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# Identification of *Lens culinaris* defense genes responsive to the anthracnose pathogen *Colletotrichum truncatum*

Vijai Bhadauria<sup>1</sup>, Kirstin E Bett<sup>1</sup>, Tengsheng Zhou<sup>2</sup>, Albert Vandenberg<sup>1</sup>, Yangdou Wei<sup>2</sup> and Sabine Banniza<sup>1\*</sup>

## Abstract

**Background:** Anthracnose of lentil, caused by the hemibiotrophic fungal pathogen *Colletotrichum truncatum* is a serious threat to lentil production in western Canada. *Colletotrichum truncatum* employs a bi-phasic infection strategy characterized by initial symptomless biotrophic and subsequent destructive necrotrophic colonization of its host. The transition from biotrophy to necrotrophy (known as the biotrophy-necrotrophy switch [BNS]) is critical in anthracnose development. Understanding plant responses during the BNS is the key to designing a strategy for incorporating resistance against hemibiotrophic pathogens either via introgression of resistance genes or quantitative trait loci contributing to host defense into elite cultivars, or via incorporation of resistance by biotechnological means.

**Results:** The *in planta* BNS of *C. truncatum* was determined by histochemical analysis of infected lentil leaf tissues in time-course experiments. A total of 2852 lentil expressed sequence tags (ESTs) derived from *C. truncatum*-infected leaf tissues were analyzed to catalogue defense related genes. These ESTs could be assembled into 1682 unigenes. Of these, 101 unigenes encoded membrane and transport associated proteins, 159 encoded proteins implicated in signal transduction and 387 were predicted to be stress and defense related proteins (GenBank accessions: JG293480 to JG293479). The most abundant class of defense related proteins contained pathogenesis related proteins (encoded by 125 ESTs) followed by heat shock proteins, glutathione S-transferase, protein kinases, protein phosphatase, zinc finger proteins, peroxidase, GTP binding proteins, resistance proteins and syringolide-induced proteins. Quantitative RT-PCR was conducted to compare the expression of two resistance genes of the NBS-LRR class in susceptible and partially resistant genotypes. One (*contig186*) was induced 6 days post-inoculation (dpi) in a susceptible host genotype (Eston) whereas the mRNA level of another (*LT21-1990*) peaked 4 dpi in a partially resistant host genotype (Robin), suggesting roles in conditioning the susceptibility and conferring tolerance to the pathogen, respectively.

**Conclusions:** Data obtained in this study suggest that lentil cells recognize *C. truncatum* at the BNS and in response, mount an inducible defense as evident by a high number of transcripts (23% of the total pathogen-responsive lentil transcriptome) encoding defense related proteins. Temporal expression polymorphism of defense related genes could be used to distinguish the response of a lentil genotype as susceptible or resistant.

**Keywords:** Lentil, Hemibiotrophy, EST, Resistance genes, Plant defense genes

\* Correspondence: [sabine.banniza@usask.ca](mailto:sabine.banniza@usask.ca)

<sup>1</sup>Crop Development Centre, University of Saskatchewan, 51 Campus Drive, Saskatoon SK S7N 5A8, Canada

Full list of author information is available at the end of the article

## Background

*Colletotrichum truncatum* (Schwein.) Andrus and W.D. Moore is the causative agent of anthracnose in legume species, including lentil (*L. culinaris* Medik.) [1]. This fungal pathogen uses sequential biotrophic- and necrotrophic-infection strategies to invade and colonize the plant hosts. Transition from the asymptomatic biotrophic phase (characterized by intracellular thick primary hyphae) to a destructive necrotrophic phase (characterized by thin filamentous secondary hyphae) referred to as the biotrophy-necrotrophy switch (BNS), is essential for anthracnose development [2]. One of the mechanisms likely to be used by hemibiotrophic pathogens like *C. truncatum* to colonize their host plants is the evasion or suppression of plant defense during the biotrophic phase, and elicitation during the BNS and necrotrophic phases.

