved in cell death, endogenous and external stimuli, cell communication and abiotic responses were up-regulated at this stage (Fig. 3B). Most of the annotated genes showing the highest transcript abundance at 144 hai have been associated previously with plant–pathogen interactions and are involved in redox homeostasis and antioxidant signalling. These included glutathione *S*-transferase, ascorbate biosynthesis genes, dismutases, catalases and peroxidases (Foyer, 2005; Table S4). In addition, transcripts of genes involved in the production of secondary metabolites (Table S2) and those related to cell death were noticeably differentially abundant. The transcript abundance of genes involved in protein degradation via ubiquitination was highest at this time point, as was the transcript abundance of the bZIP, MYB and WRKY transcription factors.

Identification of components of resistance in response to *P. infestans*

To characterize the defence mechanisms induced after pathogen infection, we first investigated the expression of putative PRRs. An analysis with each predicted PRR protein using a Hidden Markov Model (HMMER) revealed that a total of 106 genes encoding

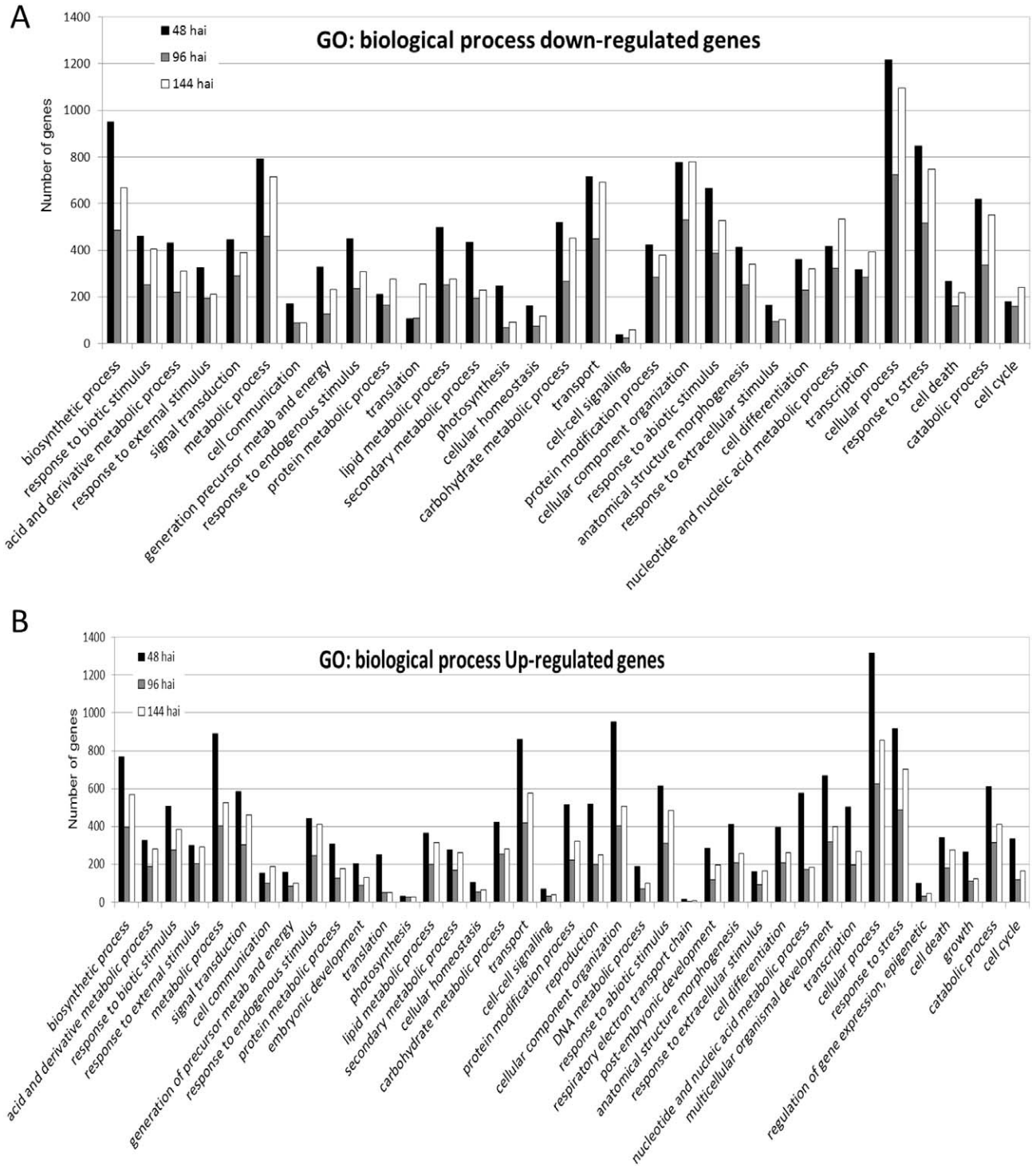

