Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2011) **12**(5), 463–477 DOI: 10.1111/J.1364-3703.2010.00687.X

Identification of differentially expressed genes in a resistant versus a susceptible blueberry cultivar after infection by *Colletotrichum acutatum*

TIMOTHY D. MILES*, BRAD DAY AND ANNEMIEK C. SCHILDER

Department of Plant Pathology, Michigan State University, 107 Center for Integrated Plant Systems, East Lansing, MI 48824, USA

SUMMARY

Anthracnose fruit rot, caused by the fungus *Colletotrichum acutatum*, is an important disease of blueberry worldwide. The cultivar Elliott is resistant, severely restricting fungal growth and sporulation relative to the susceptible cultivar Jersey. The objectives of this research were as follows: (i) to analyse pre-penetration events in 'Elliott' and 'Jersey' at different stages of fruit development; (ii) to identify putative defence genes in 'Elliott' fruit; and (iii) to monitor the timing of the oxidative burst in both cultivars. Light microscopy revealed no differences in the pre-penetration activities of *C. acutatum* on the immature fruit of both cultivars. However, at fruit ripening, conidia germinated and formed appressoria more rapidly on 'Jersey' than on 'Elliott' fruit. Using suppression subtractive hybridization, 37 differentially expressed sequence tags (ESTs) were detected in 'Elliott' versus 'Jersey' upon infection. Several of the ESTs had homology to known plant defence genes, such as a class II chitinase, pathogenesis-related protein 10 (PR10) and β-1,3-glucanase. Two putative genes involved in oxidative stress were identified: a metallothionein-like protein and monodehydroascorbate reductase. ESTs of fungal origin were also detected. Many ESTs had no homology to known genes. Using semi-quantitative and quantitative reverse transcription-polymerase chain reaction (RT-PCR), the expression of most of the candidate genes was detected earlier in 'Elliott' than in 'Jersey', some within 24 h post inoculation (hpi). Monitoring of the oxidative burst showed that the overall H_2O_2 concentration was two to three times higher in 'Elliott' than in 'Jersey' at 24 hpi. The elucidation of the basis of resistance to *C. acutatum* in blueberry will facilitate the development of new anthracnose fruit rot-resistant cultivars.

**Correspondence*: Email: milesti2@msu.edu

INTRODUCTION

Colletotrichum species are ubiquitous fungal pathogens, infecting numerous agronomically important plant species (Afanador-Kafuri *et al*., 2003; Batson and Roy, 1982; Bergstrom and Nicholson, 2000; Bernstein *et al*., 1995; Freeman *et al*., 1998, 2000; Timmer and Brown, 2000). *Colletotrichum acutatum* J.H. Simmonds causes fruit rot in a range of hosts, including almonds, apples, citrus, olives, stone fruits and strawberries (Adaskaveg and Hartin, 1997; Moral *et al*., 2008; Peres *et al*., 2005). *Colletotrichum acutatum* was first described as a separate species in 1965 (Simmonds, 1965). Although initial studies referred to *C. gloeosporioides* (Penz.) Penz. & Sacc. as the causal agent of anthracnose fruit rot on highbush blueberries (Daykin and Milholland, 1984; Hartung *et al*., 1981; Milholland, 1995), *C. acutatum* is now considered to be the primary cause of the disease in temperate regions (Peres *et al*., 2005; Polashock *et al*., 2005; Schilder *et al*., 2002; Verma *et al*., 2006; Yoshida and Tsukiboshi, 2002).

The main symptom of anthracnose on blueberries is rotting of ripe fruit in the field before harvest and in storage after harvest (Milholland, 1995). Initially, sunken areas develop on the fruit surface, followed by the formation of acervuli exuding salmon-coloured conidial masses. This disease can have a severe economic impact, with pre-harvest losses estimated at 10%–20% and post-harvest losses as high as 100% in storage (Milholland, 1995). Most blueberry cultivars are susceptible to anthracnose fruit rot. However, several resistant cultivars have been identified, including 'Elliott', which displays strong resistance in the field and in laboratory inoculation studies (Ehlenfeldt, 2003; Polashock *et al*., 2005).

