RNA-Seq derived identification of differential transcription in the chrysanthemum leaf following inoculation with *Alternaria tenuissima*

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**Abstract**

**Background:** A major production constraint on the important ornamental species chrysanthemum is black spot which is caused by the necrotrophic fungus *Alternaria tenuissima*. The molecular basis of host resistance to *A. tenuissima* has not been studied as yet in any detail. Here, high throughput sequencing was taken to characterize the transcriptomic response of the chrysanthemum leaf to *A. tenuissima* inoculation.

**Results:** The transcriptomic data was acquired using RNA-Seq technology, based on the Illumina HiSeq™ 2000 platform. Four different libraries derived from two sets of leaves harvested from either inoculated or mock-inoculated plants were characterized. Over seven million clean reads were generated from each library, each corresponding to a coverage of >350,000 nt. About 70% of the reads could be mapped to a set of chrysanthemum unigenes. Read frequency was used as a measure of transcript abundance and therefore as an identifier of differential transcription in the four libraries. The differentially transcribed genes identified were involved in photosynthesis, pathogen recognition, reactive oxygen species generation, cell wall modification and phytohormone signalling; in addition, a number of varied transcription factors were identified. A selection of 23 of the genes was transcription-profiled using quantitative RT-PCR to validate the RNA-Seq output.

**Conclusions:** A substantial body of chrysanthemum transcriptomic sequence was generated, which led to a number of insights into the molecular basis of the host response to *A. tenuissima* infection. Although most of the differentially transcribed genes were up-regulated by the presence of the pathogen, those involved in photosynthesis were down-regulated.

**Keywords:** Ornamental plant, *Alternaria tenuissima*, RNA-Seq, Cell wall modification genes

**Background**

Chrysanthemum (*Chrysanthemum morifolium* Ramat.) is the second most commercially valuable ornamental species after rose [1,2]. A serious production constraint is represented by black spot disease (causative pathogen the necrotrophic fungus *Alternaria tenuissima* (Fr.) Wiltsh) [3]. The disease is most damaging during humid, warm conditions, which makes it a year-round problem for greenhouse-based production [3,4]. Severe infections damage the commercial value of the plant, as they cause leaf necrosis, and reduce the quantity and quality of the flowers [5]. Little is known regarding the chrysanthemum/black spot host-pathogen interaction. However, in the host-pathogen system involving the model species *Arabidopsis thaliana* and the related pathogen *A. brassicicola*, the pathogenesis-related protein PR4 is significantly up-regulated [6]. In the tomato/*A. alternata* system, the ethylene (ET), jasmonate (JA) and salicylic acid (SA) signalling pathways are all activated as part of the host response [7], while the response of mint to *A. alternata* infection features many proteins related to stress and defence [8]. Finally, Egusa et al. (2009) [9] have shown that the transcription of the genes *PGIP* (polygalacturonase inhibiting protein) and *PPO* (polyphenol oxidase) is induced in the leaf of the Japanese pear...
when challenged by *A. alternata*. Plant defence responses are first activated in the organs located at the site of infection but are then extended to the uninfected systemic (distal) organs, activating a systemic acquired resistance (SAR) which is effective against a broad spectrum of pathogens in the whole plant [10-12]. However, limited studies have examined the induction of SAR in chrysanthemum/black spot host-pathogen interaction, so far. In the present study, the systemic responses are expected by surveying gene expression profiles in the noninfected (systemic) leaves.

RNA-Seq technology has been developed to enable the simultaneous sequencing of very large numbers of short reads, and in so doing has revolutionized the qualitative and quantitative analysis of the transcriptome [13-16]. When applied to cotton infected with a wilt pathogen, of the >32,000 genes identified by mapping the reads to a genomic sequence assembly, over 3,000 were found to be up- or down-regulated as part of the defence response [16]. Similarly, an analysis of the banana-*Fusarium oxysporum* interaction successfully demonstrated the up-regulation of genes involved in hormone synthesis, pathogenesis-related genes, transcription factors and signalling/regulatory genes [17]. Finally, the lettuce-*Botrytis cinerea* interaction has been shown to feature the induction of genes involved in the phenylpropanoid pathway and in terpenoid synthesis, as well as a global down-regulation of genes responsible for photosynthesis [18].

In present study, we aimed to 1) elucidate the localized responses to the infections on the inoculated site by comparing libraries generated from mock-inoculated and inoculated leaves; 2) to describe the systemic response by comparing libraries generated from neighbouring leaves from mock-inoculated and inoculated leaves. The present study reports the outcome of a RNA-Seq based analysis of the chrysanthemum-*A. tenuissima* interaction. The experiment has yielded information regarding the identity of the genes which are either up- or down-regulated as part of the defence response. The majority of the differentially transcribed (DT) genes were involved in either pathogen recognition, reactive oxygen species detoxification, cell wall modification or phytohormone signalling, but also a range of transcription factors, belonging to various families were identified. Validation of the RNA-Seq data was provided by subjecting a set of 23 of the DT genes to quantitative RT-PCR (qPCR).

**Results**

**Analysis of RNA-Seq libraries**

The major characteristics of the four libraries (Figure 1) are summarized in Table 1 and Additional file 1: Figure S1. The number of raw reads per library ranged from ~7.2 to ~7.6 million, and the total number of base pairs sequenced from 352,864,582 to 370,177,360 (Table 1) (Accession No. for library A SRS464569; Accession No. for library B SRS480632; Accession No. for library C SRS480633; Accession No. for library D SRS480635). After removal of reads including adaptor sequence, reads in which >10% of the bases were uncertain, a total of, respectively 7,524,234, 7,248,778, 7,201,318 and 7,554,640 clean reads were obtained, corresponding to 368,687,466, 355,190,122, 352,864,582 and 370,177,360 base pairs (Table 1). The proportion of clean reads was >99.30% in each library (Additional file 1: Figure S1).