Fig. 3 Change in gene ontology (GO) biological process in tomato leaves during the interaction with *Phytophthora infestans*. Frequency of down-regulated (A) and up-regulated (B) GO biological processes during the biotrophic (black), transition (grey) and necrotrophic (white) stages of the interaction.

putative LRR-RK proteins were expressed at some point during the interaction. After selecting only the best matches (≤1.2e-10), we targeted 21 putative PRRs, which could be divided into seven groups (the number of homologues is indicated in parentheses): GBP (1), FLS2 (2), EFR (4), EIX (1), LysM (5), Xa21 (4) and NbLRK1 (4). Most (67%) of these putative PRRs are currently annotated as hypothetical proteins or unnamed proteins in the tomato database; the expression profiles of some of these are shown in Fig. 5.

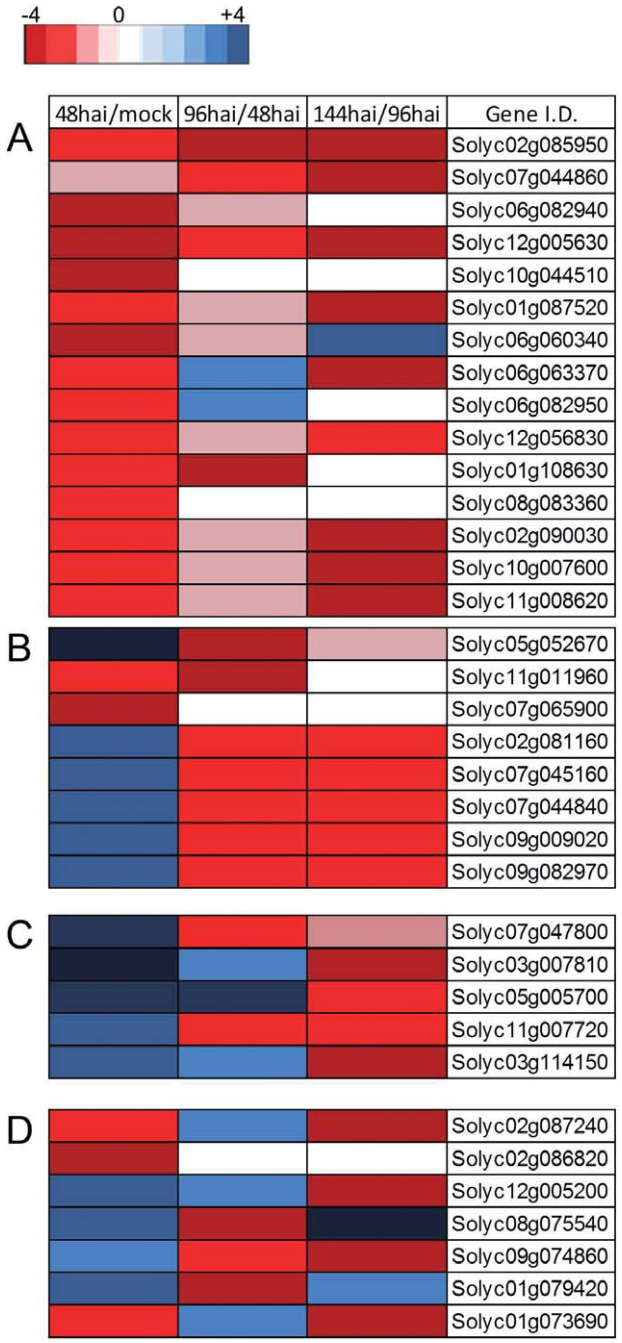

Fig. 4 Transcript abundance of primary metabolism-associated genes. Heat map of changes in transcript abundance of primary metabolism-associated genes after infection: photosynthesis-related genes (A), glycolysis (B), fermentation (C) and mitochondrial metabolism-related genes (D), generated using MapMan. The units are the $\log_2$ values of the changes from 48mock to the first 48 h after inoculation (hai), 48 to 96 hai and 96 to 144 hai. Red denotes down-regulated gene expression and blue denotes up-regulated gene expression, as shown in the colour panel indicator. Gene ID number is provided in the right column.