Studies conducted to date have investigated the histology and cytology of compatible interaction between lentil and *C. truncatum* [3,4]. However, the molecular basis of compatible and incompatible interaction is poorly understood. The generation of ESTs from pathogen-infected host tissues is an effective and rapid way to decipher the mechanisms underpinning plant-microbe interactions. Ma et al. [5] constructed a cDNA library to catalogue genes expressed during the compatible interaction between the obligate biotrophic pathogen *Puccinia striiformis* f. sp. *tritici* adapted race CYR31 (Pst; the causative agent of stripe rust) and wheat genotype Suwon 11. BLASTX analysis of 2743 unigenes from the Pst-infected host transcriptome revealed 52.8% as plant sequences and 16.3% as fungal sequences. The plant-derived unigenes were classified into 13 functional categories on the basis of cellular roles of expressed genes. The most highly represented functional group comprised enzymes involved in energy and metabolism, which included 26% of the unigenes, followed by unknown proteins (13%) and disease/defense related proteins (13%). In a similar study, Yu and colleagues [6] constructed a suppression subtracted hybridization library from the wheat genotype Suwon 11 challenged with urediniospores of non-adapted Pst race CYR23 and isolated 652 unigenes of plant origin. Seventy-seven unigenes (11%) were predicted to encode stress and resistance-related proteins, such as catalase, phenylalanine ammonia-lyase, ascorbate peroxidase, superoxide dismutase, hypersensitive induced reaction protein 1, wound-induced genes, and others. In a compatible interaction between *Arabidopsis thaliana* accession DiG and the necrotrophic pathogen *Alternaria brassicicola*, the causal agent of black spot disease of crucifers, Mukherjee et al. [7] identified differentially expressed genes by suppression subtraction hybridization. Among them were pathogenesis-related (PR) and monooxygenase genes.

Previously, we constructed a cDNA plasmid library from the anthracnose-susceptible Canadian lentil cv. Eston

infected with *C. truncatum* isolate CT-21. *In silico* analyses using BLASTX algorithm of 5000 ESTs predicted that nearly 57% of the ESTs were likely to encode proteins of plant origin, whereas 39% encoded fungal proteins [2]. In the current study, we determined the *in planta* growth phases of *C. truncatum* and characterized the lentil ESTs. Quantitative RT-PCR was used to differentiate susceptible and resistance genotypes using two resistance genes as target genes.