**Figure 1** The source of RNA libraries prepared from mock- and pathogen-inoculated chrysanthemum plants. A and B: mock inoculation, C and D: inoculation with an *A. tenuissima* spore suspension. A, C: The first and fourth true leaves without any treatment. B, the second and third mock-inoculated true leaves. D: the second and third pathogen-inoculated true leaves. Leaves were harvested at 0 h, 6 h, 24 h, 48 h and 72 h after inoculation.

**Read mapping**

A reference gene database [Raw sequence data were deposited in the NCBI Sequence Read Archive database (http://trace.ncbi.nlm.nih.gov/Traces/sra_sub/sub.cgi?) under accession number SRP029991] which included all known *Chrysanthemum morifolium* unigene sequences was used to map the RNA-Seq reads. Based on the chosen criteria, 69.75% of the clean reads recognized sequences in this database (Table 1). On a per library basis, the proportions of the clean reads uniquely mapped to the database were, respectively, 46.81%, 45.82%, 46.14% and 44.56%. In addition, the proportion of the clean reads from library D uniquely mapped to the publicly available *A. tenuissima* database was 0.02%. The number of genes identified increased with the number of reads, but above 6,000,000 reads no further genes were detected, implying full saturation of the transcriptome (Figure 2).

**GO classification of DT genes**

Of the 217 genes classified as DT genes in the contrast between library A (leaf 1 and 4 of plants mock-inoculated on leaf 2 and 3) and B (mock-treated leaf 2 and 3), 106 could be assigned a GO classification; the equivalent number for the A vs C (leaf 1 and 4 of plants inoculated by the pathogen on leaf 2 and 3) contrast was 418 out of 659, for the B vs D (pathogen-infected leaf 2 and 3) contrast 1,057 out of 1,705, and for the C vs D contrast 294 out of 494
Table 1 Summary of read mapping

| Sample ID | Total reads | Total basepairs | Total mapped reads | Perfect match <=2 bp mismatch | Unique match | Multi-position match | Total unmapped reads |
|-----------|-------------|-----------------|--------------------|--------------------------------|---------------|----------------------|---------------------|
| A         | 7524234     | 368087466       | 5429990            | 3412870             | 2017120       | 3522413              | 1907577             | 2094244             |
| B         | 7248778     | 355190122       | 5069400            | 3184769             | 1884631       | 3321526              | 1747874             | 2179378             |
| C         | 7201318     | 352864582       | 5033405            | 3149786             | 1883619       | 3322965              | 1710440             | 2167913             |
| D         | 7554640     | 370177360       | 5060651            | 3142621             | 1918030       | 3366700              | 1693951             | 2493989             |

(100.00%) (100.00%) (72.17%) (45.36%) (26.81%) (46.81%) (25.35%) (27.83%)
(100.00%) (100.00%) (69.93%) (43.94%) (26.00%) (45.82%) (24.11%) (30.07%)
(100.00%) (100.00%) (69.90%) (43.74%) (26.16%) (46.14%) (23.75%) (30.10%)
(100.00%) (100.00%) (66.99%) (41.60%) (25.39%) (44.56%) (22.42%) (33.01%)

Figure 2 Sequencing saturation analysis of each library. Sequencing saturation in the four libraries of A, B, C and D. The number of different genes detected rose as the read number was increased.
(Additional file 2: Table S1, Additional file 3: Table S2, Additional file 4: Table S3 and Additional file 5: Table S4, Additional file 6: Table S5, Additional file 7: Table S6, Additional file 8: Table S7 and Additional file 9: Table S8). Furthermore, of the 659 genes classified as DT genes in the contrast between library A and C (A vs C), 469 (71.2%) behaved similarly between A vs C and B vs D; the equivalent number for the B vs D contrast was 469 out of 1,705 (27.5%) (Additional file 7: Table S6 and Additional file 8: Table S7). For the A vs B contrast, seven genes were categorized as "cellular component", six as "molecular function" and 14 as "biological process"; the respective distributions in A vs C, B vs D and C vs D were ten, ten and 20, nine, 11 and 20, nine, nine and 18 (Figure 3). The frequency of DT genes was highest in the contrast B vs D. The commonest molecular functions of the DT genes in this contrast were binding and catalytic activity; in terms of cellular component, most were associated with cells, cell parts, macromolecular complexes, membranes, membrane parts, organelles and organelle parts; finally, in terms of biological process, the majority were associated with cellular processes, metabolic processes and the response to stimulus (Figure 3). Signalling responses to or mediated by JA, SA and ET were well represented, particularly those active in JA-mediated signalling. Some transcription factors and cell wall modification genes were also differentially transcribed, as were genes involved in secondary metabolism (phenylpropanoid pathway and terpenoid synthesis). Among the biological processes well represented among the down-regulated genes were those involved in photosynthesis. In the contrast between B and D, a small number of up-regulated genes belonged to the categories ‘cell killing’, ‘positive regulation of biological process’, ‘membrane-enclosed lumen’ and ‘receptor activity’. A higher number of genes identified in this contrast were associated with ‘response to stimulus’ and ‘signalling’ than in the contrasts A vs B, A vs C and C vs D (Figure 3).

Changes in transcription level

The distribution of unigene coverage in each sample was analysed as a way of evaluating the quality of the RNA-Seq dataset (Figure 4). The term “gene coverage” reflects the proportion of the full gene sequence represented by RNA-Seq reads. For most of the unigenes, gene coverage was >50%. The transcription level of each unigene (Additional file 10: Table S9) was derived from the number of relevant reads recovered following Mortazavi et al. (2008) [19]. DT genes (Additional file 6: Table S5, Additional file 7: Table S6, Additional file 8: Table S7, Additional file 9: Table S8) were identified using an algorithm developed by Audic et al. (1997) [20]. Between A and B, 20 genes were up- and 197 down-regulated, between A and C, the totals were 562 and 97, respectively, between B and D, 1,181 and 524, respectively and C and D, 245 and 249, respectively (Figure 5).

