Differential gene expression in tomato fruit and *Colletotrichum gloeosporioides* during colonization of the RNAi–*SlPH* tomato line with reduced fruit acidity and higher pH

Shiri Barad\(^1,2\), Noa Sela\(^3\), Amit K. Dubey\(^1\), Dilip Kumar\(^1\), Neta Luria\(^1\), Dana Ment\(^1\), Shahar Cohen\(^4\), Arthur A. Schaffer\(^4\) and Dov Prusky\(^1\)\(^*\)

**Abstract**

**Background:** The destructive phytopathogen *Colletotrichum gloeosporioides* causes anthracnose disease in fruit. During host colonization, it secretes ammonia, which modulates environmental pH and regulates gene expression, contributing to pathogenicity. However, the effect of host pH environment on pathogen colonization has never been evaluated. Development of an isogenic tomato line with reduced expression of the gene for acidity, *SlPH* (Solyc10g074790.1.1), enabled this analysis. Total RNA from *C. gloeosporioides* colonizing wild-type (WT) and RNAi–*SlPH* tomato lines was sequenced and gene-expression patterns were compared.

**Results:** *C. gloeosporioides* inoculation of the RNAi–*SlPH* line with pH 5.96 compared to the WT line with pH 4.2 showed 30% higher colonization and reduced ammonia accumulation. Large-scale comparative transcriptome analysis of the colonized RNAi–*SlPH* and WT lines revealed their different mechanisms of colonization-pattern activation: whereas the WT tomato upregulated 13-LOX (lipoxygenase), jasmonic acid and glutamate biosynthesis pathways, it downregulated processes related to chlorogenic acid biosynthesis II, phenylpropanoid biosynthesis and hydroxybenzoic acid tyramine amide biosynthesis; the RNAi–*SlPH* line upregulated UDP-D-galacturonate biosynthesis I and free phenylpropanoid acid biosynthesis, but mainly downregulated pathways related to sugar metabolism, such as the glyoxylate cycle and L-arabinose degradation II. Comparison of *C. gloeosporioides* gene expression during colonization of the WT and RNAi–*SlPH* lines showed that the fungus upregulates ammonia and nitrogen transport and the gamma-aminobutyric acid metabolic process during colonization of the WT, while on the RNAi–*SlPH* tomato, it mainly upregulates the nitrate metabolic process.

**Conclusions:** Modulation of tomato acidity and pH had significant phenotypic effects on *C. gloeosporioides* development. The fungus showed increased colonization on the neutral RNAi–*SlPH* fruit, and limited colonization on the WT acidic fruit. The change in environmental pH resulted in different defense responses for the two tomato lines. Interestingly, the WT line showed upregulation of jasmonate pathways and glutamate accumulation, supporting the reduced symptom development and increased ammonia accumulation, as the fungus might utilize glutamate to accumulate ammonia and increase environmental pH for better expression of pathogenicity factors. This was not found in the RNAi–*SlPH* line which downregulated sugar metabolism and upregulated the phenylpropanoid pathway, leading to host susceptibility.

**Keywords:** Fungal pH regulation, Induced pathogenicity, Host pH regulation

\(^*\) Correspondence: dovprusk@volcani.agri.gov.il; dovprusk@agr.gov.il
\(^1\)Department of Postharvest Science of Fresh Produce, Agricultural Research Organization, the Volcani Center, 7505101 Rishon LeZion, Israel
Full list of author information is available at the end of the article

© The Author(s), 2017. Open Access This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.
Background

Acidity is a major determinant of fruit taste and quality, in combination with sugars and flavor volatiles. Most edible fruits have acidic pH values in the range of 3–5. Previous studies identified a gene family encoding membrane proteins responsible for acidity in fruit, termed PH, and showed functionality of the gene in tomatoes [1]. This suggested its importance for the fruit acidity trait. However, the mechanism underlying this gene's modulation of pH is not fully understood. Cohen et al. [1] were the first to develop a stable RNA interference (RNAi) transgenic tomato line (RNAi–SlPH) with about 30% less citric acid and no significant differences in malic acid levels relative to the wild type (WT), resulting in tomatoes with more than a full unit higher pH [1].

The ability of postharvest pathogens to alter pH locally was initially described for Colletotrichum gloeosporioides, and then extended to some other pathogens, such as Alternaria alternata, Botrytis cinerea, Penicillium expansum, Penicillium digitatum, Penicillium italicum, Phomopsis mangiferae, Monilinia fructicola, and Fusarium oxysporum [2–10]. Attacking pathogenic fungi such as P. expansum, P. digitatum, P. italicum [7], Phomopsis mangiferae [2], B. cinerea [5], and Sclerotinia sclerotiorum [11] acidify tissue with organic acids. Fungi can also achieve ambient alkalization by actively secreting ammonia, which results from protease activation followed by amino acid deamination [12]. Ammonium accumulation has been detected in association with pathogenicity of many Colletotrichum species, including C. gloeosporioides, C. acutatum, C. higginsianum, C. graminicola, and C. coccodes [8, 13–15], as well as A. alternata [3, 4], and F. oxysporum [9]. The ammonium secreted by these species alkalizes the host tissue; its concentration can reach approximately 5 mM, as found in decayed avocado, tomato, and persimmon fruit [3, 4, 8, 13]. In each case with Colletotrichum spp., increased ammonium accumulation has been related to enhanced pathogenicity [8, 13, 16]. In the case of A. alternata, ammonium accumulation led to a 2.4 pH unit increase in several hosts—tomato, pepper, melon, and cherry [3, 4]. Interestingly, ammonia accumulation and pH increase were not correlated across host species, suggesting that pH increase in each host depends on a complex interaction that involves the buffering capacity of the tissue, nitrogen and carbon availability, and the fruit’s initial pH [4]. However, low pH has been found to activate higher ammonia production and secretion in Colletotrichum spp. [13, 17].

In both cases, i.e., alkalization and acidification of the environment via secretion of ammonia by Colletotrichum and organic acid by Penicillium, respectively, pathogenicity factors are clearly modulated, being either activated or repressed [18, 19]. P. expansum acidifies the host tissue to pH levels of 3.5 to 4.0, conditions that significantly enhance polygalacturonase (pg1) transcription [7, 19]. Similarly, in C. gloeosporioides, pelB, encoding pectate lyase, is expressed and secreted in vitro at pH levels higher than 5.7, similar to the pH values present in decaying tissue [20–22]. This suggests that postharvest pathogens modulate the expression of genes contributing to pathogenicity according to environmental pH-inducing conditions.

The development of the RNAi–SlPH tomato line, which has reduced acidity and increased pH relative to the WT [1, 23], offered the possibility of testing the effect of host changes on the pathogenicity factors affecting C. gloeosporioides colonization [24]. The published transcriptomic analyses of the tomato fruit and the pathogen responses during fungal colonization are the basis for the present analysis [24, 25]. Previously, we analyzed the importance of the small molecules secreted by the pathogen as key factors modulating environmental pH and activating fungal colonization of a single host. In the present work, transcriptomic analysis of two similar hosts—WT and a transgenic tomato line with downregulation of a single gene affecting acidity and pH—showed that pH affects not only the pathogen but also the host’s gene expression and the phenotypic host response to fungal colonization.

Results and discussion

Analysis of pH, acidity and susceptibility of RNAi–SlPH fruit colonized by C. gloeosporioides

Comparison of freshly harvested tomato fruits of the RNAi–SlPH and WT lines showed that the former has threefold less total acid than the latter (Table 1). Evaluation of the mesocarp tissue of RNAi–SlPH fruit showed significantly higher pH values than in the WT containing the SlPH gene (Table 1). Comparison of C. gloeosporioides isolate Cg-14 colonization patterns on freshly harvested WT and RNAi–SlPH fruit inoculated with a suspension of 10⁶ spore ml⁻¹ showed 2.37- and 1.54-fold enhanced colonization of the RNAi–SlPH fruit after 48 and 72 h, respectively, compared to the WT. Ammonia accumulation in the mesocarp of the RNAi–SlPH line was significantly lower than that detected in the WT mesocarp (0.002 compared to 1.255 mM). Ammonia accumulation was accompanied by an increase in pH (ΔpH of 1.5 in the WT line compared to ΔpH 0.8 in the RNAi–SlPH line), suggesting

Table 1 pH and total acids (TA) of healthy WT and RNAi–SlPH tomato lines

|          | WT       | RNAi–SlPH |
|----------|----------|-----------|
| pH       | 4.17 ± 0.04 | 5.96 ± 0.02 |
| (% TA)   | 0.85 ± 0.04 | 0.29 ± 0.02 |
that fungal colonization of the RNAi–SlPH line occurs with reduced ammonia accumulation (Fig. 1) as a result of an enhanced pathogen and/or host response induced by that strain’s higher pH level which contributes to fungal colonization.

**Proﬁling gene expression in RNAi–SlPH and WT tomato fruit during colonization by C. gloeosporioides**

Gene profiling was carried out to determine the factors in the host and pathogen response that modulate the two tomato lines differential behavior. Gene-expression profiling of tomato fruit was conducted in two replicates of non-inoculated and *C. gloeosporioides*-inoculated WT and RNAi–SlPH lines. To determine the effect of initial host pH on fungal responses, eight libraries of single-end RNA sequences (deposited in the NCBI Sequence Read Archive (SRA)) under accession no. SRP078571) were mapped to the reference genomes of tomato (*S. lycopersicum*, build 2.50) [23] and *C. gloeosporioides* isolate Cg-14 (GEO accession number GSE41844) [24] using Bowtie2 software [26]. The eight libraries consisted of: (i) two replicates of a healthy WT tomato line; (ii) two replicates of a WT tomato line infected with *C. gloeosporioides* sampled 72 h postinoculation; (iii) two replicates of a healthy RNAi–SlPH tomato line, and (iv) two replicates of an RNAi–SlPH tomato line infected with *C. gloeosporioides* 72 h postinoculation.