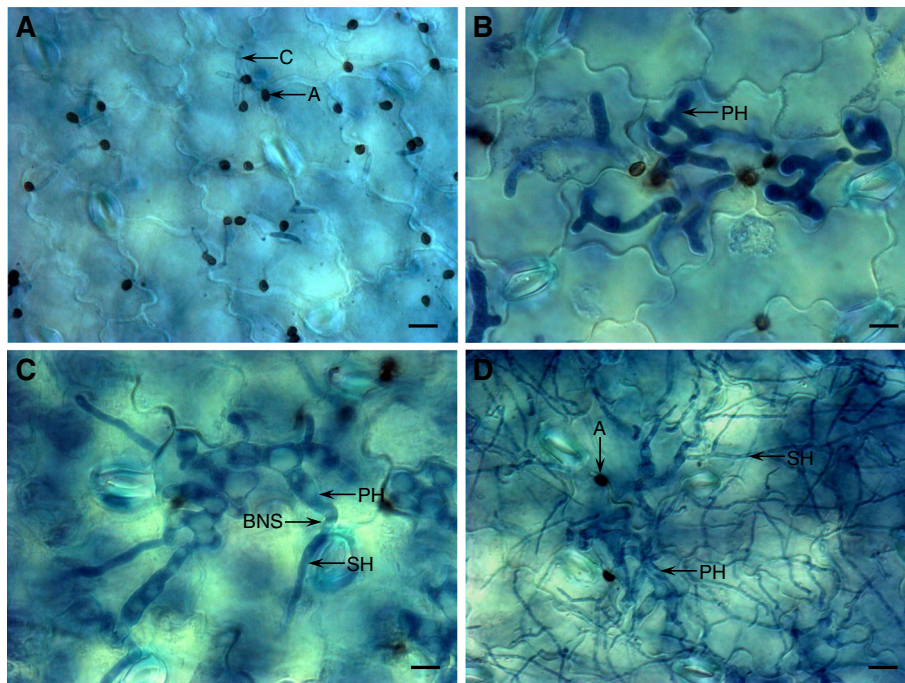
## Results

### Defining the *in planta* growth phases of *C. truncatum*

To visualize the *in planta* fungal developmental stages, we collected leaf tissues of lentil cv. Eston infected with *C. truncatum* isolate CT-21 from 16 hours post-inoculation (hpi) to 68 hpi (Figure 1A-D). At 16 hpi, over 50% of appressoria had generated infection (or penetration) pegs that in turn had penetrated the host cell cuticle and cell wall. This stage of infection is known as the appressorium penetration phase. At 44 hpi, most epidermal cells contained an average of nine thick biotrophic primary hyphae (PH) (the biotrophic phase). These PH were entirely confined to the initially infected epidermal cells. These hyphae are likely to interact with the host plasma membrane but pull away after plasmolysis, indicating a weak host-pathogen association [2]. In contrast, in other hemibiotrophic pathogens like *Magnaporthe oryzae* (the causative agent of rice blast disease), the biotrophic invasive hyphae invaginate and modify the host plasma membrane, and remain adhered with the host cell surface even after plasmolysis, indicating a tight host-pathogen association [8]. During 48-56 hpi, nearly 80% of PH of *C. truncatum* gave rise to thinner secondary hyphae that invaded the neighboring cells. This period of infection is known as the BNS phase. This morphological differentiation of thin hyphae emanating from the thick PH coincides with onset of the destructive necrotrophic phase, when the host tissues are killed and macerated by the pathogen ahead of colonization (68 hpi).

### The lentil transcriptome contains sequences encoding proteins belonging to 12 functional categories

In a previous study, we constructed a directional cDNA library in the plasmid pBluescript from mRNA isolated from lentil cv. Eston infected with isolate CT-21 of *C. truncatum* at the BNS phase (48-56 hpi). Using 5'-end single pass sequencing, 5000 ESTs were generated with an average read length of more than 600 nucleotides. *In silico* analyses of 5000 ESTs had predicted that 57% of ESTs were likely to encode proteins of plant origin, whereas 39% of ESTs were predicted to be proteins of fungal origin. The remaining ESTs (4%) were unassignable [2,9]. The 2852 ESTs of plant origin could be



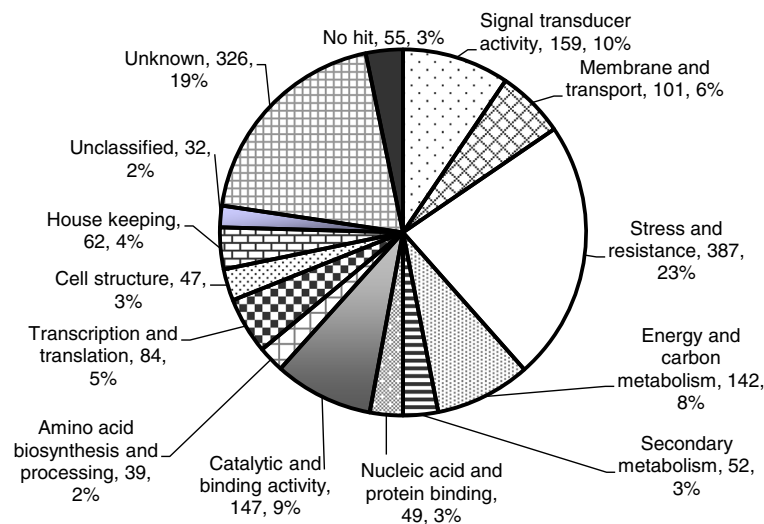
**Figure 1** Histochemical observation of the *in planta* *Colletotrichum truncatum* structures. (A) Appressorial penetration (16 hpi); (B) Biotrophic phase (44 hpi); (C) BNS phase (48-56 hpi); and (D) Necrotrophic phase (68 hpi). C, conidia; A, appressorium; BNS, biotrophy-necrotrophy switch; and SH, secondary hyphae. Bars=10  $\mu$ m.

assembled into 464 contigs and 1218 singletons, resulting in a total of 1682 unigenes.