Transcription factors, cell wall modification genes and genes involved in JA and SA signalling were all regulated by A. tenuissima inoculation

The transcription data indicated that infection by A. tenuissima regulated a number of transcription factors and genes associated with pathogenesis and JA and SA signalling. Three members of the GRAS-type transcription factor family were more abundantly transcribed in the inoculated than in the mock-inoculated leaves. When qPCR was performed on a selection of the 23 of the DT genes to validate the conclusions drawn from the RNA-Seq analysis, all the genes behaved as predicted. Most of the genes examined (the exception was the MYB transcription factor CL9570) were induced after A. tenuissima inoculation (Table 2). The qPCR analysis suggested that genes involved in cell wall modification, JA and SA signalling and transcription factors comprised a network of interactions, providing the host with a capacity to fine-tune its disease response.

Discussion

Global patterns of transcription in response to infection by A. tenuissima

The chrysanthemum genome is polyploid and large, so has not as yet benefited from comprehensive and integrated genomic and transcriptomic sequence analysis. The molecular basis of its defence response against pathogen infection is currently poorly understood, but the advent of high-throughput sequencing technology now allows an unprecedented opportunity to explore it. About 70% of the reads in each of the four RNA-Seq libraries were mappable back to known transcripts (Table 1), a proportion which is somewhat lower than achieved in the lettuce/B. cinerea system [18], probably reflecting the more comprehensive status of the lettuce transcriptome. The 30% of reads which were not mappable are presumably associated with as yet unidentified transcripts [16]. The validation through qPCR of the transcripts identified as regulated by A. tenuissima infection showed that the RNA-Seq method is well suited for the analysis of transcription induced as part of the defence response in chrysanthemum (Table 2). In all, 659 DT genes were identified in the A vs C contrast and 1,705 in B vs D (Figure 5, Tables S7, S8). A comparison with the outcomes of the lettuce/B. cinerea and A. thaliana/B. cinerea interactions [18,21,22] showed that only two genes were up-regulated in all three systems, namely Lsa004290.1, At1g74360 and Unigene7965_All (encoding an LRR protein kinase) and Lsa016859.1, At4g17500 and Unigene17395_All (ERF1). During the early phase of both the lettuce/B. cinerea and the A. thaliana/B. cinerea interactions, genes in the ET pathway are heavily involved.
Figure 3 (See legend on next page.)
in the defence response, a finding which was replicated in the chrysanthemum/A. tenuissima interaction. The RNA-Seq data further show that a large number of genes are involved in the host response between six and 72 h post inoculation, including several genes involved in the JA and SA pathways. Some of these are discussed in more detail below.

Pathogen recognition-related genes modulated by A. tenuissima infection

Plant pattern recognition receptors (PRRs) perceive microbe-associated molecular patterns (MAMPs), a set of molecular signatures encompassing whole classes of microbes. This recognition initiates a basal level of immunity (termed MAMP-triggered immunity) [23]. Receptor-like kinases (RLKs), which form the largest plant receptor family, are PRRs localized at the plasma membrane [24]. The involvement of RLKs in certain host-pathogen interactions has been well documented experimentally [18]. The RNA-Seq-based transcriptomic analysis of the lettuce/B. cinerea interaction has revealed that several types of RLK are differentially transcribed [18], and the same phenomenon was recorded in the chrysanthemum/A. tenuissima interaction. The damage caused by microbes can induce the host to synthesize MAMP-like products, termed damage-associated molecular patterns (DAMPs) [23]. Oligogalacturonides (OGs) released from the plant cell wall activate the DAMP-associated response. The induction of an OG receptor wall-associated kinase (WAK) has been suggested as being necessary for the survival of A. thaliana challenged by pathogen infection [25]. Five WAK-like kinases (encoded by Unigene52017_All, Unigene36001_All, Unigene55763_All, Unigene49198_All and Unigene12436_All) were among the DT genes detected in the B vs D contrast (Additional file 11: Table S10), and a sixth (Unigene49198_All) was identified in the A vs C contrast (Additional file 12: Table S11). Another study showed that WAKs were involved in the immune responses against B. cinerea. Furthermore, transgenic plants overexpressing WAK1 conferred resistance to B. cinerea in A. thaliana [26].

Genes encoding a second class of PRR, the leucine-rich repeat RLKs (LRR-RLKs), are known to be involved in both basal and cultivar-specific host immunity [27]. In A. thaliana, BRI1 encodes an LRR-RLK which forms a heterodimeric complex with a second LRR-RLK called BAK1 acting as a negative regulator of microbial infection-induced cell death [27]. Bak1 mutants develop spreading necrosis after the triggering of apoptosis by infection with B. cinerea [27]. The transcription of two LRR-RLK (Unigene20925_All and Unigene27322_All), four BRI-like (Unigene55939_All, Unigene15368_All, Unigene29292_All, Unigene18133_All), six BAK1 genes (Unigene37501_All, Unigene36228_All, Unigene16709_All, Unigene16958_All, Unigene14705_All, and Unigene27008_All), and two somatic embryogenesis
receptor kinase (SERK) genes (Unigene22508_All, Unigene14416_All) was modulated by A. tenuissima infection in the B vs D contrast (Additional file 13: Table S12), as were an additional two BRI-like (Unigene15368_All and Unigene4877_All), a somatic embryogenesis receptor kinase (SERK) genes (Unigene22508_All), and four BAK1 genes (Unigene36228_All, Unigene4877_All, Unigene14705_All and Unigene27008_All) in the A vs C contrast (Additional file 12: Table S11). All the above genes were up-regulated except for Unigene18133_All. In the lettuce/B. cinerea interaction, one BRI-like (Lsa034184.1) gene was up-regulated, but no BAK1 genes [18].