Hierarchical clustering analysis of the tomato lines indicated different gene-expression patterns in healthy vs. infected tissues. Overall, there were more upregulated than downregulated genes upon *C. gloeosporioides* infection in both lines (Fig. 2a). By edgeR analysis [27], 1190 and 631 genes were differentially expressed in the healthy vs. infected WT and healthy vs. infected RNAi–SlPH lines, respectively (Fig. 3), with a false discovery rate (FDR) threshold of <1e−3 and Log "Fold Change" (FC) greater than 2 or smaller than −2. Thus the WT line showed an almost sixfold increase in gene expression compared to the RNAi–SlPH line, suggesting negative regulation of gene expression in the RNAi–SlPH line.

Pearson correlation analysis between the expression levels of genes in the different samples showed a high degree of agreement between measurements conducted on replicates of each treatment, indicating the reproducibility of the results (see experimental correlation heat map in Fig. 2b). Gene-expression levels for healthy WT and RNAi–SlPH tomatoes clustered together, as did the levels for infected WT and RNAi–SlPH tomatoes. The heat map further showed that the major differences in gene expression were between infected and non-infected tomato lines, whereas only minor differences were due to introduction of the mutation in the RNAi–SlPH line.
Analysis of the differentially expressed tomato genes during C. gloeosporioides infection

To understand the importance of the specific changes in both cultivars, the differentially expressed genes in both lines were analyzed. Of the 1190 and 631 genes that were differentially expressed during fungal colonization of WT and RNAi–SIPH tomato lines, respectively, 304 (20%) were similar between lines (Fig. 3a); 886 genes were exclusively differentially expressed during infection of the WT line and may contribute to this line's reduced susceptibility compared to the RNAi–SIPH line (Fig. 3a). Of these 888 genes, 298 were upregulated and 590 were...
downregulated (Fig. 3b and c). However, only 329 genes were exclusively differentially expressed during infection of RNAi–SlPH tomato, possibly contributing to this line’s susceptibility (Fig. 3a). Of these, 59 were upregulated and 270 were downregulated (Fig. 3b and c). The difference in gene profiles between the high and low pH lines might indicate that different processes are modulated by the same pathogen under initial conditions of differential host pH.

These results suggested that some of the 298 upregulated and 590 downregulated genes in the colonized WT line might contribute to reduced fungal development (Fig. 3b). Similarly, some of the 59 upregulated and 270 downregulated genes might contribute to the increased susceptibility to colonization of the RNAi–SlPH line (Fig. 3c). Furthermore, the host response might be strongly affected by the initial pH. If we consider that tomato fruit pH can increase from harvesting at the breaker stage (initial ripening) to full maturity, pH may strongly affect host responses to pathogen on the same fruit.

Differentially regulated host pathways during infection of the WT and RNAi–SlPH lines

Using MetGenMap software [28], we discovered the changed pathways (KEGG) in each tomato line during infection.

Table 2: Upregulated and downregulated pathways in C. gloeosporioides-infected WT lines compared to RNAi–SlPH lines

| Pathway name                                      | P-value   |
|---------------------------------------------------|-----------|
| Upregulated                                      |           |
| 13-LOX and 13-HPL pathway                        | 0.00074119|
| oleate biosynthesis I (plants)                    | 0.0113267 |
| jasmonic acid biosynthesis                        | 0.0144936 |
| wax ester biosynthesis                            | 0.0170085 |
| glutamate biosynthesis IV                         | 0.0253189 |
| Downregulated                                    |           |
| phenylethanol biosynthesis                        | 2.302E-05 |
| ethylene biosynthesis from methionine             | 0.0002738 |
| suberin biosynthesis                              | 0.0005621 |
| simple coumarin biosynthesis                      | 0.0019152 |
| hydroxycinnamic acid tyramine amide biosynthesis  | 0.0022188 |
| chlorogenic acid biosynthesis II                  | 0.0027091 |
| wound-induced proteolysis I                       | 0.0086595 |
| alanine biosynthesis III                          | 0.0086595 |
| seed germination protein turnover                 | 0.0094942 |
| purine degradation                                |           |
| fatty acid α-oxidation                            | 0.0160793 |
| aesculetin biosynthesis                           | 0.0174724 |
| glutamate degradation II                          | 0.0422702 |
| t Rena C5 biosynthesis                            | 0.0422702 |

Pathways modulating host resistance: Jasmonic acid (JA) and phenylpropanoid

The most strongly upregulated pathway in the colonized WT line was the 13-LOX and 13-HPL (hydroperoxide lyase pathway) pathway involved in 13-LOX activity (Table 2). These enzymes are involved in the biosynthesis of JA during the transformation of linolenic acid to JA by a multistep process [29]. JA has been proven to be involved in plant resistance to pathogens by activating pathogenesis-related proteins such as PR-1, PR-3 and PR-8 [30]. This pathway was not significantly upregulated during infection of the RNAi–SlPH line, suggesting that this upregulation may contribute to the reduced colonization of the WT line compared to the RNAi–SlPH line.

In contrast, a pathway that was downregulated during C. gloeosporioides infection of the WT line was the chlorogenic acid biosynthesis II pathway (Table 2). Chlorogenic acid (5-O-caffeoyl-D-quinic acid) is one of the most widespread hydroxycinnamic acid derivatives in plants. Its physiological roles include defense, disease resistance as an antioxidant, and growth regulation [31]. Moreover, hydroxycinnamic acid tyramine amide biosynthesis was also downregulated in the infected WT tomato line (Table 2). Incorporation of hydroxycinnamic acid tyramine amides into the cell wall has been reported to enhance its efficiency as a barrier against pathogens by increasing its rigidity and decreasing its digestibility [32–34]. This type of response has been observed during colonization of potato by Phytophthora infestans, with the potato tyramine N-hydroxycinnamoyl transferase activity increasing upon infection, as well as upon wounding [34], and during appressorium formation at the biotrophic stage of C. gloeosporioides [25], although it may not affect the response of the WT strain. The chlorogenic acid biosynthesis II pathway was not modulated by C. gloeosporioides in the RNAi–SlPH line. It is possible that under the new environmental conditions present in this line, the fungus does not modulate this defense system and/or has a different, more efficient mechanism to enable its enhanced colonization.

Pathways modulating nitrogen metabolism: Glutamate and glutamine metabolism

An important pathway that was upregulated during colonization of the WT line by C. gloeosporioides was the glutamate biosynthesis IV pathway, along with downregulation of the glutamate degradation II pathway (Table 2). The glutamine synthetase–glutamine-
oxoglutarate aminotransferase (GS–GOGAT) cycle has been proposed to function in primary nitrogen assimilation, in which glutamate is continuously synthesized by the GOGAT glutamate synthase and metabolized by the enzyme GS [35–38]. Glutamate metabolism has a pivotal role in plant amino acid metabolism since it orchestrates crucial metabolic functions, including assimilation or dissimilation of ammonia and amino acid transamination, and it provides both the carbon skeleton and α-amino group for biosynthesis of amino acids with key roles in plant defense, such as γ-aminobutyric acid (GABA), arginine, and proline [36, 39]. It is therefore responsible for cell viability.

The host glutamate metabolism in the WT strain may function in two opposing ways in response to pathogens, either activating the host defense response, or being exploited by the pathogen to promote infection, for example by ammonia accumulation [8, 40]. It has been proposed that nitrogen accumulation in the colonized area leads to strong resistance to the pathogen. Overexpression of glutamate receptors in transgenic Arabidopsis plants increased ammonium transportation within the challenged cells, resulting in delayed senescence and increased levels of resistance against B. cinerea [41]. This may also explain the observed levels of resistance in Arabidopsis lines overexpressing arginase, a urea-generating enzyme which eventually supplies the GS–GOGAT cycle with nitrogen sources [40, 42]. This suggests that the high level of GS activity maintains the critical functionality of the GS–GOGAT cycle at the inoculated sites. Similarly, upregulation of GS1 and accumulation of glutamine at an early infection stage demonstrated that nitrogen remobilization is stimulated in infected leaves [43]. Collectively, it seems that glutamate might promote activation of the GS–GOGAT cycle to boost tolerance to the pathogen in infected tissues.

Modulation of the glutamate biosynthesis pathway during C. gloeosporioides infection of a WT line has also been reported in the interaction between Colletotrichum lindemuthianum and Phaseolus vulgaris. In that system, upon pathogen colonization, GS1 levels increase in the host during the biotrophic stage of Colletotrichum, resulting in glutamine accumulation in the phloem around the infection site. Concomitantly, C. lindemuthianum shifts to necrotrophic invasion, presumably because the increased vascular glutamine concentration is perceived as a host escape signal [40, 43]. This might explain the relatively limited colonization pattern observed here on the WT tomato compared to the RNAi–SIPH line. This timely transition in virulence strategy enables the anthracnose pathogen to trap high levels of glutamine in the phloem before the host can efficiently translocate its nitrogen reservoir out of the infected area.