To assign putative functions to the 1682 plant-derived unigenes, the sequences were queried against the non-redundant protein database using BLASTX. Fifty-five unigenes (3%) had no hit and 326 unigenes (19%) matched to either unknown or hypothetical proteins, and hence the function of 381 unigenes could not be determined. The remaining 1302 (78%) unigenes had homologies to proteins with known function in the protein database at an *E* value cutoff  $\leq 10^{-5}$  and were classified into 12 functional categories according to the *Arabidopsis* sequencing project-functional categories and the Gene Ontology. The most highly represented functional group comprised stress and resistance associated proteins and included 387 unigenes (~23%), followed by 159 proteins for signal transduction (~10%), 147 with catalytic and binding activity (~9%), 142 involved in energy and carbon metabolism (~9%), 101 associated with membrane and transport (~6%), 84 with potential role in transcription and translation (~5%), 62 house-keeping proteins (~4%), 52 secondary metabolism (~3%), 49 with nucleic acid and protein-binding activity (~3%), 47 associated with cell structure (~3%), and 39 for amino acid biosynthesis and processing (~2%). The remaining 2% of the proteins encoded by 32 unigenes could not be classified (Figure 2).

### Stress and resistance related proteins

Triggering of host defense is mainly attributed to three groups of proteins: signal perception and transduction, translocation of these signals and expression of resistance. Nearly 39% of unigenes encoded proteins implicated in these processes (Additional file 1). Proteins potentially involved in signal transducing activity were GTP-binding proteins (GTPases), protein kinases and phosphatases, zinc finger proteins, ethylene-responsive proteins, WRKY transcription factors, and calcium binding proteins. Thirteen unigenes (GenBank accessions: JG293538 through JG293544, JG293600 through JG293604 and JG293612) were predicted to encode proteins involved in GTP binding (G-proteins or GTPases). Twenty-four unique sequences (GenBank accessions: JG293549 through JG293552, JG293554 through JG293556, JG293566 through JG293571, JG293581 through JG293589, JG293591 and JG293616) showed significant similarity to protein kinases, including mitogen-activated protein kinases. We also identified 20 enzymes (GenBank accessions: JG293525 through JG293537 and JG293572 through JG293578) implicated in de-phosphorylating proteins, including 13 calcineurins. Seventeen unigenes belonged to zinc finger proteins (GenBank accessions: JG293495 through JG293510 and JG293518). Ethylene responsive proteins (GenBank accessions: JG293511 through JG293514) and WRKY transcription factors



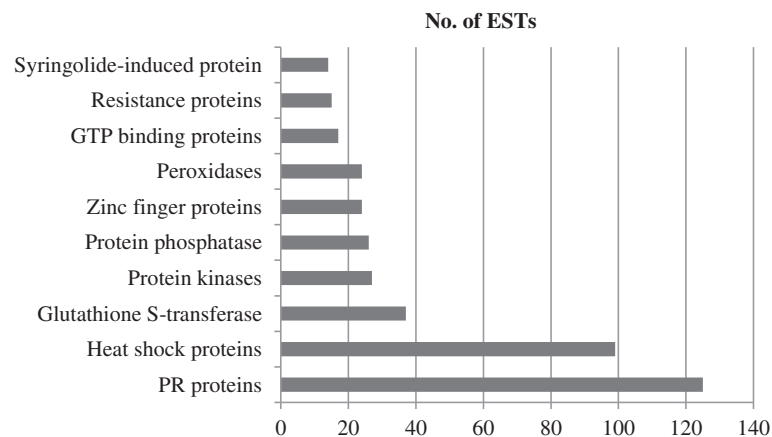
**Figure 2** The functional classification of 1682 unigenes with significant homologies with plant genes captured during the compatible interaction of *Colletotrichum truncatum* isolate CT-21 and *Lens culinaris* cv. Eston. Using BLASTX algorithm with an  $E$  value  $\leq 10^{-5}$ , these unigenes were grouped into 14 categories according to Gene Ontology hierarchy.

(GenBank accessions: JG293516, JG293517, JG294035 and JG294036) triggering resistance responses were also catalogued. The plasma membrane ABC transporters (GenBank accessions: JG293664 through JG293667) along with 97 other transport related proteins were also extracted from the infected lentil transcriptome. The expression of these host genes at the BNS of pathogen colonization indicated the manipulation of host defense responses by *C. truncatum* for accommodating the life-style transition to the necrotrophic phase. A total of 388 unigenes encoding proteins involved in the expression of stress and resistance were identified from the cDNA library. These included catalase, peroxidases, phenylalanine

ammonia-lyase, heat shock proteins, universal stress proteins, hypersensitive-induced response proteins, glutathione S-transferase and resistance proteins.