The products of genes encoding cysteine-rich receptor-like kinases (CRKs) are RLKs which contain an extracellular cysteine-rich repeat domain. These genes are reportedly activated by oxidative stress, pathogen attack and exposure to SA [18,28-30]. Although less widely researched, they have been characterized to be involved in the pathogen defense and programmed cell death in A. thaliana [28,30]. Here, three CRKs were identified as DT genes (Unigene55939_All, Unigene15489_All and Unigene14705_All) in the B vs D comparison (Additional file 14: Table S12) and a further three (Unigene4299_All, Unigene14705_All and Unigene15489_All) in the A vs C comparison (Additional file 15: Table S14). Thus, PRR-RLKs are clearly involved in the chrysanthemum host response to A. tenuissima infection. The early activation of these genes may reflect their transcription as an attempt by the host to recognize MAMPs/DAMPs.

ROS (reactive oxygen species) detoxification genes modulated by A. tenuissima infection
MAMPs are known to trigger the production of ROS in response to pathogen infection, largely derived from NADPH oxidase activity (commonly referred to as “respiratory burst oxidase homologues” (rboh)) [17,31]. Two chrysanthemum rboh homologues were recognized: Unigene300_All (homologue of rbohD) and Unigene45792_All (rbohF); both were differentially transcribed in the B vs D (Additional file 16: Table S15) and A vs C contrasts (Additional file 17: Table S16). The lettuce rbohD homologue (Lsa002796.1) was induced 48 h after infection with B. cinerea, but its rbohF homologue (Lsa018309.1) was not up-regulated [18]. In A. thaliana, both rbohD and rbohF are required for ROS detoxification [32]. Two further pathogen-inducible α-dioxygenases (Unigene32071_All and Unigene12359_All) (Additional file 16: Table S15) were differentially transcribed in the B vs D contrast, and one (Unigene32071_All) in A vs C (Additional file 17: Table S16); this class of gene was also up-regulated in the lettuce/B. cinerea system; its product is involved in protecting the cell against oxidative stress [18].

Genes associated with photosynthesis were mostly down-regulated by A. tenuissima infection
DT genes involved in photosynthesis were uniformly down-regulated in the B vs D contrast, with the sole exception of Unigene2020_All (Additional file 18: Table S17). In the contrast C vs D, four photosynthesis-related DT genes were detected, and all were down-regulated (Additional file 19: Table S18). The response mirrors the outcomes in the lettuce/B. cinerea and lettuce/Verticillium dahliae systems [18,33], as well as in other documented plant-pathogen interactions [34-39]. Unigene6198_All, predicted to be involved in the determination of the plant’s circadian clock, was down-regulated in both the B vs D (Additional file 18: Table S17) and C vs D contrasts (Additional file 19: Table S18). Similar examples of the suppression of such genes by pathogen infection have been described in both lettuce [18] and A. thaliana [40]. In the case of A. thaliana, B. cinerea infection appears to dampen the oscillating transcription of certain core clock components, leading to the suggestion that the pathogen attempts to dampen the host’s defence response,
**Table 2** Genes differentially transcribed in ‘Zaoyihong’ leaves in response to mock-inoculation (B) and *A. tenuissima* inoculation (D)

| GeneID          | Function                  | Annotation                                | B-RPKM | D-RPKM       | B-Relative  | D-Relative  |
|-----------------|---------------------------|-------------------------------------------|--------|---------------|-------------|-------------|
|                 |                           |                                           |        | level of gene expression |            | level of gene expression |            |
| Unigene6575_All | Transcription factors     | MYB family transcription factor           | 16.44  | 78.47         | 38.91 ± 4.27| 128.30 ± 8.75|
| Unigene16113_All| MYB family transcription factor |                                           | 4.16   | 15.43         | 2.12 ± 0.25 | 5.33 ± 0.11 |
|                 |                           | MYB family transcription factor           | 40.79  | 16.56         | 20.65 ± 0.61| 9.22 ± 0.34 |
| CL9570          | Ethylene-responsive transcription factor |                                         | 19.13  | 95.07         | 52.19 ± 1.11| 134.46 ± 9.55|
| Unigene29332_All| Ethylene-responsive transcription factor |                                         | 8.06   | 29.67         | 1.93 ± 0.16 | 8.52 ± 0.21 |
| Unigene17395_All| Ethylene-responsive transcription factor |                                         | 29.14  | 105.39        | 18.36 ± 1.63| 59.55 ± 2.61|
| Unigene271_All  | WRKY family transcription factor |                                           | 140.72 | 1061.25       | 91.14 ± 4.36| 766.75 ± 123.16|
| Unigene12209_All| WRKY family transcription factor |                                           | 45.59  | 291.26        | 35.82 ± 2.34| 253.35 ± 8.63|
| Unigene37669_All| WRKY family transcription factor |                                           | 100.02 | 506.41        | 56.99 ± 3.64| 305.58 ± 11.64|
| Unigene23051_All| WRKY family transcription factor |                                           | 251.80 | 974.58        | 159.19 ± 15.47| 722.79 ± 37.85|
| Unigene20543_All| WRKY family transcription factor |                                           | 18.01  | 101.86        | 14.91 ± 1.26| 78.02 ± 3.25 |
| Unigene4479_All | WRKY family transcription factor |                                           | 14.71  | 43.49         | 14.59 ± 1.37| 39.91 ± 1.47 |
|                  | NAC transcription factor |                                           | 18.01  | 101.86        | 14.91 ± 1.26| 78.02 ± 3.25 |
|                  | NAC transcription factor |                                           | 33.29  | 99.47         | 18.1 ± 0.11 | 22.31 ± 2.11 |
| Unigene41060_All| GRAS family transcription factor |                                           | 17.43  | 94.16         | 49.72 ± 5.56| 166.22 ± 8.29|
| Unigene20543_All| GRAS family transcription factor |                                           | 72.90  | 271.62        | 52.64 ± 4.53| 169.66 ± 7.79|
| Unigene1471_All | GRAS family transcription factor |                                           | 15.68  | 38.84         | 63.39 ± 4.99| 285.75 ± 22.55|
|                  | JA signalling pathway     | JAZ gene                                  | 20.57  | 97.20         | 56.24 ± 2.76| 254.24 ± 17.19|
| Unigene23699_All| MYC2 transcription factor |                                           | 71.44  | 27.57         | 34.38 ± 2.78| 14.16 ± 0.74 |
| CL10952         | Vegetative storage protein |                                           | 65.82  | 133.03        | 114.24 ± 4.80| 224.06 ± 16.05|
|                  | SA signalling pathway     | NPR1 gene                                 | 20.05  | 45.55         | 147.74 ± 18.02| 187.23 ± 7.05 |
| Unigene52251_All| TGA transcription factor |                                           | 7.64   | 49.89         | 3.88 ± 0.44 | 21.00 ± 0.79 |
|                  | Cell wall modification    | Polygalacturonase-inhibiting protein      | 1.03   | 30.96         | 1.01 ± 0.07 | 15.04 ± 1.08 |
| CL11209         | Polyphenol oxidase         |                                           | 87.35  | 485.99        | 99.99 ± 26.96| 499.20 ± 24.85|