The transcript abundance of most of these putative receptors increased after *P. infestans* infection. However, within each category, the putative homologues showed differences in expression patterns at different phases of infection (Fig. 5).

The list of transcripts showing differential abundance included a total of 108 putative genes encoding resistance-like proteins with Toll-interleukin-like receptor/coiled coil (TIR/CC)-NBS-LRR domains, including specific examples associated with resistance to bacteria (*Pto* and *RPM1*; *Pseudomonas syringae*; Chang *et al*., 2002; Grant *et al*., 1995), oomycetes [the tomato homologues of *RPP8* and *RPP13* (*Hyaloperonospora arabidopsidis*; Bittner-Eddy and Beynon, 2001; Cooley *et al*., 2000) and a homologue to *RGA4* (*P. infestans*; Song *et al*., 2003)] and viruses (*N*-like gene; Whitham *et al*., 1996). The transcript abundance of most of these genes increased during the infection time course at least until 144 hai (Fig. 5). The transcript abundance of other components of the *R*-gene-mediated defence response, such as *Rar1* (Muskett *et al*., 2002), *EDS1* (Falk *et al*., 1999) and *NPR1*-like (Rairdan and Delaney, 2002), also increased early in the interaction.

DISCUSSION

Analysis of the tomato transcriptome during the interaction with *P. infestans* using the 454 pyrosequencing platform identified nearly 94 000 tomato unigenes, 24 093 of which were singletons. This number is substantially higher than the approximately 35 000 genes that are currently predicted in the tomato genome (The Tomato Genome Consortium, 2012). It is likely that many of these sequences are the product of alternative splicing or, alternatively, the unmatched sequences may reflect incomplete coverage of the tomato genome sequence, non-coding RNAs, fusion transcripts or untranslated region (UTR) sequences that were not included in the predicted genes. It is important to note that the number of unmatched sequences is consistent with other annotated genomes (cucumber, Guo *et al*., 2010; humans, Mane *et al*., 2009; Arabidopsis, Weber *et al*., 2007; *P. infestans*, this study; Zuluaga *et al*., 2015).

This study provides insights into the highly complex network of changes that occur in tomato during various stages of the hemibiotrophic infection, demonstrating that almost 10 000 genes encoding proteins in 400 pathways are responsive to inoculation/infection. Such information indicates a dramatic change in plant host physiology, even during the biotic stage of the interaction, when no symptoms of infection were apparent. Substantial reprogramming of both primary and secondary plant metabolism is suggested, with inferred metabolic changes of particular note including reduction in photosynthesis- and glycolysis-related transcripts, concomitant with an increase in transcripts related to starch degradation, fermentation and mitochondrial metabolism. This indicates a fundamental switch, wherein carbon is obtained from sink, rather than source, host