#### Abundance dynamics of transcripts encoding stress and defense related proteins

On the basis of copy number or frequencies of ESTs in the cDNA library, the relative abundance of transcripts was determined for ten classes of genes encoding proteins implicated in stress and defense (Figure 3). The highest number of transcripts was found for genes encoding pathogenesis related protein encoding genes (125 transcripts), followed by genes encoding heat shock proteins (99 transcripts),



**Figure 3** Distribution of transcripts encoding the 10 most abundant classes of stress and defense associated proteins of *Lens culinaris* induced during the biotrophic-necrotrophic switch (BNS) of *Colletotrichum truncatum* infection.

glutathione S-transferase (37 transcripts), protein kinases (27 transcripts), protein phosphatase (26 transcripts), zinc finger proteins (24 transcripts), peroxidase (24 transcripts), GTP binding proteins (17 transcripts) and resistance proteins (15 transcripts) and syringolide-induced proteins (14 transcripts).

PR proteins included thaumatin like proteins (53 transcripts), glucanases (6 transcripts) and chitinases (42 transcripts). One of the prominent defense responses associated with hypersensitive cell death response (HR) is the accumulation of PR proteins [10-12], some of which may have chitinase and  $\beta$ -1, 3-glucanase activity [13]. The PR protein encoding genes are induced earlier and at higher levels in incompatible plant-pathogen interactions, compared to compatible interactions [10-12]. Their higher transcript levels during the BNS of the compatible lentil - *C. truncatum* interaction indicates the involvement of HR to initiate the transition process. Forty-five unique sequences assembled from 99 ESTs encoding heat shock proteins (HSPs), including small HSPs, HSP70, HSP80 and HSP90. HSPs are induced in response to biotic and abiotic stresses, such as pathogens, heat, cold and wound [14]. Genes encoding catalase, peroxidase and glutathione S-transferase involved in antioxidant activity were also identified from the *C. truncatum*-infected transcriptome. In addition, 27 ESTs were matched to serine/threonine protein kinases, including mitogen activated (MAP) protein kinases, whereas 26 transcripts were similar to protein phosphatases, including calcineurin. ESTs encoding proteins responsible for recognition and transduction of signals to culminate in a host defense response were present in the host transcriptome. Among them were protein kinase (27 transcripts) and phosphatases (26 transcripts), GTPases (15 transcripts) and zinc finger proteins (24 transcripts). Fifteen transcripts encoded motifs characteristic for disease resistance (R) proteins, including nucleotide-binding site and leucine-rich repeats (NBS-LRR). Fourteen ESTs were matched to syringolide-induced proteins that were found to be induced after treatment with the syringolide elicitors produced by the bacterium *Pseudomonas syringae* [15].

#### Quantitative RT-PCR profiling of disease resistance genes

To investigate how the mRNA levels of disease resistance genes alter in susceptible (Eston) and partially resistant (CDC Robin) genotypes of *L. culinaris* during the interaction with *C. truncatum* isolate CT-21, two corresponding time-courses of infection (1 through 7 days post-inoculation [dpi]) were conducted. The expression of two R (NBS-LRR class) gene homologs *contig 186* and *LT21-1990* was analyzed using qRT-PCR. The *contig 186* and *LT21-1990* exhibited transcriptional peaks in Eston (17-fold) and CDC Robin (29-fold), respectively. The NBS-LRR class of R proteins is involved

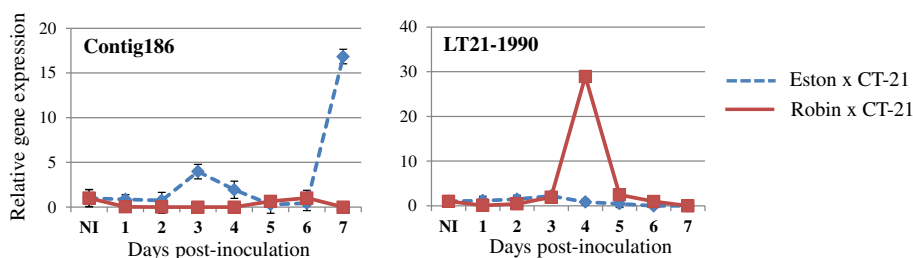
in either direct or indirect perception of pathogen effectors, resulting in a HR that is sufficient to halt the ingress of biotrophic pathogens. A 17-fold-induction of *Contig 186* (TIR [toll/interleukin-1 receptor]-NBS-LRR) mRNA level was observed in CT-21-infected Eston leaf tissues harvested at 7 dpi, suggesting that HR triggered during recognition merely facilitates the infection process considering that the fungus is already in the destructive necrotrophic phase at this stage of infection. No transcriptional change was detected in CT21- infected CDC Robin plants. However, *LT21-1990* (CC [coiled coil]-NBS-LRR) expression peaked at 4 dpi in the partially resistant genotype CDC Robin whereas expression in Eston remained unaltered (Figure 4). This suggests post-invasion resistance mechanisms may play a role in limiting the proliferation of *C. truncatum* in lentil tissues.