All these genes were significantly up- or down-regulated (P value < 0.05). The RPKM data were obtained from the RNA-Seq analysis and the relative level of transcript abundance from the qPCR analysis.
because a set of genes associated with plant immune responses was revealed to be regulated by the plant's circadian clock [41].

Genes associated with cell wall protection affected by A. tenuissima infection
Both PGIP and PPO were up-regulated in response to A. tenuissima infection (Table 2). PGIP and PPO are known to respond to various cues [42-48], including the presence of A. solani [42], A. triticina [49], A. macrospora [50], Sclerotinia graminicola [51] and Colletotrichum lindemuthianum [52]. PGIP transcript abundance increased over the period 6–24 h after inoculation with A. tenuissima (Table 2). Many fungi secrete endo-polygalacturonase, an enzyme which degrades the polysaccharides present in the plant cell wall. Host genes involved in the early defence response include those which help to reinforce the cell wall, and thereby inhibit pathogen entry. PGIP's role in mediating resistance to A. alternata infection has been shown in both rough lemon and Japanese pear [9,53], while the growth of B. cinerea is restricted in transgenic tomato heterologously expressing pear PGIP [54]. PPO transcription in chrysanthemum was enhanced after inoculation with A. tenuissima (Table 2). Therefore, it was speculated that CmPGIP (Unigene6575_All and a homologue of AtPGIP), was prominently transcribed from 48 h after inoculation with A. tenuissima (Table 2). Therefore, it was speculated that CmPGIP (Unigene6575_All) may be involved in defence response to the necrotrophic fungus A. tenuissima through the regulation of secondary cell wall biosynthesis. Modifications to the plant cell wall were already recognized as the potential mechanism of resistance, which was previously provided by the reports on the response of plants to fungal challenges [67].

JA and SA signalling pathway-related genes involved in the response to A. tenuissima infection
CmJAZ (Unigene11800_All), CmMYC2 (Unigene3689_All), CmVSP (CL10952), CmNPR1 (Unigene23699_All) and CmTGA (Unigene22521_All) were all induced by A. tenuissima infection (Table 2). NPR1 is a major component of SA signalling, functioning as a co-activator of the TGA transcription factors known to regulate the transcription of various SA-responsive genes [57]. JAZ (jasmonate ZIM domain) proteins repress JA signalling by binding to transcriptional regulators such as MYC2. The degradation of JAZ proteins relieves the JAZ-mediated repression of the JA signalling pathway and thereby activates a large number of JA responsive genes [57,58]. JA and SA both play an important part in host defence against herbivorous insects and microbial pathogens. In A. thaliana, VSP2 and PR-1 are, respectively, JA- and SA-responsive proteins [58]. Overall, a number of genes within the JA and SA signalling pathways are involved in the defence response of chrysanthemum against A. tenuissima infection.

Transcription factors responding to A. tenuissima infection
Transcription factors are central to the control of the timing and placement of defence response gene expression [59]. Their mode of action is to first recognize and then bind to regulatory elements located in the promoter region of their target genes, thereby activating or de-activating their transcription. Here, five classes of transcription factor (MYB, AP2/ERF, WRKY, NAC and GRAS) were identified among the DT genes responding to A. tenuissima infection. In A. thaliana, AtMYB30, AtMYB44 and AtMYB96 are all involved in the triggering of apoptosis and therefore resistance against biotrophic bacterial pathogens such as Pseudomonas syringae [60-64]. AtMYB108 is required for resistance against B. cinerea and A. brassicicola [65]. The transcription of AtMYB38 has been associated with secondary cell wall formation [66]. Here, one CmMYB copy (Unigene6575_All and a homologue of AtMYB38), was prominently transcribed from 48 h after inoculation with A. tenuissima (Table 2). Therefore, it was speculated that CmMYB (Unigene6575_All) may be involved in defence response to the necrotrophic fungus A. tenuissima through the regulation of secondary cell wall biosynthesis. Modifications to the plant cell wall were already recognized as the potential mechanism of resistance, which was previously provided by the reports on the response of plants to fungal challenges [67].

Conclusions
In this study, we characterized the leaf transcriptome of chrysanthemum and provided the comparative DT genes
involved in the interaction between chrysanthemum and A. tenuissima. These findings provide a substantial contribution to existing sequence resources of chrysanthemum, and a strong basis for further characterization of gene expression profiles in the interaction of chrysanthemum and A. tenuissima. The majority of the DT genes were those involved in pathogen recognition, reactive oxygen species detoxification, cell wall modification, phytohormone signalling, and transcription factors belonging to various families were also identified, which will improve our understanding of the molecular mechanisms underlying direct response and induced systemic resistance of chrysanthemum to A. tenuissima.