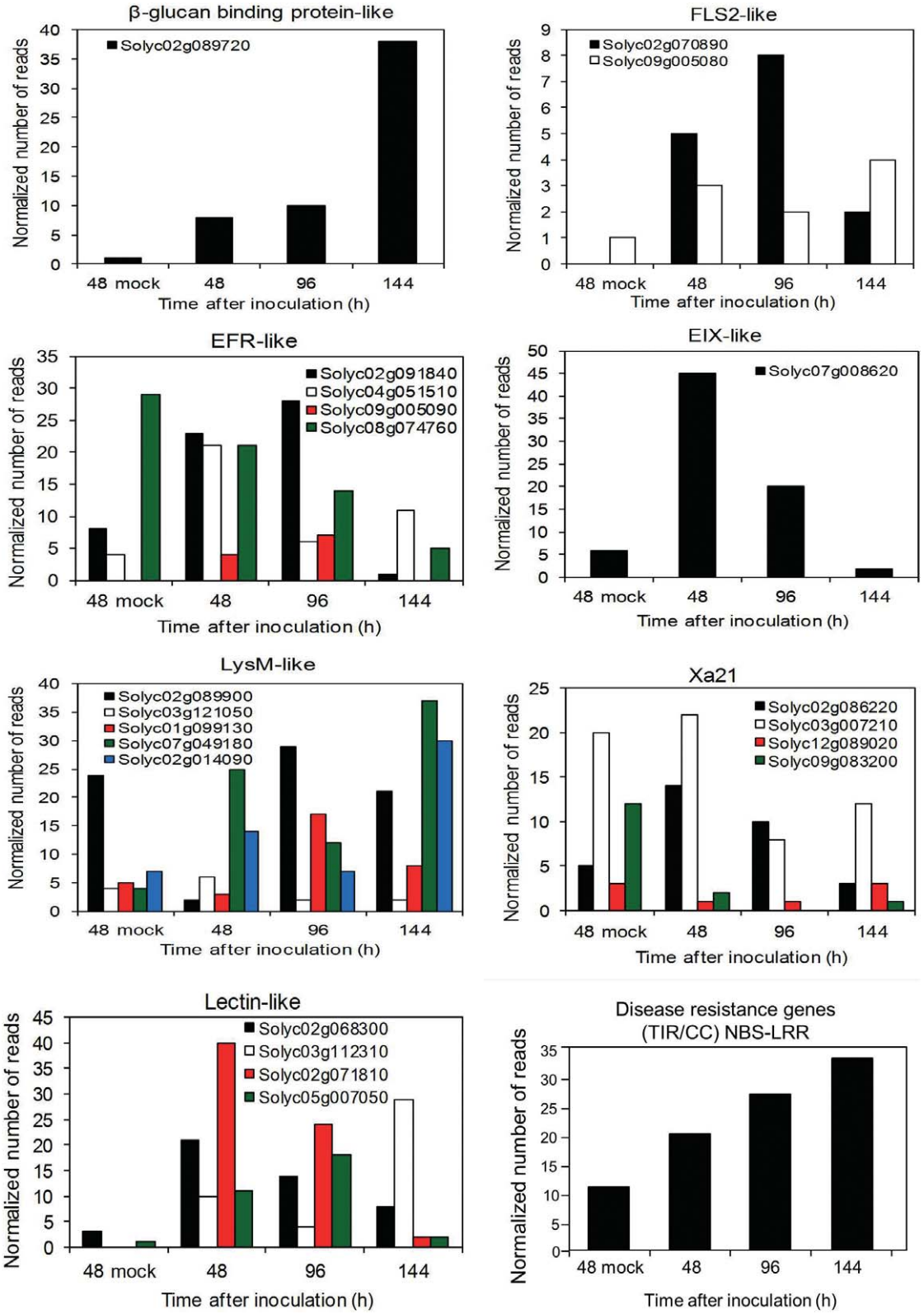

Fig. 5 Expression profiles of putative pathogen recognition receptors (PRRs) and putative resistance genes after pathogen infection, derived from the RNA-Seq data. Units are normalized to the number of reads. Gene numbers are indicated. EFR, elongation factor-Tu (EF-Tu) receptor; EIX, ethylene-inducing xylanase; FLS2, Flagellin-sensing 2; (TIR/CC)-NBS-LRR, (Toll-interleukin-like receptor/coiled coil)-nucleotide-binding site-leucine-rich repeat.

tissues. This finding is consistent with previous reports describing compatible interactions with both biotrophs and necrotrophs (Berger *et al*., 2007; Bolton, 2009; Jupe *et al*., 2013). Our data suggest that such changes occur during the biotrophic phase in a hemibiotrophic interaction. We again note that the reduction in photosynthesis-related gene expression at 48 hai is unlikely to be directly related to plant cell death, as no symptoms were apparent at this point. In addition, a group of transcription factor transcripts was accumulated throughout the infection, consistent with that observed in tomato on infection with *P. capsici* (Jupe *et al*., 2013).

Our data reveal the activation of multiple defence response mechanisms after pathogen infection. Putative tomato PRRs involved in basal defence were identified, most of which were annotated as hypothetical proteins and collectively showed variation in the timing of expression. This is comparable with a recent study of the tomato–*P. capsici* interaction, in which PAMP defence was induced on infection (Jupe *et al*., 2013). The abundance of some of the putative PRR transcripts increased throughout the interaction (e.g. GBP-like involved in oomycete recognition in soybean against *P. sojae*; Fliegmann *et al*., 2005). This late accumulation of transcripts, up to 144 hai, supports the idea of a potential sustained activation of PRRs (Lu *et al*., 2009). FLS2 and EFR have been implicated in bacterial perception by recognizing bacterial flagellin and bacterial EF-Tu, respectively (Gomez-Gomez and Boller, 2000; Zipfel *et al*., 2006). Although EFR has been found only in the Brassicaceae family (Segonzac and Zipfel, 2011), the identification of a putative EFR-like transcript in tomato is consistent with a report by Boller and Felix (2009), who found LRR-RK homologues to EFR encoded in the poplar and rice genomes.