#### Discussion

In this study, we catalogued the *C. truncatum*-infected lentil transcriptome of the susceptible cv. Eston induced at the BNS. To date, EST-based studies have been conducted to identify stress and defense associated genes expressed during the compatible and incompatible interactions between wheat and the obligate biotrophic pathogen *Puccinia striiformis* f. sp. *tritici*, the causal agent of stripe rust [5,6], *Arabidopsis thaliana* and necrotrophic pathogen *Alternaria brassicicola* [7], and rice and *M. oryzae* [16,17]. There has been no previous EST-based analysis of the lentil genes induced at the BNS of pathogen infection.

Nearly 57% of ESTs were identified as being of plant origin in the *C. truncatum*-infected lentil transcriptome and were clustered into 1682 unigenes. Among them, 647 unigenes were predicted to be implicated in stress and defense processes of host plants, including those involved in signal (pathogen molecules) perception or recognition (*R*-genes), translocation (serine/threonine protein kinases) and expression of resistance (*PR*-genes). The presence of a high number of such transcripts indicates that *C. truncatum* induces host defense at the BNS phase, most likely to facilitate the transition from biotrophy to necrotrophy by causing HR. Rather than restricting pathogen growth as would be the case for a biotrophic pathogen, HR may facilitate fungal invasion during the necrotrophic phase.

It is likely that *C. truncatum* exposes itself to the host defense system by unmasking conserved pathogen molecules like pathogen associated molecular patterns and by secreting cell death elicitors and toxins during the BNS phase. The pathogen conserved features (signals) are likely to be recognized by host receptors like extracellular (LRR) and cytoplasmic (NBS-LRR) R-proteins (JG293831 and JG293836). Receptor-mediated perception of signals may lead to a cascade reaction through the phosphorylation and



**Figure 4** Quantitative RT-PCR analysis of the mRNA levels of two putative resistance genes (*contig 186* and *LT21-1990*) in susceptible (cv. Eston) and partially resistant (cv. CDC Robin) *Lens culinaris* genotypes during interaction with *Colletotrichum truncatum* CT-21. *Lens culinaris EF1- a* was used as a reference gene for normalizing mRNA levels of target genes following inoculation of *C. truncatum* CT-21. The relative gene expression (fold change) was calculated from 3 biological replicates using the  $2^{-\Delta\Delta CT}$  method [51]. Time-points of infection (1 through 7 days post-inoculation) and relative gene expression are shown on X- and Y, respectively. NI, non-inoculated.

dephosphorylation of MAPKs (JG293551 and JG293556), MAPKKs (JG293552 and JG293555) and MAPKKKs (JG293550 and JG293553) [18,19]. In addition, we identified 13 discrete unigenes encoding G-proteins, including small GTPases Ras (JG293600 and JG293602), Rab (JG293603), Rho (JG293604) and Ran (JG293612) GTPases. Signal transducing GTPases in plants include monomeric (small GTPase) and heterotrimeric G-proteins. Small GTPases are ubiquitous components of signaling systems in animals [20] and yeast [21], and one group of their effectors is MAPKKK [22] and, hence, MAPK cascades. This indicates that serine/threonine protein kinases and GTPase may be involved in the signal transduction between lentil and *C. truncatum* during the hemibiotrophic infection process. These MAPKs may phosphorylate WRKY transcription factors (JG293516, JG293517, JG294035 and JG294036) as shown for tobacco transcription factor WRKY1 by the MAP kinase SIPK. Coexpression of SIPK and WRKY1 in tobacco resulted in accelerated cell death, suggesting that SIPK and WRKY1 participate in the HR [23]. To date, a number of transcription factors, including TGA, WRKY, Myb, and EREBP, have been shown to act downstream of MAPKs [19,24-27]. WRKY transcription factors have been involved in the regulation of plant defense gene expression [28-30]. Their cognate binding site, the W box, can be found in the promoters of genes associated with resistance, such as *PR* genes [26,31-34]. A number of recent studies have pointed out the involvement of WRKY transcription factors in regulating the expression of *PR* genes potentially by direct binding in *Arabidopsis* [35-37].