Methods

Plant materials, A. tenuissima inoculum preparation and inoculation

The chrysanthemum variety ‘Zaoyihong’ was obtained from the Chrysanthemum Germplasm Resource Preserving Centre, Nanjing Agricultural University, China. Uniform cuttings were propagated in sand, and rooted seedlings transplanted into a 2:1 mixture of garden soil and vermiculite without fertilizer supplementation. The plants were grown under a 16 h photoperiod with a day/night temperature of, respectively, 25°C and 18°C. The relative humidity was maintained at 68-75% [3]. A. tenuissima conidia were isolated from diseased chrysanthemum plants, and cultured on potato dextrose agar at 25°C. An aqueous suspension of 10^6 spores per ml was prepared with a few drops of Triton X-100 added as a wetting agent [3]. The surface of the second and third true leaves of 20 day old root cuttings was punctured with a needle (approximately 0.30 mm diameter), and a 10 μl droplet of spore suspension was placed on the puncture site. Mock treatments comprised 10 μl droplets of sterile distilled water. After inoculation, the plants were held at 100% relative humidity and 25°C in the dark for 24 h, and then illuminated with 120 μmol m^-2 s^-1 cool white fluorescent light with a 12 h photoperiod. The leaves of three seedlings were sampled for each treatment at 0 h, 6 h, 24 h, 48 h and 72 h after inoculation. The samples collected at defined time points of each treatment were pooled for RNA-seq.

RNA extraction

Four separate libraries (A-D, see Figure 1) were prepared. Extracts of the second and the third true leaves of

| Table 3 Primers of quantitative reverse transcription-polymerase chain reaction for validation of the digital gene expression data |
|---------------------------------------------------------------|
| **Gene ID** | **Primer F (5'-3')** | **Primer R (5'-3')** | **Basic annotation** |
| Unigene6575_All | CGGAATCAAGAAAGGGTGCAT | ATTCATCCATCGCTGAGC | MYB family transcription factor |
| Unigene16113_All | CGTTGTGACGAGAGGAGTTGA | CTTCTTGGATGGAATTGAG | MYB family transcription factor |
| CL9570 | AAAATGCTGATTGCTGGAG | AAGACCATTTGTCAGCAAG | MYB family transcription factor |
| Unigene29332_All | CAATTCATCGACAGGGGCT | TGCTCTGACATCCTGAGTT | Ethylene-responsive transcription factor |
| Unigene17395_All | ACAGATCCCAACAAGAAGGT | TTGACACTTTTGAACGAAAG | Ethylene-responsive transcription factor |
| Unigene271_All | CTTAATCTGCTGCTGCTGTC | TTAGGGGATGAGGAGCGGT | Ethylene-responsive transcription factor |
| Unigene7360_All | CACCCTCTGTTAGTGAGGAGT | TGAAGATCCATCCTGATCAA | WRKY family transcription factor |
| Unigene12209_All | GTGCTGGAAGTTGTGGTTT | GGCTTACACAGGGTTCACG | WRKY family transcription factor |
| Unigene37863_All | GGATCTCTCAGGGGGAGTTT | GACAACGACTCAACCACCC | WRKY family transcription factor |
| Unigene37669_All | TAGATGCAACATACGGCAAGG | TTTCAGCAGAAAAGGTGTTT | WRKY family transcription factor |
| Unigene23051_All | ACCTACAAACATCAGGCTCGC | CTGCAATATGTTGTCCTAAC | NAC transcription factor |
| Unigene4479_All | CGCAACAAATATCCTGACAT | AGCGAGCGAAATCTGATTGG | NAC transcription factor |
| CL1412 | AGTTTGCCCCACACCATCTG | CGGTTTTCTGAGCCTGTG | NAC transcription factor |
| Unigene41060_All | GTAATGCTGACGATGGTTTG | CTATAATGTTGGCCGGTTTC | GRAS family transcription factor |
| Unigene20543_All | CTTGTGCGAATCCAGCTG | GACCCCTGCTGAAAAGATGA | GRAS family transcription factor |
| Unigene11471_All | TCGAGGGAAGAAGAACACTA | GTTCTTACAAAGGCGGACAT | GRAS family transcription factor |
| Unigene11800_All | AAATTGTTGCGTTTTCGCTG | GATCTTTGGGGAAGCTGGC | JAZ gene |
| Unigene3689_All | CATTAGTAAAACAAGGCCT | AACCAAGCCTCTACACGC | MYC2 transcription factor |
| CL10952 | CCAATGACCAAGAACACTA | TTGAATACGGGCCTTCATCAC | Vegetative storage protein |
| Unigene23699_All | CAAAGACATGGAGTTGACG | TCTAGATGACATGCAGGCC | NPR1 gene |
| Unigene52251_All | CCGCTTGTAGAAGACTGAT | CGTGTTGAGTGTGAGGGG | TGA transcription factor |
| CL11209 | GCGCTATATCAGGTCTGACC | CCGTGAGGTGGTTGAGGT | Polygalacturonase-inhibiting protein |
| Unigene9160_All | TGAACACCAAAGAGATGGGA | CCTACACTTTGTCCACTGTT | Polyphenol oxidase |
mock-treated and pathogen-infected plants gave rise to, respectively, libraries B and D, while extracts of the first and the fourth noninfected (systemic) true leaves of plants gave rise to libraries A and C. Total RNA was isolated using a Total RNA Isolation System (Takara, Japan), following the manufacturer’s recommendations. The quality of the total RNA (RNA Integrity Number > 6.5 and 28S:18S > 1.0) was verified using a 2100 Bioanalyzer RNA Nano chip device (Agilent, Santa Clara, CA, USA) and its concentration ascertained using an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE). The standards applied were 1.8 ≤ OD260/280 ≤ 2.2 and OD260/230 ≥ 1.8. At least 10 μg RNA was pooled in an equimolar fashion from each of the three sampled plants [73].