These results indicate that during the coevolution of pathogenic fungi with plants, they adapted to the modifications in plant nitrogen content caused by biotic stress, ultimately turning the metabolic changes in the plant to their benefit [44, 45]. This suggests that C. gloeosporioides exploits nitrogen metabolism differently in the two tomato lines. During infection of the WT and its enhanced glutamate synthesis, the fungus upregulates genes involved in glutamate metabolism to α-ketoglutarate (fungal gdh2) (Table 3, Fig. 6). This might result in continuous glutamate degradation by the fungus during infection and ammonia accumulation; at the same time, the host uses this process to escape attack. In the case of the infected RNAi–SIPH line, there is no upregulation of glutamate biosynthesis, thereby blocking the host defense system, and enabling fungal development without ammonia accumulation. This suggests that ammonia accumulation via fungal degradation of glutamate determines not only the necrotrophic stage but also the host response to the dynamics of glutamate transformation and the preservation of cell viability.

### Pathways modulating sugar metabolism

The RNAi–SIPH tomato showed upregulation and downregulation of sugar metabolic pathway genes during colonization by C. gloeosporioides: differential upregulation of the UDP-galacturonate biosynthesis pathway as well as downregulation of the glyoxylate cycle.

### Table 3 Upregulated and downregulated pathways in C. gloeosporioides-infected RNAi–SIPH lines compared to WT lines

| Pathway name                              | P-value     |
|-------------------------------------------|-------------|
| Upregulated                               |             |
| matairesinol biosynthesis                 | 0.0049779   |
| UDP-D-galacturonate biosynthesis I         | 0.0165126   |
| (from UDP-D-glucuronate)                   |             |
| free phenylpropanoid acid biosynthesis    | 0.0246834   |
| gibberellin inactivation                   | 0.0408552   |
| Downregulated                             |             |
| glyoxylate cycle                          | 0.0012551   |
| glycolate and glyoxylate degradation II    | 0.0015753   |
| cytokinin degradation                     | 0.003706    |
| superpathway of glyoxylate cycle          | 0.0070379   |
| wax ester biosynthesis II                  | 0.0117932   |
| anthocyanin biosynthesis (delphinidin 3-O-glucoside) | 0.0181857 |
| L-arabinose degradation II                 | 0.0209389   |
| anthocyanin biosynthesis (pelargonidin 3-O-glucoside, cyanidin 3-O-glucoside) | 0.0218204 |
| leucopelargonidin and leucocyanidin biosynthesis | 0.0263502 |
| leucodelphinidin biosynthesis              | 0.0263502   |
| glycogen degradation I                     | 0.0320831   |
| melibiose degradation                      | 0.0414508   |
glyoxylate degradation, superpathway of glyoxylate cycle, glycogen degradation, and arabinose degradation, suggesting a very specific decrease in sugar metabolism (Table 3). The glyoxylate cycle converts acetyl-CoA to succinate for the synthesis of carbohydrates [46]; organisms with a glyoxylate cycle therefore gain metabolic versatility because it allows cells to utilize simple carbon compounds as a carbon source when complex sources such as glucose are not available. Glyoxylate derivatives can accumulate in plants under stress; they react with DNA, oxidize membrane lipids, modify proteins or influence the transcription of stress-related genes, thereby causing cellular and developmental problems that lead to host susceptibility [47–49]. The drop in glyoxylate pathway activity in RNAi–SIPH fruit may result in decreased levels of malic and citric acid. This reduction in organic acids may lead to enhanced neutralization of the tissue environment, and upregulation of the C. gloeosporioides transcription factor regulating pH-affected genes pacC and pelB, which are expressed at high pH levels without ammonia accumulation. This negative regulation of pH environment (i.e., not by increasing ammonia but by reducing organic acid accumulation) might explain the increased colonization of C. gloeosporioides in the RNAi–SIPH strain in the presence of reduced ammonia accumulation. This possibility is supported by the recent suggestion by Bi and coworkers [50] that C. gloeosporioides produces both ammonia and gluconic acid under different host/sugar conditions.

Inversely regulated pathways during infection of the WT and RNAi–SIPH lines: Phenylpropanoid pathway

The phenylpropanoids are not only indicators of plant stress responses upon variations in light or mineral treatment; they are also key mediators of plant resistance as they are formed during the initial response to infection [32, 51, 52]. In our case, C. gloeosporioides repressed the phenylpropanoid pathway in the WT line, but upregulated it in the RNAi–SIPH line (Tables 2 and 3). Phenylalanine ammonia-lyase (PAL) is the first enzyme of the phenylpropanoid pathway, which synthesizes trans-cinnamic acid, a precursor of salicylic acid (SA) [53]. SA is a plant signal for the activation of defense responses and enhances host cell death [54]. During the interaction of soybean and the pathogen *Pseudomonas syringae* pv. *glycinea*, addition of physiological concentrations of SA enhanced the induction of defense gene transcripts, H₂O₂ accumulation, and hypersensitive cell death by an avirulent strain of the pathogen [55]. In our case, the necrotrophic stage of C. gloeosporioides may thrive under cell-death conditions. This is probably the contribution of the phenylpropanoid pathway to the enhanced pathogenicity in the RNAi–SIPH line, whereby overexpression of those genes that contribute to host cell death shifts the balance from resistance to susceptibility, as observed in this specific line. Future analysis of cell viability should fully confirm the present suggestions.

Differentially regulated fungal genes during infection of the WT and RNAi–SIPH lines

Hierarchical clustering analysis of fungal gene expression during pathogenicity on WT and RNAi–SIPH tomatoes indicated different expression patterns on each infected line. Overall, there were more upregulated than downregulated C. gloeosporioides genes during infection of the WT vs. RNAi–SIPH line (Fig. 4a). Using edgeR analysis [27], 645 genes were differentially expressed in C. gloeosporioides-infected WT and RNAi–SIPH lines with threshold described in the manuscript.
gloeosporioides during infection of the WT compared to infection of the RNAi–SIPH line, with a FDR threshold <0.05 and FC greater than 2 or smaller than −2. Among the differentially expressed genes, 415 were downregulated during infection of the RNAi–SIPH line and 230 were upregulated (Fig. 4).

Pearson correlation analysis of the expression levels of C. gloeosporioides transcripts in the different samples can serve for quality control of experimental reproducibility. The analysis showed strong agreement between measurements conducted on replicates of the various treatments, indicating high reproducibility of the results (see experimental correlation heat map in Fig. 4b).

Modulation of fungal gene ontology (GO) categories during infection of RNAi–SIPH tomato lines

The key GO response in C. gloeosporioides during infection of the RNAi–SIPH line was upregulation of fungal nitrate metabolic processes. These processes are activated when the preferred products—ammonia, glutamine, asparagine or glutamate—are not available. In planta, nitrogen availability seems to be limiting [56], indicating the fungus’ need for nitrate metabolism (Tables 4 and 5). Utilization of nitrate requires de novo synthesis of nitrate and nitrite reductase, which requires both nitrogen derepression and specific induction by nitrate. Fungal nit regulation is important for pathogenicity in Colletotrichum because it contributes to ammonia accumulation [13]. However, in our case, ammonia did not accumulate with the RNAi–SIPH line. Another possible explanation for the lack of fungal ammonia secretion with the RNAi–SIPH line is downregulation of ammonia transporters that are not active at the host’s higher pH. While ammonia accumulation was upregulated during infection of the WT, it was downregulated during infection of the RNAi–SIPH line (Fig. 1). This may result from inhibition of fungal export of ammonia and possible accumulation of ammonia inside the hyphae, as occurs in the spores of Δmep (ammonia permease) strains [57]. We did not monitor ammonia accumulation in C. gloeosporioides developing on the RNAi–SIPH tomato; however, downregulation of mepB was observed in this line (Table 3), suggesting that possibility. Furthermore, strong inhibition of NAD+-specific glutamate dehydrogenase (GDH2, encoding ammonia synthesis, JN660152) and ammonia transporter (AMET), which are downregulated at pH 6.0–7.0, supports the behavior in the RNAi–SIPH line [8, 58]. Thus, the differential activation of nitrogen metabolism by the pathogen is probably modulated differently in the WT and RNAi–SIPH lines. These results suggested that the pathogen enhances colonization at a higher pH in the (RNAi–SIPH line without ammonia accumulation as a result of the decrease in mepB expression. Together, the lack of ammonia accumulation, the concurrent drop in glyoxylate pathway activity and the possible reduction of organic acid accumulation may contribute to fungal colonization by C. gloeosporioides.

Modulation of fungal GO categories during infection of WT tomato lines

Upregulation of the GABA (Gamma-Aminobutyric Acid) metabolic process in C. gloeosporioides while infecting the WT suggested its contribution as a carbon and nitrogen source to spore/sporulation metabolism, as described for other fungi under similar pH conditions [59, 60]. GABA synthesis has been associated with acidic pH in different plant and fungal systems [59], either in response to cytosolic acidification—probably as a pH-regulatory mechanism, or during growth under acidic conditions [59]. Enhanced GABA synthesis might be one of the mechanisms employed by the fungus to utilize carbon and nitrogen sources under the acidic pH of the WT tomato [61], and it is consequently not expressed in the neutral RNAi–SIPH line.