Host–pathogen interactions have been well characterized in only a few *Colletotrichum*–fruit pathosystems, including avocado (Prusky *et al*., 2000), citrus (Brown, 1977; Timmer and Brown, 2000), mango (Hassan *et al*., 2007), strawberry (Arroyo *et al*., 2005, 2007; Brown *et al*., 2008; Casado-Diaz *et al*., 2006) and pepper (Anand *et al*., 2009; Kim *et al*., 2004, 2008; Ko *et al*., 2005; Mahasuk *et al*., 2009). Following direct penetration of host

© 2011 THE AUTHORS
MOLECULAR PLANT PATHOLOGY © 2011 BSPP AND BLACKWELL PUBLISHING LTD

tissues, *Colletotrichum* species generally have two different host colonization strategies depending on the tissue or host being colonized: intracellular hemibiotrophy and subcuticular intramural necrotrophy. Intracellular hemibiotrophy is the direct invasion of the initial host cell by a primary infection vesicle, followed by the proliferation of primary and secondary hyphae; this strategy is common in *Colletotrichum* spp. infecting bean (O'Connell *et al*., 1985), pea (O'Connell *et al*., 1993), sorghum (Wharton and Julian, 1996) and tobacco (Shen *et al*., 2001). In subcuticular intramural necrotrophy, *Colletotrichum* spp. will invade the plant superficially under the cuticle, generally producing necrotrophic hyphae sooner, proliferate intramurally and only later invade the host cell intracellularly. This type of strategy is typical for infection by *C. capsici* on cowpea (Pring *et al*., 1995) and cotton (Roberts and Snow, 1984). *Colletotrichum acutatum* is known to use both infection strategies on almond (Dieguez-Uribeondo *et al*., 2005), olive (Gomes *et al*., 2009) and strawberry (Curry *et al*., 2002).

A recent study of the infection process on ripe blueberry fruit has shown that *C. acutatum* exhibits different infection strategies depending on the cultivar being colonized (Wharton and Schilder, 2008). In the susceptible cultivar Jersey, the host–pathogen interaction was described as intracellular hemibiotrophy and, in the resistant cultivar Elliott, as intramural necrotrophy. A build-up of phenolic compounds, degradation of fungal hyphae and an accumulation of anthocyanins were noted in 'Elliott', suggesting an active resistance response (Miles *et al*., 2009; Wharton and Schilder, 2008). In addition, conidium germination and appressorium formation were slower on 'Elliott' than on 'Jersey' fruit (Wharton and Schilder, 2008). The infection process on immature fruit tissues of either cultivar was not investigated.

A variety of defence mechanisms in other *Colletotrichum*–plant interactions has been observed, including the production of reactive oxygen species (Brown *et al*., 2008), host-derived cell wall-degrading enzymes (Casado-Diaz *et al*., 2006; Goodwin *et al*., 2004; Lafitte *et al*., 1993; Wijesundera *et al*., 1989), enzymes involved in secondary metabolism (Anand *et al*., 2009; Campos *et al*., 2003; Lahey *et al*., 2004), host cell wall modification (Kim *et al*., 2004; Stanghellini and Aragaki, 1966), and preformed and induced antifungal compounds (Droby *et al*., 1986; Prusky *et al*., 2000). On infection of pepper fruits (*Capsicum annuum* L.) by *C. gloeosporioides*, many defence-related proteins, such as cytochrome P450 (Oh *et al*., 1999a), defensin, thionin-like protein (Oh *et al*., 1999b), thaumatin-like protein (Kim *et al*., 2002) and esterase (Ko *et al*., 2005), are induced in incompatible interactions. As most of these defence responses can be monitored at the transcriptional level, a broad genetic screen can provide insights into the type of defence mechanism involved in a particular plant pathosystem.