cDNA library construction and Illumina sequencing
Each total RNA extract was first treated with RNase-free DNase I (TaKaRa, Dalian, China) to remove contaminating DNA, and the mRNA content was concentrated by capturing on magnetic oligo (dT) beads. The mRNA was fragmented to a size of ~200 bp using a fragmentation buffer, and the resulting fragments used to synthesize the first cDNA strand by priming with random hexamers. The second strand was generated using a Super-Script Double-Stranded cDNA Synthesis kit (Invitrogen, Camarillo, CA), purified via magnetic beads, the ends repaired and a single adenine base added to the 3’ ends. Sequencing adaptors were then ligated to the fragments, and agarose gel electrophoresis used to select the range of fragments suitable for PCR amplification. Sequencing using an Illumina HiSeq™ 2000 platform was performed at the Beijing Genomics Institute (Shenzhen, China; http://www.genomics.cn/index.php), following the manufacturer’s protocols.

Treatment of sequence data
Raw reads were saved as .fastq files, and filtered to remove adaptor sequences, reads in which the proportion of non-called bases was > 10% and reads in which low quality (≥ 5) bases represented > 50% of the read. The remaining reads were mapped onto the set of chrysanthemum unigene sequences using SOAPaligner/SOAP2 [74]. A maximum of two mismatches was permitted for the purpose of alignment. The frequency of occurrence of individual reads was normalized to RPKM (reads per kb per million reads) [19].

Identification of DT genes
Following Audic and Claverie (1997) [20], a stringent algorithm was developed to identify DT genes. The FDR (false discovery rate) provides a criterion to determine the P-value threshold in multiple tests and analyses by manipulating the FDR value. Here, differential transcription was declared provided that the P-value was < 0.05, the FDR ≤ 0.001 and the absolute value of log2 induction ratios of treated samples compared with mock-treatment or control (CK) was ≥ 1.0. Standard gene ontology (GO) was used to describe DT gene functionality, and a hypergeometric test was used to map the DT genes to GO terms based on the BGI WEGO (Web Gene Ontology Annotation Plot, http://wego.genomics.org.cn/cgi-bin/wego/index.pl).

Quantitative real-time PCR (qPCR) validation
The transcription of 23 selected candidate genes was determined using quantitative real time PCR (qPCR). The samples collected at different time points were pooled, which were used for qPCR analysis, contaminating DNA removed by RNase-free DNase I treatment and the first cDNA strand synthesized using a Super RT kit (BioTeke, Beijing, China). A set of gene-specific primer pairs (sequences given in Table 3) was designed using Primer3 software [18,75]. qPCRs were based on SYBR Green I (TOYOBO’, Japan) implemented in a Rotor-Gene 3000 device (Corbett, Australia). The chrysanthemum EF1α gene was used as a reference. Each 25 μl qPCR reaction contained 10 μl SYBR Green PCR master mix, 0.2 μM of each primer and 10 ng cDNA, and the amplification regime consisted of an initial denaturation of 95°C/60 s, followed by 40 cycles of 95°C/15 s, 55°C/15 s, 72°C/20 s. Transcript abundances are given as the mean ± SE of three replicates. Relative transcription levels were calculated using the 2−ΔΔCt method [76].

Additional files

**Additional file 1: Figure S1.** Composition of the raw reads in the four RNA libraries. "Clean" reads are those remaining after removal of adaptor sequences, reads in which the proportion of non-called bases was >10% and reads in which low quality (≤ 5) bases represented >50% of the reads. The numbers in parentheses indicate the percentage of each type of read present.

**Additional file 2: Table S1.** GO classification of the genes differentially transcribed in the contrast between libraries A and B.

**Additional file 3: Table S2.** GO classification of the genes differentially transcribed in the contrast between libraries A and B.

**Additional file 4: Table S3.** GO classification of the genes differentially transcribed in the contrast between libraries A and B.

**Additional file 5: Table S4.** GO classification of the genes differentially transcribed in the contrast between libraries A and B.

**Additional file 6: Table S5.** Genes differentially transcribed in the contrast between libraries A and B. The criteria applied for assigning significance were: P-value < 0.05, FDR ≤ 0.001, and estimated absolute | log2Ratio(B/A) | ≥ 1. Genes listed in descending order of absolute log2Ratio(B/A). Genes retrieved from the Chrysanthemum Reference Sequence Database. Annotation of unigene sequences performed using BlastX (E < 10). The “GeneLength” column gives the length of exon sequence. A- and B-expression: frequency of unigene transcripts in libraries A and B, respectively. A- and B-RPKM: reads per kb per million reads for each unigene in libraries A and B, respectively. Log2 Ratio (B/A): the ratio between the RPKM in B and the RPKM in A. Up-Down- Regulation(B/A), P-value and FDR of each gene are also shown. KEGG.
Additional file 7: Table S5. Genes differentially transcribed in the contrast between libraries A and C. The criteria applied for assigning significance were: 1. **P**-value < 0.05, FDR ≤ 0.001, and estimated absolute |log2Ratio(C/A)| ≥ 1. Genes listed in descending order of absolute |log2Ratio(C/A)|. GeneIDs retrieved from the Chrysanthemum Reference Sequence Database. Annotation of unigene sequences performed using BlastX (E < 10). The "GeneLength" column gives the length of exon sequence. A- and C expression: frequency of unigene transcripts in libraries A and C, respectively. Log2 Ratio(C/A): the ratio between the RPKM in C and the RPKM in A, Up-Down-Regulation(D/C), P-value and FDR of each gene are also shown. KEGG annotation according to the KEGG database by BLAST. Blast nr: identification of homologues in GenBank. GO Component, GO Function and Go Process: ontology information of Cellular Components, Molecular Function and Biological Processes of Gene-corresponding GO terms. "-": no hit.