Carbohydrate active enzymes (CAZymes)

The diverse complex carbohydrates that contribute to C. gloeosporioides virulence are controlled by a panel of enzymes involved in their assembly (glycosyltransferases) and breakdown (glycoside hydrolases, polysaccharide lyases, carbohydrate esterases), collectively designated CAZymes [62]. In plant pathogens, CAZymes promote synthesis, degradation, and modification of carbohydrates, playing an important role in the breakdown of plant cell walls and in host–pathogen interactions [63]. C. gloeosporioides uses different sets of enzymes to attack the tomatoes with the different pHs by degrading complex carbohydrates of the host to simple monomers that can be utilized as nutrients [64, 65].

Using the CAZyme analysis toolkit [66], we identified 80 upregulated CAZymes during infection of line RNAi–SIPH and 107 upregulated CAZymes during infection of the WT line (Fig. 5). This indicates that similar numbers of genes encoding CAZyme activities were regulated in both tomato lines, suggesting that the number of differentially expressed CAZymes is not the limiting factor in increased colonization of the RNAi–SIPH compared to the WT line by C. gloeosporioides.
| GO term                      | Gene description                                                                 |
|-----------------------------|----------------------------------------------------------------------------------|
| nitrogen compound transport | choline transport                                                                |
|                             | amino acid permease                                                              |
|                             | purine-cytosine permease                                                         |
|                             | choline transport                                                                |
|                             | —NA—                                                                             |
|                             | choline transport                                                                |
|                             | ncs1 nucleoside transporter                                                      |
|                             | urea active transporter                                                          |
|                             | amino-acid permease inda1                                                        |
|                             | amino acid permease                                                              |
|                             | c6 finger domain                                                                 |
|                             | amino-acid permease inda1                                                        |
|                             | amino acid                                                                      |
|                             | urea transporter                                                                 |
|                             | hypothetical protein [Tuber melanosporum Mel28]                                  |
|                             | cytosine-purine permease                                                         |
|                             | amino acid                                                                      |
|                             | amino acid permease                                                              |
|                             | mfs peptide putative                                                             |
|                             | peptide transporter ptr2-a                                                       |
|                             | ammonium transporter mep1                                                        |
|                             | dicarboxylic amino acid permease                                                 |
|                             | amino acid permease                                                              |
|                             | ammonium transporter                                                             |
|                             | large neutral amino acids transporter small subunit 1                            |
|                             | ammonium transporter mep2                                                        |
|                             | uridine permease                                                                 |
|                             | amino-acid permease inda1                                                        |
|                             | proline-specific permease                                                        |
|                             | amino acid permease family                                                       |
|                             | amino acid permease                                                              |
|                             | uracil permease                                                                  |
|                             | peptide transporter ptr2-a                                                       |
|                             | ncs1 nucleoside transporter family protein                                        |
|                             | choline transport protein                                                        |
|                             | polyamine transporter tpo5                                                        |
|                             | ammonium transporter                                                             |
|                             | amino acid permease                                                              |
|                             | uracil permease                                                                  |
|                             | ncs1 allantioate transporter                                                      |
|                             | ncs1 allantioate transporter                                                      |
|                             | tpa: amino acid transporter                                                      |
| GO Category | Gene Products |
|-------------|--------------|
| anion transmembrane transport | choline transport, amino acid permease, choline transport, —NA—, choline transport, amino-acid permease inda1, amino acid permease, c6 finger domain, amino-acid permease inda1, amino acid, amino-acid permease inda1, proline-specific permease, amino acid permease family, amino acid permease, amino acid, amino acid permease, choline transport protein, polyamine transporter tpo5, amino acid permease, dicarboxylic amino acid permease, amino acid permease, mitochondrial phosphate carrier protein, tpa: amino acid transporter, large neutral amino acids transporter small subunit 1 |
| ion transmembrane transport | choline transport, amino acid permease, choline transport, —NA—, choline transport, membrane zinc transporter, amino-acid permease inda1, amino acid permease, potassium transporter, c6 finger domain, amino-acid permease inda1, amino acid, p-type calcium ATPase, amino acid permease, ammonium transporter mep1, dicarboxylic amino acid permease, amino acid permease, ammonium transporter, —NA—, large neutral amino acids transporter small subunit 1 |
| Table 5 | Upregulated fungal GO categories during *C. gloeosporioides* infection of the WT lines (Continued) |
|---------|------------------------------------------------------------------------------------------------|
|         | ammonium transporter mep2                                                                     |
|         | potassium uptake transporter                                                                 |
|         | amino-acid permease inda1                                                                     |
|         | proline-specific permease                                                                      |
|         | amino acid permease family                                                                     |
|         | amino acid permease                                                                           |
|         | choline transport protein                                                                     |
|         | polyamine transporter tpo5                                                                     |
|         | ammonium transporter                                                                          |
|         | amino acid permease                                                                           |
|         | mitochondrial phosphate carrier protein                                                         |
|         | tpa: amino acid transporter                                                                    |
|         | oxidation-reduction process                                                                   |
|         | general amidase                                                                               |
|         | cytochrome p450                                                                                |
|         | glyoxylate reductase                                                                           |
|         | short chain                                                                                    |
|         | short-chain                                                                                   |
|         | flavin-containing amine                                                                        |
|         | cytochrome p450                                                                                |
|         | FAD dependent                                                                                  |
|         | D-amino acid                                                                                  |
|         | 2-deoxy-D-gluconate 3-dehydrogenase                                                            |
|         | aryl-alcohol dehydrogenase                                                                     |
|         | 3-ketoacyl-acyl carrier protein                                                                 |
|         | polyketide synthase                                                                            |
|         | glycerate dehydrogenase                                                                        |
|         | aldehyde dehydrogenase                                                                         |
|         | D-amino-acid oxidase                                                                           |
|         | sorbitol dehydrogenase                                                                         |
|         | FAD binding domain-containing protein                                                           |
|         | trichothecene c-15 hydroxylase                                                                  |
|         | trichothecene c-15 hydroxylase                                                                  |
|         | short-chain dehydrogenase reductase                                                             |
|         | copper amine oxidase                                                                           |
|         | xanthine dehydrogenase                                                                         |
|         | integral membrane protein                                                                      |
|         | cytochrome p450 monoxygenase                                                                   |
|         | integral membrane protein                                                                      |
|         | superoxide dismutase                                                                           |
|         | chlorocatechol -dioxygenase                                                                     |
|         | NADPH dehydrogenase                                                                            |
|         | hypothetical protein [Podospora anserina S mat+]                                               |
|         | 2og-fe oxygenase superfamily protein                                                            |
|         | cysteine dioxygenase                                                                            |
|         | cytochrome b5                                                                                  |
| Upregulated fungal GO categories during *C. gloeosporioides* infection of the WT lines (Continued) |
|------------------------------------------------------------------------------------------|
| isocitrate dehydrogenase                                                                  |
| terpene synthase metal binding domain protein                                           |
| benzoate 4-monoxygenase cytochrome p450                                                   |
| copper amine oxidase                                                                     |
| naphthalene -dioxygenase subunit alpha                                                   |
| succinate-semialdehyde dehydrogenase                                                    |
| Fe-containing alcohol                                                                    |
| copper amine oxidase                                                                     |
| short chain dehydrogenase                                                                |
| cytochrome p450                                                                           |
| alcohol dehydrogenase                                                                   |
| FAD binding domain protein                                                               |
| aldehyde dehydrogenase family                                                            |
| l-ornithine 5-monoxygenase                                                               |
| short-chain dehydrogenase reductase family                                               |
| proline oxidase                                                                          |
| potassium uptake transporter                                                              |
| peroxisomal copper amine oxidase                                                         |
| benzoate 4-monoxygenase cytochrome p450                                                   |
| pigment biosynthesis protein ayg1                                                         |
| FAD dependent oxidoreductase                                                             |
| histidine acid phosphatase                                                                |
| alpha-ketoglutarate dependent xanthine dioxygenase                                       |
| NAD-specific glutamate dehydrogenase                                                     |
| amino acid transporter                                                                   |
| NAD-specific glutamate dehydrogenase                                                     |
| bifunctional purine biosynthesis protein                                                 |
| FAD dependent oxidoreductase superfamily                                                |
| short-chain dehydrogenase reductase sdr                                                  |
| alcohol dehydrogenase                                                                    |
| glycine dehydrogenase                                                                    |
| aldehyde dehydrogenase                                                                   |
| short-chain dehydrogenase                                                                |
| l-amino-acid oxidase                                                                    |
| NADP-specific glutamate dehydrogenase                                                    |
| aerobactin siderophore biosynthesis protein iucb                                         |
| endoribonuclease l-psp                                                                   |
| cytochrome p450 oxido-reductase                                                          |
| ferric-chelate reductase                                                                  |
| cytochrome b2                                                                             |
| aerobactin siderophore biosynthesis protein iucb                                         |
| short chain dehydrogenase                                                                |
| glyoxylate reductase                                                                     |
| short-chain dehydrogenase reductase sdr                                                  |
| peroxisomal dehydratase                                                                   |
Table 5  Upregulated fungal GO categories during *C. gloeosporioides* infection of the WT lines (Continued)