Defence responses in fruit to infection by *Colletotrichum* spp. have been studied mostly in immature fruit during the latent (or quiescent) phase of the infection (Prusky *et al*., 1991), and are typical of ontogenic resistance. As avocado fruits mature, the pectin lyase activity of *C. gloeosporioides* increases as the inhibitor epicatechin slowly decreases in concentration. In addition, antifungal diene compounds are degraded by fruit lipoxygenases, and the fruit gradually become increasingly susceptible to infection (Prusky *et al*., 2000). With the exception of the *C. gloeosporioides*–*Capsicum annuum* system, to our knowledge, no research has been published on the defence mechanisms in ripe fruit at different stages of the infection process. Casado-Diaz *et al*. (2006) studied the resistance response of ripe strawberry fruit to *C. acutatum* by comparing naturally infected fruit with uninfected fruit without regard to infection timing. The *C. acutatum*–blueberry pathosystem is of interest because the defence response occurs in ripe fruit and, moreover, the infection strategy of the pathogen changes depending on host susceptibility (Wharton and Schilder, 2008).

A better understanding of the basis of resistance in the *C. acutatum*–blueberry pathosystem will aid in the development of alternative management strategies for a major blueberry disease that is currently controlled primarily with fungicides (Schilder *et al*., 2002). Although anthracnose fruit rot resistance is an objective of blueberry breeding programmes, no genotypic or phenotypic markers are currently available for rapid resistance screening of blueberry breeding lines. The evaluation of plants for anthracnose fruit rot resistance can only be accomplished when they bear fruit in sufficient quantities for inoculation, usually at 2–3 years of age. In previous studies, anthracnose fruit rot resistance in blueberries was not correlated with resistance to foliar infection (Ehlenfeldt *et al*., 2006) or the production of antimicrobial fruit volatiles (Polashock *et al*., 2007). The identification of anthracnose fruit rot resistance mechanisms and associated molecular markers could improve resistance screening procedures. The goal of this research was to compare the infection process by *C. acutatum* in fruit of the resistant cultivar Elliott versus the susceptible cultivar Jersey at different stages of fruit development, and to analyse the molecular mechanisms that underpin host plant resistance to anthracnose fruit rot in 'Elliott' blueberries.

RESULTS

Inoculation of *C. acutatum* at different stages of fruit development

Detached fruit inoculation experiments demonstrated a host resistance phenotype that was described previously by Wharton and Schilder (2008). In the susceptible cultivar Jersey, conidial masses were seen as early as 5 days after inoculation and, at 8 days, started to coalesce and cover the entire surface of the fruit (Fig. 1A,C). The acervulus diameter ranged from 56 to 224 μm,