One of the earliest plant responses to a pathogen is the induction of oxidative stress and ROS, which have been associated with calcium signalling, papillae formation, reinforcement of the cell wall, HR induction (Heath, 1998; Richberg *et al*., 1998) and SAR (Bolwell and Daudi, 2009; Torres *et al*., 2006). After pathogen infection, we noted a decrease in transcript abundance of genes associated with the production of ROS, which continued through the transition stage. A similar decline in ROS during the biotrophic stage was detected in a study investigating gene expression conducted in the field in the highly compatible *P. infestans*–tomato interaction (*US17*-M82; Cai *et al*., 2013). This contrasts with the *P. infestans* (*US17*)–tomato interaction involving the partially resistant IL6-2, where ROS were highly expressed as early as 36 hai (Cai *et al*., 2013). Recently, it has been shown that the cysteine protease effector HopN1 of the hemibiotrophic pathogen *Pseudomonas syringae* pv. *tomato* DC 3000 inhibits ROS production (Rodríguez-Herva *et al*., 2012), which is consistent with the suppression of plant cell death observed by López-Solanilla *et al*. (2004). The mechanism for ROS repression in the *P. infestans*–tomato interaction is not yet known, but we note that *P. infestans* expresses a cysteine protease at 48 hai that has a signal peptide, which suggests that it might be secreted (see Zuluaga *et al*., 2015). Given the activity of *P. syringae* HopN1, it would be interesting to investigate a potential association with ROS production and the suppression of cell death during the biotrophic stage.

Pathogens that are able to suppress or evade basal defence encounter another layer of defence, mediated by *R* genes. In this study, the transcript abundance of 108 *R*-like genes (homologues of TIR- and CC-NBS-LRR genes) increased after pathogen infection. However, in this case, they were not able to prevent pathogen infection. This might be because none of these *R*-gene products recognized any of the effectors secreted by this genotype of *P. infestans* or, if they were recognized, the defence responses triggered in the gene-for-gene interaction were blocked by another pathogen effector(s), as described by Halterman *et al*. (2010) for *ipiO1-RB*. Taken together, our data indicate that the plant activates an array of defence mechanisms by 48 hai, but, in this compatible interaction, *P. infestans* is able to overcome or avoid such defences.

Another important aspect of plant defence responses is their regulation by phytohormones, such as SA and JA. It has been reported that plants challenged by hemibiotrophic pathogens induce the SA pathway at early stages of the interaction and the ET/JA pathways at later stages (Glazebrook, 2005). In this study, although we measured a significant increase in SA levels at 48 hai, JA levels were also highly induced at the biotrophic stage and continued to increase throughout the interaction, including the necrotrophic stage. This correlated with patterns of related transcript accumulation. Accordingly, we inferred that the resistance pathways regulated by these phytohormones were induced, but the plant was unable to arrest pathogen growth. Similar results have been reported for the *Arabidopsis* response against a hemibiotrophic pathogen *Colletotrichum higginsianum*: after inoculation with a virulent strain, the expression of both *PR1* and *PDF1.2* genes was induced, suggesting that the SA and JA pathways were activated (Liu *et al*., 2007).

We note that the study generated data that are consistent with previous reports for genes that have been investigated, and our measurements of both JA and SA are also consistent with the RNA-seq data. Results using semi-quantitative PCR for certain genes (see Zuluaga *et al*., 2015) are also consistent with the RNA-seq data. We conclude that the RNA-seq profiling strategy is an effective means to identify genes potentially involved in the interaction.

CONCLUSIONS

A key outcome of this study was the characterization of the tomato leaf transcriptome over a time course of a compatible interaction with *P. infestans*. The data provide insights into the orchestration of global changes in plant host physiology, metabo-

lism and the activation of different components of the defence arsenal. Transcripts of genes for both PTI and ETI were abundantly expressed by 48 hai, indicating such responses during biotrophy, but also the suppression or evasion of these responses by the pathogen, as evidenced by the absence of symptoms. In addition, SA and JA levels increased after inoculation, suggesting that defences mediated by these phytohormones were also induced. A difference in JA response between tomato and potato during a compatible interaction with *P. infestans* was suggested as, although we determined that JA was induced in tomato throughout this compatible interaction with *P. infestans*, this was not the case in a compatible interaction between *P. infestans* and potato, where Gobel *et al*. (2002) found that JA did not accumulate. Future investigations will build on this dataset and target the functional significance of candidate regulatory and structural genes that have been revealed through this study to be expressed at specific stages of infection.