| Category                                                                 | Description                                                                 |
|--------------------------------------------------------------------------|-----------------------------------------------------------------------------|
| FAD binding domain-containing protein                                    |                                                                             |
| FAD binding domain-containing protein                                    |                                                                             |
| gtp-binding protein                                                      |                                                                             |
| glycerate-and formate-dehydrogenase                                      |                                                                             |
| copper amine oxidase                                                     |                                                                             |
| cytochrome b2                                                             |                                                                             |
| cytochrome b2                                                             |                                                                             |
| salicylate hydroxylase                                                   |                                                                             |
| multicopper oxidase                                                      |                                                                             |
| 3-oxoacyl-(acyl-carrier-protein) reductase                               |                                                                             |
| transcriptional activator protein acu-membrane copper amine              |                                                                             |
| ——NA—                                                                   |                                                                             |
| dimethylglycine oxidase                                                  |                                                                             |
| sarcosine oxidase                                                        |                                                                             |
| FAD dependent oxidoreductase superfamily                                 |                                                                             |
| succinate-semialdehyde dehydrogenase                                     |                                                                             |
| aldehyde dehydrogenase                                                   |                                                                             |
| rieske 2fe-2 s family protein                                            |                                                                             |
| short chain dehydrogenase                                                |                                                                             |
| rieske 2fe-2 s family protein                                            |                                                                             |
| ankyrin repeat-containing protein                                         |                                                                             |
| short chain dehydrogenase                                                |                                                                             |
| saccharopine dehydrogenase                                               |                                                                             |
| 2og-fe oxygenase                                                         |                                                                             |
| FAD binding domain containing protein                                    |                                                                             |
| major facilitator superfamily transporter                                |                                                                             |
| hypothetical protein GLRG_11952 [Glomerella graminicola M1.001]           |                                                                             |
| aminobenzoyl-glutamate utilization protein b                             |                                                                             |
| 12-oxophytodienoate reductase                                            |                                                                             |
| ——NA—                                                                   |                                                                             |
| Mfs                                                                      |                                                                             |
| FAD binding domain-containing protein                                    |                                                                             |
| benzoate 4-monoxygenase cytochrome p450                                   |                                                                             |
| FAD binding domain protein                                               |                                                                             |
| FAD dependent oxidoreductase superfamily                                 |                                                                             |
| cytochrome p450 alkane                                                   |                                                                             |
| 2og-fe oxygenase family                                                  |                                                                             |
| oxidoreductase domain containing protein                                 |                                                                             |
| c6 transcription                                                         |                                                                             |
| 2og-fe oxygenase family                                                  |                                                                             |
| short-chain dehydrogenase reductase                                       |                                                                             |
| 3-hydroxyacyl-NAD binding                                                |                                                                             |
| alcohol dehydrogenase                                                    |                                                                             |
| FAD binding domain-containing protein                                    |                                                                             |
### Table 5 Upregulated fungal GO categories during C. gloeosporioides infection of the WT lines (Continued)

| Category                                      | GO Terms                                                                 |
|-----------------------------------------------|--------------------------------------------------------------------------|
| FAD binding domain-containing protein         | alcohol dehydrogenase, Aldehyde                                           |
|                                               | cytochrome p450 alkane, short chain dehydrogenase, n-alkane-inducible cytochrome p450 |
|                                               | streptomycin biosynthesis protein, methylmalonate-semialdehyde dehydrogenase |
|                                               | flavin dependent, Carbonyl                                                |
|                                               | 2og-fe oxygenase superfamily protein                                     |
|                                               | short chain dehydrogenase reductase family                               |
|                                               | homoisocitrate dehydrogenase                                              |
|                                               | flavin-binding monooxygenase                                              |
|                                               | phosphoadenosine phosphosulfate reductase                                |
|                                               | phosphoadenosine phosphosulfate reductase                                |
|                                               | sarcosine oxidase                                                        |
|                                               | D-lactate mitochondrial precursor                                        |
|                                               | FAD-binding domain protein                                                |
| ammonium transmembrane transport              | ammonium transporter                                                     |
|                                               | ammonium transporter mep2                                                |
|                                               | ammonium transporter mep1                                                |
|                                               | ammonium transporter                                                     |
| gamma-aminobutyric acid metabolic process     | succinate-semialdehyde dehydrogenase                                    |
|                                               | 4-aminobutyrate aminotransferase                                         |
|                                               | 4-aminobutyrate aminotransferase                                         |
|                                               | 4-aminobutyrate aminotransferase                                         |
|                                               | succinate-semialdehyde dehydrogenase                                    |
| ion transport                                 | choline transport                                                        |
|                                               | amino acid permease                                                      |
|                                               | choline transport                                                        |
|                                               | —NA—                                                                     |
|                                               | choline transport                                                        |
|                                               | membrane zinc transporter                                                |
|                                               | urea active transporter                                                  |
|                                               | amino-acid permease inda1                                                |
|                                               | amino acid permease                                                      |
|                                               | potassium transporter                                                    |
|                                               | có finger domain                                                         |
|                                               | amino-acid permease inda1                                                |
|                                               | amino acid                                                              |
|                                               | hypothetical protein [Tuber melanosporum Mel28]                          |
|                                               | p-type calcium ATPase                                                    |
|                                               | plasma membrane h + —ATPase pma1                                         |
| Organonitrogen compound catabolic process | amino acid |
|-----------------------------------------|------------|
|                                         | amino acid permease |
|                                         | ammonium transporter mep1 |
|                                         | dicarboxylic amino acid permease |
|                                         | amino acid permease |
|                                         | zip zinc transporter |
|                                         | udp-galactose transporter |
|                                         | ammonium transporter |
|                                         | —NA— |
|                                         | large neutral amino acids transporter small subunit 1 |
|                                         | ammonium transporter mep2 |
|                                         | potassium uptake transporter |
|                                         | amino-acid permease inda1 |
|                                         | proline-specific permease |
|                                         | amino acid permease family |
|                                         | amino acid permease |
|                                         | choline transport protein |
|                                         | polyamine transporter tpo5 |
|                                         | ammonium transporter |
|                                         | amino acid permease |
|                                         | mitochondrial phosphate carrier protein |
|                                         | tpa: amino acid transporter |
|                                         | urea active transporter |
|                                         | proline oxidase |
|                                         | —NA— |
|                                         | methylmalonate-semialdehyde dehydrogenase |
|                                         | urease |
|                                         | glycosyl family |
|                                         | succinate-semialdehyde dehydrogenase |
|                                         | dimethylglycine oxidase |
|                                         | NAD-specific glutamate dehydrogenase |
|                                         | succinate-semialdehyde dehydrogenase |
|                                         | dihydropyrimidinase |
|                                         | 4-aminobutyrate aminotransferase |
|                                         | NAD-specific glutamate dehydrogenase |
|                                         | aldehyde dehydrogenase |
|                                         | 3-hydroxyacyl-NAD binding |
|                                         | carbamoyl-phosphate synthase |
|                                         | guanine deaminase |
|                                         | glycine dehydrogenase |
| Aspartate family amino acid metabolic process | L-serine dehydratase |
|                                         | homocysteine synthase |
|                                         | L-asparaginase |
|                                         | —NA— |
|                                         | threonine dehydratase |
Validation of host and fungal gene expression

To validate the differential expression of specific genes identified by the RNA-Seq analysis, quantitative (q) RT-PCR analyses were performed for key genes of interest. Analysis of the WT tomato lines showed the expected increase in expression of the LOX and glutamate synthase genes that modulated the relative resistance of the WT line compared to the RNAi–SlPH line. In contrast, the WT line showed downregulated expression of genes encoding cytochrome P450 and PAL related to downregulation of the phenylpropanoid pathway in the WT, which also explains the reduced colonization of *C. gloeosporioides* on the WT line (Fig. 6a).

An interesting outcome of the comparison of *C. gloeosporioides* colonization on the WT vs. RNAi–SlPH line is the similar expression pattern of *pacC* in the fungus on both lines, indicating that the lower pH of the WT line increases when it is inoculated, as detected by the accumulation of ammonia (Fig. 1), to a value similar to that of the RNAi–SlPH line, leading to similar expression of *pacC* during colonization. However, the enhanced susceptibility of RNAi–SlPH was accompanied by upregulation of *pelB* and in contrast, downregulation of glutamate degradation, as reflected by the downregulation of *gdh2* and the ammonia transporter *amet* (Fig. 6b), further explaining the reduced ammonia accumulation in the RNAi–SlPH line.

Conclusions

*SlPH* has been found to play a significant role in tomato fruit acidity, with its modulation of tomato pH levels [1]. The transgenic RNAi–SlPH tomato showed reduced organic acid accumulation and a higher pH level than the WT tomato, and it was more susceptible to *C. gloeosporioides* attack. Our aim was to analyze the effect of the fruit’s initial pH on the host and pathogen gene profiles during fungal colonization. Gene-expression profiling

Table 5 Upregulated fungal GO categories during *C. gloeosporioides* infection of the WT lines (Continued)

| GO Category | Description |
|-------------|-------------|
| cellular amino acid catabolic process | benzoate 4-monooxygenase cytochrome p450 |
| | dimethylglycine oxidase |
| | argininosuccinate synthase |
| | threonine ammonia-lyase precursor |
| | 4-aminobutyrate aminotransferase |
| | aldehyde dehydrogenase |
| | 3-hydroxyacyl-NAD binding |
| | D-amino-acid oxidase |
| | s-adenosylmethionine synthetase |
| | phosphoadenosine phosphosulfate reductase |
| | phosphoadenosine phosphosulfate reductase |
| | aminobenzoyl-glutamate utilization protein b |
| | 5-methyltetrahydropropiomaleimide-cysteine methyltransferase |
| | dimethylglycine oxidase |
| | NAD-specific glutamate dehydrogenase |
| | succinate-semialdehyde dehydrogenase |
| | 4-aminobutyrate aminotransferase |
| | NAD-specific glutamate dehydrogenase |
| | proline oxidase |
| | aldehyde dehydrogenase |
| | methylmalonate-semialdehyde dehydrogenase |
| | 3-hydroxyacyl-NAD binding |
| | carbamoyl-phosphate synthase |
| | succinate-semialdehyde dehydrogenase |
| | glycine dehydrogenase |
| | gamma-aminobutyric acid catabolic process |
| | succinate-semialdehyde dehydrogenase |
| | 4-aminobutyrate aminotransferase |
| | succinate-semialdehyde dehydrogenase |
revealed that fruit pH affects the host’s defense strategy as well as the fungus’ pathogenesis strategy.