© 2011 THE AUTHORS
 MOLECULAR PLANT PATHOLOGY © 2011 BSPP AND BLACKWELL PUBLISHING LTD

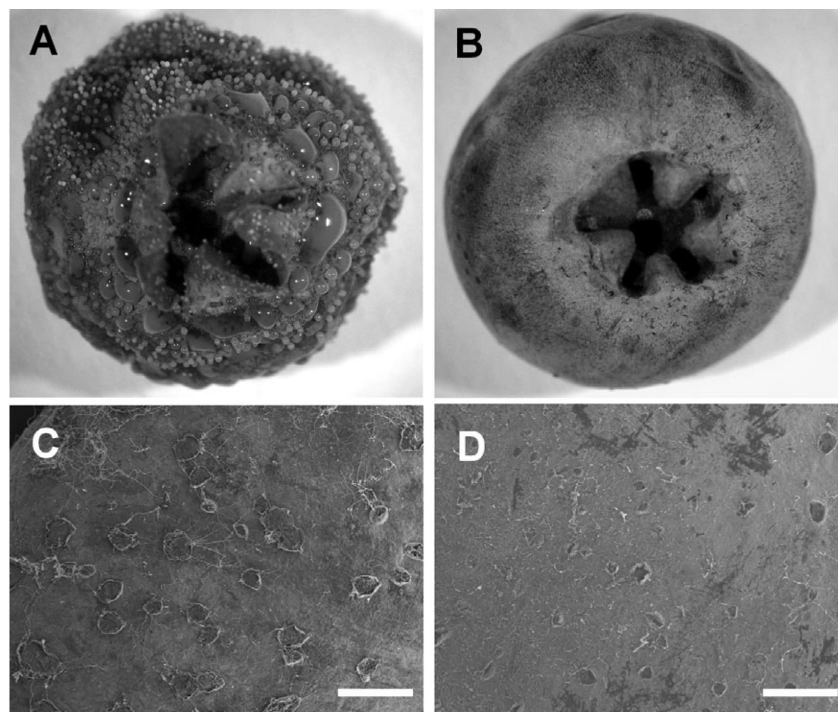

Fig. 1 Symptoms and signs of *Colletotrichum acutatum* infection of highbush blueberry fruit. Fruit appearance 8 days after inoculation in the susceptible cultivar Jersey (A) and the resistant cultivar Elliott (B). Scanning electron micrograph of acervuli on the fruit surface of 'Jersey' (C) and 'Elliott' (D). Bar, 0.5 mm.

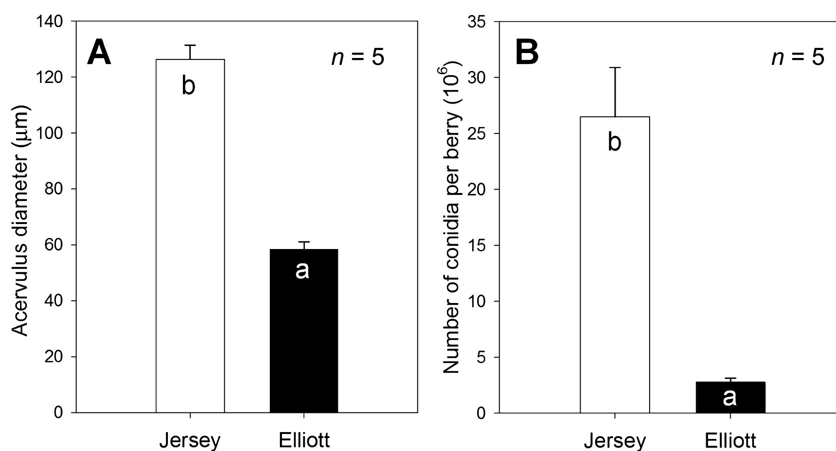

Fig. 2 (A) Average diameter of acervuli of *Colletotrichum acutatum* on ripe fruit of the susceptible blueberry cultivar Jersey (open bars) and resistant cultivar Elliott (filled bars) ($n = 50$). (B) Quantity of *C. acutatum* conidia produced on 'Jersey' (open bars) and 'Elliott' (filled bars) after 8 days of incubation ($n = 5$). Error bars denote the standard error of the mean. Means with the same letter are not significantly different from each other according to Student's paired *t*-test ($P \leq 0.05$).

with an average of 126 μm (Fig. 2A), and an average of 2.65×10^7 conidia were produced per berry (Fig. 2B). In the resistant cultivar Elliott, berries remained firm and acervuli were not visible until 8 days after inoculation (Fig. 1B). Acervuli were few and constricted, with small orange cirrhi. The average acervulus diameter was less than half that on 'Jersey' fruit: 58 μm with a range of 26–106 μm (Fig. 1D). About 10-fold fewer conidia (2.8 $\times 10^6$ per berry) were produced in 'Elliott' compared with 'Jersey' (Fig. 2B).

The examination of the pre-penetration activities of *C. acutatum* on immature fruit of both cultivars showed similar rates of conidium germination and appressorium formation. However, on ripe fruit, significantly more conidia had germinated and formed melanized appressoria by 24 h post inoculation (hpi) on 'Jersey' than on 'Elliott' (Fig. 3). As the formation of melanized appressoria indicates attempted penetration of the plant epidermis, the infection process appears to advance more rapidly in 'Jersey' than in 'Elliott'. However, by 48 hpi, 96%–100% of germinated conidia had produced melanized appressoria, regardless of fruit developmental stage or cultivar.