EXPERIMENTAL PROCEDURES

Plant material

Four-week-old tomato (*Solanum lycopersicum*, cv. M82) glasshouse-grown plants were used for inoculation with *P. infestans*. Natural light was supplemented with 400-W high-pressure sodium lamps for 12 h and temperatures were maintained between 24 and 29 °C. Plants were grown in a soil-less mix (Cornell mix) consisting of a 1 : 1 (v/v) peat–vermiculite mix supplemented with nitrogen, phosphorus and potassium (0.4 kg of each per cubic metre of mix).

Inoculum preparation and *P. infestans* isolate

Phytophthora infestans US-11 (US050007), a tomato-specialized isolate, was grown on detached tomato leaflets in order to ensure that the inoculum was robust and *P. infestans* was virulent, as axenically grown *P. infestans* inoculum loses virulence through time (Mizubuti *et al*., 2000). Sporangia were harvested in distilled water and the concentration was adjusted to 4000 sporangia/mL using a haemocytometer. Subsequently, the sporangia were incubated at 4 °C for 1 h to release zoospores. After 1 h at 4 °C, a mixture of sporangia and zoospores is typically produced as most of the sporangia germinate indirectly (forming zoospores), which we note is analogous to a typical field infection. Inoculation involved the application of a 20-μL drop of this mixture of sporangia and zoospores to the abaxial side of the leaflet, which was then placed in a Petri dish containing water agar as a moist chamber.

Assessment of the biotrophic, transition to necrotrophic and necrotrophic phases

The interaction between *P. infestans* and tomato was studied in a time course spanning 7 days in order to define the biotrophic, transition to necrotrophic and necrotrophic phases. To define the three pathogenicity stages, samples were collected every 12 h and analysed using three methods (described by Zuluaga *et al*., 2015) to define the three pathogenicity stages: (i) macroscopic observation of the tomato symptoms after *P. infestans* inoculation; (ii) microscopic observation of pathogen development *in planta* using trypan blue to stain *P. infestans* (Chung *et al*., 2010); and (iii) the use of *P. infestans* molecular markers that are specifically expressed at the biotrophic and necrotrophic stages of infection.

Tissue collection and RNA extraction for 454 sequencing

Tissue was collected from *P. infestans*-inoculated leaves at 48, 96 and 144 hai. These time points corresponded to the biotrophic, transition and necrotrophic stages, respectively (see Results). Mock-inoculated tissue (treated with a 20-μL drop of water) was collected at 48 h after the droplet had been added. Leaf discs from the drop inoculation sites were harvested using a paper hole puncher (diameter, 7 mm) and immediately frozen in liquid nitrogen. The use of small 7-mm-diameter leaf discs ensured that only necrotic tissue was assayed at the 144-hai time point (see Zuluaga *et al*., 2015). Twenty-five tomato plants per time point were used in each experiment. The experiment was repeated four times and the leaf discs from the four experiments were pooled (100 plants per time point). The pooled plant tissue was ground in liquid nitrogen using a mortar and pestle. Total RNA was extracted using the hot-phenol protocol of Perry and Francki (1992), as modified by Gu *et al*. (2000).

mRNA isolation and RNA amplification

mRNA was isolated from 250 ng of total RNA and amplified using a TargetAmp™ One-Round aRNA Amplification Kit 103 (Epicentre Biotechnologies Madison, WI, USA). First, poly-A RNA was transcribed from total RNA to generate first-strand cDNAs. The reaction was primed with a synthetic oligo (dT) primer containing a phage T7 RNA polymerase promoter sequence at its 5' end. The first-strand cDNA synthesis was catalysed by SuperScript III reverse transcriptase (Invitrogen Grand Island, NY, USA), generating a cDNA:RNA hybrid. The RNA component of the cDNA:RNA hybrid was digested using the RNase H enzyme, and the RNA fragments primed the second-strand cDNA synthesis. The resulting product was a double-stranded cDNA containing the T7 transcription promoter in an orientation that generated antisense RNA (aRNA). High yields of aRNA were produced in a rapid *in vitro* transcription reaction (amplified RNA) using the double-stranded cDNA (Epicentre Biotechnologies).