Each host followed a different strategy in response to fungal attack. One of the key processes upregulated in the infected WT vs. RNAi–SIPH tomatoes was the 13-LOX pathway, resulting in JA biosynthesis, a known host defense response. Interestingly, the glutamate biosynthesis IV pathway was upregulated, while the glutamate degradation II pathway was downregulated. We suggest that the pathogen induced production of glutamate by the plant as a preferred nitrogen source for the fungus to accumulate ammonia, and to increase the environmental pH for better expression of the pathogenicity factors pelB and pacC. This may indicate that the host–pathogen interaction affects the host metabolism according to acid content and pH.

In contrast, the RNAi–SIPH tomato showed different modulated pathways. There were significant changes in sugar metabolism, in particular downregulation of glyoxylate degradation, which might have led to reduced organic acid accumulation in the RNAi–SIPH line and might have caused cellular and developmental problems, affecting host susceptibility. This suggests that modulation of the glyoxylate cycle contributes to this line’s susceptibility to C. gloeosporioides. Furthermore, the phenylpropanoid pathway showed differential modulation in the two lines: upregulation during infection of the RNAi–SIPH line and downregulation during infection of the WT line. This pathway may eventually lead to cell death, improving fungal colonization ability in the necrotrophic stage. This may contribute to the RNAi–SIPH line’s susceptibility and the WT’s resistance to C. gloeosporioides pathogenicity. Thus, the present work indicates that environmental pH has a significant effect on the “balance” of activated pathways and mechanism(s) leading to the final host response, and pathogen colonization outcome.

Gene-expression analysis of the fungal transcriptome revealed that the fungus also responds differently to each tomato line. Upregulation of the nitrogen metabolic process, involving nitrate/nitrite reductase, was a key induced factor in the pathogen colonizing the RNAi–SIPH line. These genes contribute to Colletotrichum pathogenicity [13]. On the other hand, the nitrogen transport process and GABA metabolism were upregulated in C. gloeosporioides colonizing the WT line. In this case, several genes involved in ammonia transport contributed to C. gloeosporioides pathogenicity (gdh2, amet, mepB) [9, 57].

In summary, enhanced colonization of tissue via pH modulation does not solely affect fungal pathogenicity. This is the first report of pH modulation—effected either locally by the fungus or genetically by affecting the expression of a single gene—determining the strategic response of the host to the challenging Colletotrichum colonization. On the pH-neutral RNAi–SIPH tomato, the major pathogenicity factors are probably modulated directly by PACC and include pelB expression, which may increase virulence compared to the WT acidic line where the fungus has to increase the pH using several
ammonia transporters to activate pathogenicity factors in the postharvest pathogen.

Methods
Fungal isolates, plant cultivars and fruit inoculation
Single-spore cultures of Colletotrichum gloeosporioides (Penz.) isolate Cg-14 were obtained from a decayed avocado fruit [67]. The isolate was thoroughly analyzed in tomato infections [25]. Cultures were grown at 27 °C in the dark, and maintained on M3S plates. Conidia were harvested with 10 ml sterile distilled water supplemented with 0.01% (v/v) Triton X-100. Cells were visualized with a model BX60F-3 microscope (Olympus America Inc., Melville, NY, USA) and counted with a hemocytometer (Brand, GMBH, Wertheim, Germany).

Tomato (Solanum lycopersicum var. MP-1) plants were grown in the greenhouse under standard conditions. Transgenic SlPH tomatoes, impaired in the PH ortholog and obtained as described by Cohen et al. [1], and the WT strain were compared.

Tomato fruit was inoculated by placing 5 µl of a conidial suspension containing 10⁶ spore ml⁻¹ on each of four 2-mm deep and 1-mm diameter wounds spaced evenly in a circle around the upper part of the stem end of 7–10 fruits. Following inoculation, the fruits were incubated for 72 h (unless otherwise specified) at 25 °C in covered plastic containers containing wet paper towels. Samples of the colonized tissue of the infected fruit were collected and frozen with liquid nitrogen for subsequent RNA analysis. Control tissue was obtained from healthy fruits of the RNAi–SlPH and WT lines.

To determine pH and acidity content in the fruit, juice from four to five tomatoes (20 g tissue from each tomato in four replicates) was extracted using a mortar and pestle and filtered through a gauze pad. Fruit pH was determined in the juice. Subsequently, acid content of the fruit juice was determined using a Metrohm titrator (678 EP/KF processor, Switzerland).

Analysis and quantification of total ammonia production and pH changes effected by C. gloeosporioides
Tomato tissue pH was measured in the homogenized solution with a Hamilton double-pore slim electrode connected to a Thermo Orion Model 720A Plus pH meter. Ammonia was detected calorimetrically with an ammonia test kit (Merck, Darmstadt Germany); its concentration was determined in 5-ml samples of 10-fold-diluted supernatant of the homogenized fruit tissue, according to the manufacturer’s instructions. Ammonia concentration was analyzed in the healthy and colonized tissue, using healthy tissue extract as a control for the amount of ammonia present in the colonized tissue of the fruit. Concentrations were reported in millimolar ammonia per gram fresh weight (FW).

RNA extraction
Total RNA was extracted from tomato fruit according to Yang et al. [68], with minor modifications: aliquots were taken from pooled samples of four to five inoculation areas on each tomato. The samples were ground to a fine powder in liquid nitrogen and transferred into 50-ml centrifuge tubes with 10 ml cetyltrimethylammonium bromide-RNA extraction buffer as described by [68]. The mixture was shaken for 3 min and then incubated at 65 °C for 15 min. The centrifuged sample was subjected to a second extraction under similar conditions. Following centrifugation, LiCl was added to a final concentration of 2.5 M and RNA was allowed to precipitate overnight at 4 °C. The precipitated RNA was pelleted at 4 °C for 30 min at 10,000xg, washed with 70% ethanol, and resuspended at 65 °C for 3 min in STSE buffer comprised of 10 mM Tris pH 8, 1 M NaCl, 1 mM EDTA pH 8, and 0.5% (w/v) SDS and the samples were further treated as described before [50, 68]. Finally the RNA was
further treated with Turbo DNAse (Ambion, Austin, TX, USA) and kept at −80 °C until use.

Preparation of libraries
A 500-ng aliquot of total RNA from each of eight samples was processed with the Truseq Stranded Total RNA with Ribo Zero Plant (RS-122-2401) (Illumina, San Diego, CA, USA). Libraries were evaluated with Qubit and TapeStation software (Agilent Technologies, Palo Alto, CA, USA), and sequence libraries were constructed with barcodes to enable sample multiplexing. The results showed 13.2–16.9 million single-end 50-bp reads. These were sequenced on one lane of an Illumina HiSeq 2500 V3 instrument at the Bioinformatics Unit of the Weizmann Institute of Science, Rehovot, Israel.

Bioinformatics analysis of RNA-Seq data
Eight libraries of single-end, 50-nucleotide-long total RNA-Seq reads were generated using Illumina HiSeq 2500. The libraries contained the following sequences: (i) duplicates of healthy WT tomato tissue, line 6421, with 15,127,651 and 13,235,200 reads, respectively; (ii) duplicates of healthy transgenic tomato tissue, line 6422, with 16,905,007 and 14,389,826 reads, respectively; (iii) duplicates of C. gloeosporioides-infected WT tomato tissue, line 6421, with 15,292,125 and 15,272,104 reads, respectively; (iv) duplicates of C. gloeosporioides-infected transgenic tomato tissue, line 6422, with 14,749,785 and 16,162,329 reads, respectively. The datasets are available at the NCBI Sequence Read Archive (SRA) under accession no. SRP078571.

Genome assemblies of tomato (S. lycopersicum, build 2.50 [23]) and C. gloeosporioides isolate Cg-14 (GEO accession no. GSE41844 [24]) were used as reference sequences. Bowtie2 software was used to align the RNA-Seq outputs against the transcriptome of C. gloeosporioides [26]. The libraries were aligned against the C. gloeosporioides genome and tomato genome (downloaded from NCBI accession no. PRJNA176412).

RSEM software was used for transcript quantification and the edgeR package for calculation of the differential gene expression for of the RNA-Seq data [27, 69]. The annotations of C. gloeosporioides were taken from GEO accession no. GSE41844 [24]. GO-enrichment was analyzed by Fisher's Exact Test with multiple testing correction of FDR [70]. We compared the infected WT line with the infected RNAi–SlPH line, and therefore 'upregulated in RNAi–SlPH' means higher expression of the corresponding transcripts in the RNAi–SlPH line as compared to the WT line. Heat map and clustering of the genes were visualized by using the R software ggplots2 package [71].

We used a FDR threshold of <0.05 and logFC greater than 1 or smaller than −1 for analysis of C. gloeosporioides genes, and a FDR threshold of <1e-3 and logFC greater than 2 and smaller than −2 for tomato genes.