Suppression subtractive hybridization (SSH) of 'Elliott' and 'Jersey' cDNA libraries

We constructed two representative subtracted cDNA libraries of ripe fruit from 'Elliott' and 'Jersey', each containing five discrete

© 2011 THE AUTHORS
MOLECULAR PLANT PATHOLOGY © 2011 BSPP AND BLACKWELL PUBLISHING LTD

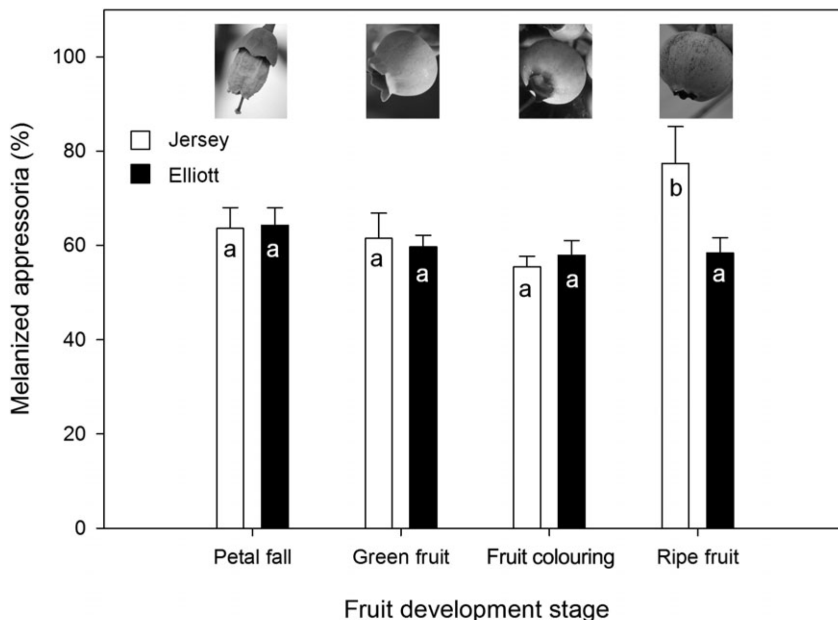

Fig. 3 Percentage of *Colletotrichum acutatum* conidia that had formed melanized appressoria at 24 h post inoculation on blueberry fruit at different stages of development in the susceptible cultivar Jersey (open bars) and resistant cultivar Elliott (filled bars). Error bars denote the standard error of the mean ($n = 5$). Within each development stage, means with the same letter are not significantly different from each other according to Student's paired *t*-test ($P \leq 0.05$).

time points (0, 24, 48, 96, 144 hpi) following inoculation with *C. acutatum*. The pooled subtracted 'Elliott' cDNA library was cloned into the vector pGEM-T-Easy, and we screened 1056 clones against the pooled subtracted 'Jersey' library. Using Southern blot analysis, 37 clones were found to be differentially expressed (Fig. 4). DNA sequence analysis using TBLASTX identified significant homology of these clones with genes producing proteins involved in a variety of cellular processes. The 37 clones were grouped according to their putative physiological function, including defence-related (five clones), abiotic stimuli and development responses (four clones), protein synthesis and processing (three clones), signal transduction and post-translational regulation (one clone) and hypothetical proteins (nine clones) (Tables 1 and S1, see Supporting Information). As was the case in similar studies conducted to date, a large number (41%) of DNA sequences had no significant homology to known genes. In addition, the theoretical isoelectric point (p*I*) of the most homologous protein was calculated for each putative plant-expressed sequence tag (EST). A class II chitinase (EST01) and a β-1,3-glucanase (EST08) were found to be fairly basic (p$I = 8.82$ and 9.37, respectively). However, other homologous proteins were found to be acidic, such as metallothionein-like protein (EST05) and pathogenesis-related protein 10 (PR10) (EST10) (p$I = 4.83$ and 5.36, respectively).