Phytopathogens secrete effector proteins to facilitate colonization and to suppress host defenses. Some of which function in the apoplast and others are transported to the host cell cytoplasm [38,39]. Apoplastic effectors interfere with host plant defense processes, e.g., by inhibiting plant proteases and lytic enzymes. Cytoplasmic effectors may be recognized by intracellular receptors like NBS-LRR type resistance proteins. This recognition may be direct as

shown for Pto and AvrPto (tomato resistance gene and *Pseudomonas syringae* effector, respectively) interaction [40] or indirect. Indirect recognition is based on detection of altered conformation of virulence targets by R-proteins (Guard hypothesis) [41]. In both cases, the second stage of resistance after perception involves the transduction of recognition signal to the nucleus and to pre-formed enzyme complexes in the cell, resulting in the onset of active defenses, including generation of reactive oxygen species (ROS), cell wall reinforcement, production of antimicrobial compounds like phytoalexins, defense gene activation and programmed cell death (HR) [42,43]. However, at the BNS, rather than restricting the pathogen development, this active defense merely facilitates the transition of the hemibiotroph to the necrotrophic phase, which may require highly focused and transient cell death/necrosis. Expression profiling of two unique sequences encoding proteins of the NBS-LRR class of resistance proteins showed that *contig 186* and *LT21-1990* were induced in susceptible and partially resistant genotypes, respectively. Temporal expression of resistance genes differentiates the response of a genotype as susceptible or resistant.

Genes encoding potential ROS generating and scavenging enzymes like type 3 peroxidase (JG293954), catalase (JG294070), ascorbate peroxidase (JG294047) and glutathione S-transferase (JG293849 through JG293868) were also present in the cDNA library, suggesting that ROS may play an important role in the lentil response to *C. truncatum* during the BNS. The transition from biotrophy to necrotrophy may be facilitated by ROS production and programmed cell death as the necrotrophic phase of the pathogen requires assimilation of nutrients from dead or necrotized host tissues [44,45]. We also identified eight discrete unigenes encoding proteins induced by syringolide in the *C. truncatum*-infected lentil transcriptome. Syringolides are low molecular weight glycolipid elicitors secreted by the bacterial plant pathogen *P. syringae* pv. *glycinea* strains carrying an active

allele of *avr* gene D (*avrD*) [46]. These elicitors are believed to translocate into the host cell by passive diffusion where they bind to a cytoplasmic 34-kDa protein (p34) that is present in both resistant (carrying *Rpg4* resistance gene) and susceptible (carrying the recessive allele *rpg4*) soybean cultivars, suggesting that p34 acts as a guard and that the interaction conforms to the guard hypothesis. Syringolides elicit HR in *Rpg4* soybean cultivars, but not in *rpg4* cultivars [47]. It is likely that *C. truncatum* secretes syringolide-like proteins at the BNS that manipulate or alter guard-like proteins (virulence targets) of lentil (JG293986 through JG293993). Such interactions may activate the cognate R proteins, resulting in the HR cell death, which likely signals the switch to necrotrophy.

## Conclusions

We catalogued and characterized the lentil transcriptome induced during the BNS phase of *C. truncatum* infection in the susceptible lentil cv. Eston. The presence of high numbers of transcripts encoding defense related proteins indicates that the pathogen triggers a cascade of defense responses, which culminate into cell death and as a result, the pathogen proliferates by switching the lifestyle to necrotrophy and colonize plant tissues. The data obtained in this study also suggest that the timing of induction in defense responses is the determining factor for susceptibility or resistance of a genotype to pathogens.