Gene-expression analysis by qRT-PCR
Real-time qRT-PCR was performed with the StepOne-Plus System (Applied Biosystems, Grand Island, NY, USA). PCR amplification was performed with 3.4 μl of cDNA template in 10 μl reaction mixture containing 6.6 μl mix from the SYBR Green Amplification Kit (ABgene, Surrey, UK) and 300 nM primers. Additional file 1 lists the forward and reverse primers for each of the indicated genes. The PCR was carried out as follows: 10 min at 94 °C, and 40 cycles of 94 °C for 10 s, 60 °C for 15 s, and 72 °C for 20 s. The samples were subjected to melting-curve analysis: efficiencies were close to 100% for all primer pairs, and all products showed the expected sizes of 70 to 100 bp. All of the samples were normalized to endogenous control gene-expression levels, and the values were expressed as the change (increasing or decreasing) in level relative to a calibrator sample. Results were analyzed with StepOnePlus software v.2.2.2. Relative quantification was performed by the ΔΔCT method. The ΔCT value was determined by subtracting the C_T results for the target gene from those for the endogenous control gene—18S for C. gloeosporioides and TIP41 for tomato analysis—and normalizing against the calibration sample to generate the ΔΔCT values. Each experiment was performed in triplicate, and three different biological experiments were conducted. One representative set of results is presented as mean values of 2^-ΔΔCT ± SE for each treatment.

Additional file
Additional file 1: Table S1. Primers for validations. (DOCX 12 kb)

Abbreviations
13-HPL pathway: 13-hydroperoxide lyase pathway; CAZymes: Carbohydrate active enzymes; GABA: Gamma-Aminobutyric Acid; GO: Gene ontology; GS–GOGAT: GLutamine synthetase–glutamine-oxoglutarate aminotransferase; JA: Jasmonic acid; Lox: Lipoxygenase; PAL: Phenylalanine ammonia-lyase; pg1: Polygalacturonase; SA: Salicylic acid; SRA: Sequence read archive

Acknowledgements
Not applicable.

Funding
We acknowledge funding for the research by the US/Israel Binational Agricultural Research Fund (BARD), grant no. IS-4773-14, and the Israel Science Foundation granted to DP, and used to hire SB, NL and DM. The founding agencies did not have any involved in the design of the studies or in their analysis or interpretations.

Availability of data and materials
RNA-Seq data have been deposited in the NCBI SRA database under accession no. SRP078571.
Authors’ contributions
SB drafted the manuscript. NS performed the bioinformatics analyses and drafted the manuscript. AKD performed the experiments and validations. DK performed qRT-PCR analyses. NL performed the RNA extractions. DM performed the inoculation experiments. SC and AAS developed the RNAi mutants with differential concentrations of organic acids. DP conceived and supervised the project, and drafted the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate
Plant Materials were developed in Israel by SC and AAS and grown at the ARO greenhouses in Israel. All tomato materials is originated from breading programs under the direction of AAS.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details
1 Department of Postharvest Science of Fresh Produce, Agricultural Research Organization, the Volcani Center, 7505101 Rishon LeZion, Israel. 2 Department of Plant Pathology and Microbiology, The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, 76100 Rehovot, Israel. 3 Department of Plant Pathology and Weed Research, ARO, the Volcani Center, 50250 Bet Dagan, Israel. 4 Department of Plant Sciences, Agricultural Research Organization, the Volcani Center, 50250 Bet Dagan, Israel.

Received: 10 November 2016 Accepted: 25 July 2017

Published online: 04 August 2017

References
1. Cohen S, Itkin M, Yeselson Y, Tzuri G, Portnoy V, Harel-Baja R, Lev S, Saar U, Davidovitz-Rikanati R, Baranes N. The PH gene determines fruit acidity and contributes to the evolution of sweet melons. Nat Commun. 2014;5.
2. Davidzon M, Alkan N, Kobiler I, Prusky D. Acidification by gluconic acid of mango fruit tissue during colonization via stem end infection by Phomopsis mangiferae. Postharvest Biol Technol. 2010;55(2):71–7.
3. Eshel D, Lichter A, Dinor A, Prusky D. Characterization of Alternaria alternata glucanase genes expressed during infection of resistant and susceptible persimmon fruits. Mol Plant Pathol. 2002;3(5):347–58.
4. Eshel D, Miyara I, Ailing T, Dinoor A, Prusky D. pH regulates endoglucanase expression and virulence of Alternaria alternata persimmon fruit. Mol Plant-Microbe Interactions. 2002;15(8):774–9.
5. Manteau S, Abouna S, Lambert B, Legendre L. Differential regulation by ambient pH of putative virus factor secretion by the phytopathogenic fungus Botrytis cinerea. FEBS Microbiol Ecol. 2003;49(3):150–66.
6. Prusky D, McEvey JL, Leverenz B, Conway WS. Local modulation of host pH by Colletotrichum species as a mechanism to increase virulence. Mol Plant-Microbe Interactions. 2001;14(9):1105–13.
7. Prusky D, McEvey JL, Salthrer R, Conway WS, Jones R. Relationship between host acidification and virulence of Penicillium spp. on apple and citrus fruit. Phytopathology. 2004;94(1):44–51.
8. Miyara I, Shafsan H, Davidzon M, Sherman A, Prusky D. pH regulation of ammonia secretion by Colletotrichum gloeosporioides and its effect on appressorium formation and pathogenicity. Mol Plant-Microbe Interact. 2010;23(3):304–16.
9. Miyara I, Shnalideman C, Meng X, Vargas WA, Diaz-Minguez JM, Sherman A, Thon M, Prusky D. Role of nitrogen-metabolism genes expressed during pathogenicity of the alkalinizing Colletotrichum gloeosporioides and their differential expression in acidifying pathogens. Mol Plant-Microbe Interact. 2012;25(9):1251–63.
10. Rollins JA, Dickman MB. pH signaling in Sclerotinia sclerotiorum: identification of a pac/RIM1 homolog. Appl Environ Microbiol. 2001;67(1):75–81.
11. Bateman D, Beer S. Simultaneous production and synergistic action of oxalic acid and polygalacturonase during pathogenesis by Sclerotium rolfsii. Phytopathology. 1965;55(11):1204–11.
12. Jennings DH. Some perspectives on nitrogen and phosphorus metabolism in fungi. In: Boddy L, Machant R, Read DJ, editors. Nitrogen and phosphorus utilization by fungi. Cambridge: Cambridge University Press; 1989. p. 1–31.
13. Alkan N, Fluur R, Sherman A, Prusky D. Role of ammonia secretion and pH modulation on pathogenicity of Colletotrichum coccodes on tomato fruit. Mol Plant-Microbe Interact. 2008;21(12):1058–66.
14. Dieguez-Uribondo J, Forster H, Adaskaveg JE. Visualization of localized pathogen-induced pH modulation in almond tissues infected by Colletotrichum acutatum using confocal scanning laser microscopy. Phytopathology. 2008;98(11):1171–8.
15. O’Connell RJ, Thon MR, Hacquard S, Ailing T, Prusky D. Effect of ammonia production by Colletotrichum gloeosporioides on pectin activation, pectate lyase secretion, and fruit pathogenicity. Appl Environ Microbiol. 2006;72(3):1034–9.
16. Prusky D, Alkan N, Mengitse T, Fluur R. Quiescent and Necrotrophic lifestyle choice during postharvest disease development. Annu Rev Phytopathol. 2013;
17. Barad S, Sela N, Kumar D, Kumar-Dubey A, Glam-Matana N, Sherman A, Prusky D. Fungal and host transcriptome analysis of pH-regulated genes during colonization of apple fruits by Penicillium expansum. BMC Genomics. 2016;17(1):330.
18. Prusky D, Gold S, Keen NT. Purification and characterization of an endopolygalacturonase produced by Colletotrichum gloeosporioides. Physiol Plant Pathol. 1989;35(2):121–33.
19. Yakoby N, Benc-Moualem D, Keen NT, Dinoor A, Pines O, Prusky D. Colletotrichum gloeosporioides pefB is an important virulence factor in avocado fruit-fungus interaction. Mol Plant-Microbe Interact. 2001;14(8):988–95.
20. Yakoby N, Kobiler I, Dinoor A, Prusky D. pH regulation of pectate lyase secretion modulates the attack of Colletotrichum gloeosporioides on avocado fruits. Appl Environ Microbiol. 2000;66(3):1025–30.
21. Sato S, Tabata S, Hirakawa H, Asamizu E, Shirasawa K, Isobe S, Kanoke T, Nakamura Y, Shibata D, Aoki K, et al. The tomato genome sequence provides insights into fleshy fruit evolution. Nature. 2012;485(7400):635–41.
22. Alkan N, Meng X, Friedlander G, Neuvensi E, Suko S, Sherman A, Thon M, Fluur R, Prusky D. Global aspects of pacR regulation of pathogenicity genes in Colletotrichum gloeosporioides as revealed by transcriptome analysis. Mol Plant-Microbe Interact. 2012;26(11):1345–58.
23. Alkan N, Friedlander G, Ment D, Prusky D, Fluur R. Simultaneous transcriptome analysis of Colletotrichum gloeosporioides and tomato fruit pathosystem reveals novel fungal pathogenicity and fruit defense strategies. The New Plantologist. 2015;205(2):801–15.
24. Langmead B, Salzberg SL. Fast gapped-read alignment with bowtie 2. Methods. 2012;94(4):357–9.
25. Robinson MD, McCarthy DJ, Smyth GK. EdgeR: a bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 2010;26(1):139–40.
26. Joung JG, Corbett AM, Fellman SM, Tiernan DM, Klee HJ, Giovannoni JJ, Fei Z, Plant GenOMAP: an integrative analysis system for plant systems biology. Plant Physiol. 2009;151(4):1758–68.
27. Farmer EE, Ryan CA. Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. Plant Cell. 1992;4(2):129–34.
28. Raymond P, Farmer EE. Jasmonate and salicylate as global signals for defense gene expression. Current Opinion in Plant Biology. 1998;1(5):404–11.
29. Kuhel T, Koch U, Heller W, Wellmann E. Chlorogenic acid biosynthesis: characterization of a light-induced microbial 5-O-(4-carboxyloxy)-quininate/shikimate 3′-hydroxylase from carrot (Daucus Carota L.) cell suspension cultures. Arch Biochem Biophys. 1987;258(1):226–32.
30. Nicholson RL, Hammerschmidt R. Phenolic compounds and their role in disease resistance. Annu Rev Phytopathol. 1992;30(1):369–89.
33. Fry SC. Cross-linking of matrix polymers in the growing cell wall of angiosperms. An Rev Plant Physiol. 1998;49(3):165–86.