Expression profiling and origin determination of selected 'Elliott' clones

Eight ESTs with significant homology (E value $< 1.0 \times 10^{-5}$) to known protein and gene sequences in the translated nucleotide database at the National Center for Biotechnology Information (NCBI) were further analysed (Table 1). Specific primers were designed for the analysis of temporal expression profiles through semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) (Table 2). Among these ESTs were a class II chitinase (EST01), monodehydroascorbate reductase (EST03), ubiquitin-conjugating enzyme (EST04), metallothionein-like protein (EST05), β-1,3-glucanase (EST08), PR10 (EST10), dihydroflavonol 4-reductase (EST11) and a hypothetical protein from *Mus musculus* (EST13). EST13 was chosen because it had weak homology to a barley (*Hordeum vulgare*) cysteine proteinase inhibitor (accession number CAG38123; *E* value, 4.3; maximum identity, 27%) using BLASTX and, according to the CATH version 3.3 classification, it structurally contains a domain common in other plant cysteine proteinase inhibitors known as superfamily 3.10.450.10 (*E* value, 1.4×10^{-5}) (Orengo *et al*., 1997). In all cases, EST expression increased at an earlier time point in the host–pathogen interaction in 'Elliott' than in 'Jersey'. Many of the ESTs in 'Elliott' were detected as early as 24 hpi, whereas others were detected at 48 hpi (Fig. 5A). None of the ESTs was detected prior to inoculation, except for dihydroflavonol 4-reductase (EST11), which was constitutively expressed. In addition, the initial total RNA preparation after a DNAse treatment yielded no measurable amplification when visualized through gel electrophoresis, indicating that the genomic DNA was eradicated from the samples following DNAse treatment. Moreover, sequencing of the semi-quantitative RT-PCR products indicated that the designed primers were amplifying their intended targets.