## Methods

### Plant and fungal materials

Lentil plants of the susceptible Canadian cultivar Eston [48] and the partially resistant cultivar CDC Robin [49], and the *C. truncatum* isolate CT-21 (compatible with Eston, restricted growth in CDC Robin) were maintained as described previously [2,3]. For infection assays, fungal spores (conidia) were collected from 10-day-old oatmeal agar cultures by flooding Petri dishes with sterile water. Three-week-old lentil plants and fungal spore suspensions ( $4 \times 10^4$  conidia/mL) were used in attached and detached infection assays [4].

### Visualization of the *in planta* fungal structures

A detached infection assay with lentil cv. Eston and CT-21 was conducted, and infected lentil leaf tissues collected at various time-points were fixed in a solution of 60% methanol, 30% chloroform, and 10% acetic acid until required. Fixed samples were re-hydrated and stained in trypan blue solution as described previously [50]. The leaves then were mounted in 30% glycerol on glass slides and viewed at 40X magnification using an axioplan microscope (Carl Zeiss Microscopy, Thornwood, NY, USA).

### EST analysis and functional categorization of lentil transcriptome

A BNS-specific cDNA library in the plasmid pBluescript® II XR was constructed from *C. truncatum*-infected lentil (cv. Eston) leaf tissues and 5000 ESTs were generated [2]. For this study, lentil ESTs were trimmed for vector, adaptor, and low quality sequence regions using DNAMAN software (Lynnon Corporation, Pointe-Claire, Quebec, Canada). The resulting sequences were clustered using Sequencher (Gene Codes, Ann Arbor, MI, USA) into unique sequences (unigenes). A similarity search was performed to assign putative functions to these unigenes using the BLASTX algorithm with an *E* value threshold  $\leq 10^{-5}$ . The putative functions of unigenes with plant origin were classified according to Beavn et al. [51] and Ashburner et al. [52].

### Intact plant infection time-courses, RNA isolation and quantitative RT-PCR

Lentil cultivars Eston and CDC Robin were sprayed with *C. truncatum* CT-21, and incubated under controlled conditions conducive to anthracnose infection as described elsewhere [4]. Leaf tissues were harvested at 24 hours intervals until 7 dpi, frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until required. Total RNA was extracted using phenol and chloroform extraction followed by LiCl precipitation [53], and the genomic DNA (gDNA) was removed from total RNA using RNase-free amplification grade DNase I (Invitrogen life technologies, Carlsbad, CA, USA). Two micrograms of gDNA-free total RNA were used to synthesize cDNA with SuperScript reverse transcriptase (Invitrogen, Carlsbad, CA, USA) following the protocol of the supplier, and the resulting cDNA was diluted 20 times in DNase/RNase-free ultra pure water (Invitrogen life technologies, Carlsbad, CA, USA). Quantitative RT-PCR analyses were conducted on the CFX96 platform (Bio-Rad, Hercules, CA, USA) complying with the MIQE guidelines [54].

*Lens culinaris* EF1- $\alpha$  was used as the reference gene as it shows no alternation in its transcript levels in lentil following *C. truncatum* inoculation. The mRNA levels of selected target genes (*contig 186* and *LT21-1990*) at various time-points were normalized, and the normalized expression of target genes in non-infected lentil tissues was used as calibrator (considered as 1). The relative gene expression (fold change) was calculated from 3 biological replicates using the  $2^{-\Delta\Delta\text{CT}}$  method [55]. Primers used in the study are listed in the Additional file 2.

### Additional files

**Additional file 1:** Defense associated lentil unigenes expressed at the *in planta* biotrophic-necrotrophic switch of *Colletotrichum truncatum* infection.

**Additional file 2: List of primers.** Oligonucleotide sequence of reference and target genes used in qRT-PCR.

#### Competing interests

The authors declare no competing interests.

#### Authors' contributions

SB, VB, KB, AV and YW conceived and designed the study. VB conducted the experiments. SB, VB, KB and AV wrote the manuscript. VB and TZ conducted the data analysis. All authors read and approved the final manuscript.

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#### Author details

<sup>1</sup>Crop Development Centre, University of Saskatchewan, 51 Campus Drive, Saskatoon SK S7N 5A8, Canada. <sup>2</sup>Department of Biology, University of Saskatchewan, 112 Science Place, Saskatoon SK S7N 5E2, Canada.

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