Additional file 8: Table S7. Genes differentially transcribed in the contrast between libraries B and D. The criteria applied for assigning significance were: 1. **P**-value < 0.05, FDR ≤ 0.001, and estimated absolute |log2Ratio(D/B)| ≥ 1. Genes listed in descending order of absolute |log2Ratio(D/B)|. GeneIDs retrieved from the Chrysanthemum Reference Sequence Database. Annotation of unigene sequences performed using BlastX (E < 10). The "GeneLength" column gives the length of exon sequence. B- and D expression: frequency of unigene transcripts in libraries B and D, respectively. Log2 Ratio(D/B): the ratio between the RPKM in D and the RPKM in B, Up-Down-Regulation(D/B), P-value and FDR of each gene are also shown. KEGG annotation according to the KEGG database by BLAST. Blast nr: identification of homologues in GenBank. GO Component, GO Function and Go Process: ontology information of Cellular Components, Molecular Function and Biological Processes of Gene-corresponding GO terms. "-": no hit.

Additional file 9: Table S8. Genes differentially transcribed in the contrast between libraries C and D. The criteria applied for assigning significance were: 1. **P**-value < 0.05, FDR ≤ 0.001, and estimated absolute |log2Ratio(C/D)| ≥ 1. Genes listed in descending order of absolute |log2Ratio(C/D)|. GeneIDs retrieved from the Chrysanthemum Reference Sequence Database. Annotation of unigene sequences performed using BlastX (E < 10). The "GeneLength" column gives the length of exon sequence. C- and D expression: frequency of unigene transcripts in libraries C and D, respectively. Log2 Ratio(C/D): the ratio between the RPKM in D and the RPKM in C, Up-Down-Regulation(D/C), P-value and FDR of each gene are also shown. KEGG annotation according to the KEGG database by BLAST. Blast nr: identification of homologues in GenBank. GO Component, GO Function and Go Process: ontology information of Cellular Components, Molecular Function and Biological Processes of Gene-corresponding GO terms. "-": no hit.

Additional file 10: Table S9. The transcription level of each unigene derived from the number of relevant reads recovered in the four libraries. The "GeneLength" column gives the length of exon sequence.

Additional file 11: Table S10. The differential transcription of Wall-associated receptor kinase-like (WAK-like) genes in the contrast B vs D. The criteria applied for assigning significance were: 1. **P**-value < 0.05, FDR ≤ 0.001, and estimated absolute |log2Ratio(B/D)| ≥ 1. RPKM: reads per kb per million reads.

Additional file 12: Table S11. The differential transcription of Wall-associated receptor kinase-like (WAK-like), brassinosteroid insensitive 1 (BRI-like), somatic embryogenesis receptor kinase (SERK), and BRI-associated receptor kinase 1 (BRAK) genes in the contrast A vs C. The criteria applied for assigning significance were: 2. **P**-value < 0.05, FDR ≤ 0.001, and estimated absolute |log2Ratio(A/C)| ≥ 1. RPKM: reads per kb per million reads.

Addiional file 13: Table S12. The differential transcription of leucine-rich repeat receptor-like kinase (LRR-RLK), brassinosteroid insensitive 1 (BRI)-associated receptor kinase 1 (BAK1), and somatic embryogenesis receptor kinase (SERK) genes in the contrast B vs D. The criteria applied for assigning significance were: 1. **P**-value < 0.05, FDR ≤ 0.001, and estimated absolute |log2Ratio(B/D)| ≥ 1. RPKM: reads per kb per million reads.

Additional file 14: Table S13. The differential transcription of cytokine-rich receptor-like protein kinase (CRK) genes in the contrast B vs D. The criteria applied for assigning significance were: 2. **P**-value < 0.05, FDR ≤ 0.001, and estimated absolute |log2Ratio(B/D)| ≥ 1. RPKM: reads per kb per million reads.

Additional file 15: Table S14. The differential transcription of cytokine-rich receptor-like protein kinase (CRK) genes in the contrast A vs C. The criteria applied for assigning significance were: 1. **P**-value < 0.05, FDR ≤ 0.001, and estimated absolute |log2Ratio(A/C)| ≥ 1. RPKM: reads per kb per million reads.

Additional file 16: Table S15. The differential transcription of respiratory burst oxidase, and alpha-dioxigenase genes in the contrast B vs D. The criteria applied for assigning significance were: 2. **P**-value < 0.05, FDR ≤ 0.001, and estimated absolute |log2Ratio(B/D)| ≥ 1. RPKM: reads per kb per million reads.

Additional file 17: Table S16. The differential transcription of respiratory burst oxidase, and alpha-dioxigenase genes in the contrast A vs C. The criteria applied for assigning significance were: 2. **P**-value < 0.05, FDR ≤ 0.001, and estimated absolute |log2Ratio(A/C)| ≥ 1. RPKM: reads per kb per million reads.

Additional file 18: Table S17. The differential transcription of photosynthesis and circadian rhythm-related genes in the contrast B vs D. The criteria applied for assigning significance were: 1. **P**-value < 0.05, FDR ≤ 0.001, and estimated absolute |log2Ratio(B/D)| ≥ 1. RPKM: reads per kb per million reads.

Additional file 19: Table S18. The differential transcription of photosynthesis and circadian rhythm-related genes in the contrast C vs D. The criteria applied for assigning significance were: 1. **P**-value < 0.05, FDR ≤ 0.001, and estimated absolute |log2Ratio(C/D)| ≥ 1. RPKM: reads per kb per million reads.

Additional file 20: Table S19. High-throughput sequencing metadata.
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