cDNA synthesis and FLX-454 sequencing

The first and second strands of cDNA were synthesized using the SuperScript® choice system for cDNA synthesis (Invitrogen), following the manufacturer's instructions. For each sample, first-strand cDNA was synthesized from three reactions of 5 μg of the amplified aRNA for a total of 15 μg of aRNA per sample, using 100 ng of random hexamers in each reaction. Once the second strand had been synthesized, the cDNA was cleaned using a PureLink™ PCR purification kit (Invitrogen) following the manufacturer's instructions, and a minimum of 9 μg of cDNA was recovered. cDNA library construction and 454 sequencing took place at the Cornell University Life Sciences Core Laboratories Center.

cDNA sequence processing and assembly

The raw 454 sequence files in SFF format were base called using the Pyrobayes base caller (Quinlan *et al*., 2008). The sequences were then processed to remove low-quality regions and adaptor sequences using the programs LUCY (Chou and Holmes, 2001) and SeqClean. The resulting high-quality sequences were then screened against the NCBI UniVec database, *E. coli* genome sequences. Sequences shorter than 30 bp were discarded. To distinguish *Phytophthora* transcript sequences from those of tomato, the cDNA sequences were aligned to the tomato genome in the SGN database. Sequences with at least 90% sequence identity and 50% length coverage were regarded as derived from tomato. The resulting tomato cDNA sequences were then assembled into unigenes using iAssembler (Zheng *et al*., 2011).

Unigene annotation and pathway prediction

Tomato unigenes were compared with sequences in GenBank using BLASTX non-redundant protein (Kent, 2002) and UniProt databases with a cut-off value of 1e-5. The unigene sequences were also translated into proteins using ESTScan (Iseli *et al*., 1999), and the translated protein sequences were then compared with InterPro and Pfam domain databases with a cut-off value of 1e-5. The GO terms were assigned to each unigene based on the GO terms annotated to corresponding homologues in the UniProt database (Camon *et al*., 2004), as well as those to InterPro and Pfam domains using interpro2go and pfam2go mapping files, respectively, provided by the GO website. Biochemical pathways were predicted from the tomato unigenes using Pathway Tools (Karp *et al*., 2002), and pathways that were altered on pathogen infection were identified using the Plant MetGenMAP system (Joung *et al*., 2009).

Identification of differentially expressed genes

Following cDNA sequence assembly, digital expression information of each unigene was derived following normalization to the total number of sequenced transcripts per sample. The 454 reads were normalized with the calculation: number of reads of a unigene from the specific sample $\times$ 100 000 (that is, the number of reads if 100 000 reads are collected)/total number of reads collected from that specific sample. The significance of differential gene expression was determined using the R statistic described in Stekel *et al*. (2000), and the resulting raw *P* values were adjusted for multiple testing using the FDR (Benjamini and Hochberg, 1995). Genes with a fold change greater than 2.0 and FDR $<$ 0.05 were considered to be differentially expressed. GO terms enriched in the set of differentially expressed genes were identified using GO::TermFinder (Boyle *et al*., 2004), requiring *P* values adjusted for multiple testing to be $<$0.05.

Hormone quantification

Jasmonate and salicylate quantification was performed as described by Thaler *et al*. (2010). Briefly, tomato leaves (100 mg) from mock-inoculated plants and plants inoculated with *P. infestans* were collected and frozen in liquid N_2 at 48, 96 and 144 hai. Frozen samples were transferred into 2-mL screw cap tubes containing 900 mg zirconia/silica beads (BioSpec, Bartelsville, OK, USA) and 1 mL of extraction buffer (1-propanol–H_2O–HCl, 2 : 1 : 0.002, v/v/v). d4-SA and d5-JA (CDN Isotopes, Point-Claire, QC, Canada) were added as internal standards, and samples were analysed on a triple-quadrupole LC-MS/MS system (Quantum Access, Thermo Scientific, Waltham, MA, USA).

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CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1 Filtered file with normalized number of reads from the tomato RNA-Seq data.

Table S2 Differentially expressed tomato genes at the three pathogenicity stages.

Table S3 Differentially expressed pathways at the three pathogenicity stages.

Table S4 Gene ontology (GO) classification of the differentially expressed pathways at the three pathogenicity stages.