34. Schmidt A, Grimm R, Schmidt J, Scheel D, Strack D, Rosahl S. Cloning and expression of a potato cDNA encoding hydroxycinnamoyl-CoA:tyramine N-(hydroxycinnamoyl) transferase. J Biol Chem. 1999;274(7):4273–80.

35. Cren M, Hirai B. Glutamine synthetase in higher plants regulation of gene and protein expression from the organ to the plant. Cell Plant Cell Physiol. 1999;40(1):187–93.

36. Forde BG, Lea PJ. Glutamate in plants: metabolism, regulation, and signalling. J Exp Bot. 2007;58(3):2339–58.

37. Lam HM, Coschigano K, Oliveira I, Melo-Oliveira R, Coruzzi G. The molecular-genetics of nitrogen assimilation into amino acids in higher plants. Annual Reviews of Plant Biology. 1996;47(1):569–93.

38. Miflin BJ, Lea PJ. The pathway of nitrogen assimilation in plants. Phytochemistry. 1976;15(6):873–85.

39. Galli G, Tang G, Zhu X, Gakire B. Lysine catabolism: a stress and development super-regulated metabolic pathway. Curr Opin Plant Biol. 2001;4(3):261–6.

40. Seifi HS, Van Bockhaven J, Angenon G, Höfte M. Glutamate metabolism in plant disease and defense: friend or foe? Mol Plant-Microbe Interact. 2013;26(5):475–85.

41. Kang S, Kim HB, Lee H, Choi JY, Heu S, Oh CJ, Kwon SJ, An CS. Overexpression in Arabidopsis of a plasma membrane-targeting glutamate receptor from small radish increases glutamate-mediated Ca2+ influx and delays fungal infection. Molecules and Cells. 2006;21(3):418–27.

42. Brauc S, De Vooght E, Claeyss M, Geuns JM, Hofte M, Angenon G. Overexpression of arginase in Arabidopsis Thaliana influences defence responses against Botrytis Cinerea. Plant Biology (Stuttgart, Germany). 2012;14(Suppl. 1):39–45.

43. Tavernier V, Cadiou S, Pageau K, Laugé R, Reisdorf-Cren M, Langin T, et al. Purification of pectate lyase produced by Colletotrichum gloeosporioides. Mol Plant Pathol. 2002;3(5):371–80.

44. Hammond-Kosack KE, Parker JE. Deciphering plant-pathogen communication: fresh perspectives for molecular resistance breeding. Curr Opin Biotechnol. 2003;14(2):177–93.

45. Parniske M. Intracellular accommodation of microbes by plants: a common factor involved in the resistance of unripe avocado fruits to anthracnose. Mol Plant-Microbe Interact. 1994;7(2):293–7.

46. Yang G, Zhou R, Tang T, Shi S. Simple and efficient isolation of high-quality total RNA from Hibiscus Tiliaceus, a mangrove associate and its relatives. Prep Biochem Biotechnol. 2008;38(3):257–64.

47. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Royal Stat Soc. 1995;57(1):289–300.

48. Wattad C, Dinoor A, Prusky D. Purification of pectate lyase produced by Colletotrichum gloeosporioides and its inhibition by epicetamine: a possible factor involved in the resistance of unripe avocado fruits to anthracnose. Mol Plant-Microbe Interact. 1994;7(2):293–7.

49. Lam H-M, Coschigano K, Oliveira I, Melo-Oliveira R, Coruzzi G. The molecular-genetics of nitrogen assimilation into amino acids in higher plants. Annual Reviews of Plant Biology. 1996;47(1):569–93.

50. Allan WL, Clark SM, Hoover GJ, Shelp BJ. Role of plant glyoxylate reductases during stress: a hypothesis. Biochem J. 2009;423(1):15–22.

51. Howard J, Aigner L, Fink G, Ditta G, Gubler U, Schaller G. Effective transformation of the Arabidopsis thaliana genome. Science. 2001;293(5529):619–22.

52. Dixon RA, Achnine L, Kota P, Liu CJ, Reddy M, Wang L. The glyoxylate cycle enables plants and bacteria to grow on acetate. Biochemistry. 5th edition. 2002. Section 17.4.

53. Schindler D, Lea PJ. Glutamate in plants: metabolism, regulation, and signalling. J Exp Bot. 2007;58(3):2339–58.

54. Espeso E, Fluhr R, et al. Carbon regulation of environmental pH by secreted small molecules that modulate pathogenicity in phytopathogenic fungi. Mol Plant Pathol. 2015; doi:10.1111/mpp.12355.

55. Shnaiderman C, Miyara I, Kobler I, Sherman A, Prusky D. Differential activation of ammonium transporters during the accumulation of ammonia by Colletotrichum gloeosporioides and its effect on appressoria formation and pathogenicity. Mol Plant-Microbe Interact. 2013;26(3):345–55.

56. Divon HH, Ziv C, Davydov O, Yarden O, Fluhr R. The global nitrogen regulator, FNR1, regulates fungal nutrition-genes and fitness during Fusicoccum oxysporum pathogenesis. Mol Plant Pathol. 2006;7(6):485–97.

57. Shnaiderman C, Miyara I, Kobler I, Sherman A, Prusky D. Differential activation of ammonium transporters during the accumulation of ammonia by Colletotrichum gloeosporioides and its effect on appressoria formation and pathogenicity. Mol Plant-Microbe Interact. 2013;26(3):345–55.

58. Miyara I, Shafirn H, Kramer Haimovich H, Rollins J, Sherman A, Prusky D. Multi-factor regulation of peptate lyase secretion by Colletotrichum gloeosporioides pathogenic on avocado fruits. Mol Plant Pathol. 2008;9(3):281–91.

59. Kumar S, Punekar NS. The metabolism of 4-amino butyrate (GABA) in fungi. Mycol Res. 1997;101(4):403–9.

60. Mead O, Thynne E, Winterberg B, Solomon PS. Characterising the role of GABA and its metabolism in the wheat pathogen Stagonospora nodorum. Plos One. 2013;8(11):e79368.

61. Sanchez-Tores P, Gonzalez-Candelas L. Isolation and characterization of genes differentially expressed during the interaction between apple fruit and Penicillium expansum. Mol Plant Pathol. 2003;4(6):447–57.

62. Lombard V, Golacosta Ramulu H, Drula E, Coutinho PM, Henriussit B. The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Res. 2014;42(D1):D490–5.

63. Zhao Z, Liu H, Wang C, Xu J-R. Comparative analysis of fungal genomes reveals different plant cell wall degrading capacity in fungi. BMC Genomics. 2013;14(1):274.

64. Marchet-Houben M, Ballester A-R, de la Fuente B, Harries E, Marcos JF, Gonzalez-Candelas L, Gábaldon T. Genome sequence of the necrotrophic fungus Penicillium digitatum, the main postharvest pathogen of citrus. BMC Genomics. 2012;13(1):646.

65. Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henriussit B. The carbohydrate-active Enzymes database (CAZy): an expert resource for glycogenomics. Nucleic Acids Res. 2009;37(Database issue):233–8.

66. Park BH, Kapinets TV, Syd MH, Leuze MR, Uberbacher EC. CAZy enzymes for the analysis of functional groups. Nucleic Acids Res. 2009;37(Database issue):233–8.

67. Wattad C, Dinoor A, Prusky D. Purification of peptate lyase produced by Colletotrichum gloeosporioides and its inhibition by epicetamine: a possible factor involved in the resistance of unripe avocado fruits to anthracnose. Mol Plant-Microbe Interact. 1994;7(2):293–7.

68. Yang G, Zhou R, Tang T, Shi S. Simple and efficient isolation of high-quality total RNA from Hibiscus Tiliaceus, a mangrove associate and its relatives. Prep Biochem Biotechnol. 2008;38(3):257–64.

69. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics. 2011;12(1):323.

70. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Royal Stat Soc. 1995;57(1):289–300.

71. Team RDC. R: a language and environment for statistical computing. R Foundation for Statistical Computing: Vienna; 2009.

72. Venny. An interactive tool for comparing lists with Venn diagrams. (2007-2015). http://bioinfogp.cnb.csic.es/tools/venny/index.html. Accessed 9 Mar 2015.