© 2011 THE AUTHORS

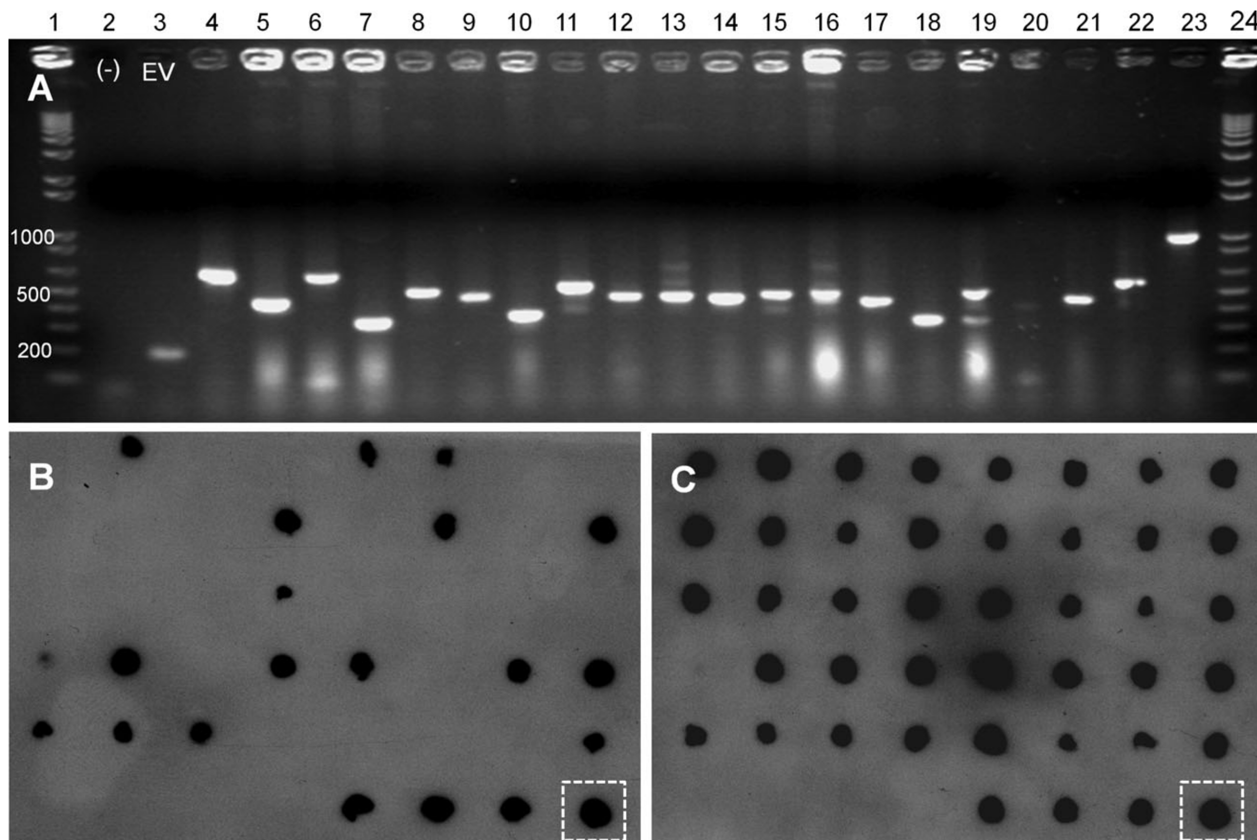

Fig. 4 Suppression subtractive hybridization to detect differential expression of genes in 'Elliott' (resistant) compared with 'Jersey' (susceptible) blueberry fruit after inoculation with *Colletotrichum acutatum*. (A) Colony polymerase chain reaction (PCR) products obtained from the forward-subtracted 'Elliott' cDNA library with T7 and Sp6 primers used for dot blot analysis and visualized through gel electrophoresis. Lanes 1 and 24, 1-kb+ DNA ladder; lane 2, negative control; lane 3, EV = empty vector; lanes 4–23, PCR products from the forward-subtracted 'Elliott' cDNA library. (B) PCR products from the forward-subtracted 'Elliott' library hybridized with the reverse-subtracted 'Jersey' library DIG probe. (C) PCR products from the forward-subtracted 'Elliott' library hybridized with the forward-subtracted 'Elliott' DIG probe. Broken lines surround the actin PCR product which served as an internal standard.

The origin (plant or fungal) of the eight ESTs was then determined by PCR using the putative actin and fungal internal transcribed spacer (ITS) as controls. PCR products for all eight of the ESTs were only seen when using the genomic DNA of the host plant as the template for PCR (Fig. 5B). No PCR products were observed in the reaction with fungal genomic DNA, except for the control ITS1F–ITS4 reaction. This indicates that all of the selected ESTs originated from the host plant.

Using quantitative RT-PCR, selected ESTs were also detected earlier in 'Elliott' than in 'Jersey' (Fig. 6). Throughout the experiments, the expression of putative actin remained relatively constant. For this reason, the putative actin gene was treated as an endogenous control and the expression of the candidate genes was normalized relative to actin. In general, normalized expression levels began to increase at 24 hpi (EST01, EST03, EST04, EST08, EST10, EST11 and EST13) and 48 hpi (EST05) in 'Elliott'. If there was expression in 'Jersey', it visibly increased at 48 hpi (EST11) and 144 hpi (EST01, EST05, EST10 and EST13). There was virtually no expression of any of the selected ESTs in the water-treated control of either cultivar (Fig. 6).

Oxidative burst in green and ripe fruit after inoculation with *C. acutatum*

Based on the identification and temporal expression of genes putatively involved in the oxidative burst response, we monitored the accumulation of H_2O_2, an indicator of reactive oxygen species, in inoculated epidermal peels of green and ripe fruit of both cultivars. Measurements were taken at 0, 12, 18, 24 and 48 hpi with *C. acutatum*. Overall, H_2O_2 levels were significantly lower in green fruit than in ripe fruit (Fig. 7). An increase in H_2O_2 was observed at 18 hpi and a peak at 24 hpi in both cultivars, regardless of fruit maturity stage. However, H_2O_2 levels were two

© 2011 THE AUTHORS

Table 1 Characteristics and predicted physiological function of differentially expressed sequence tags (ESTs) from ripe fruit of highbush blueberry cultivars Elliott versus Jersey after inoculation with *Colletotrichum acutatum*.

ID code*	Sequence size (bp)†	Redundancy‡	Genbank accession no.	Homologous gene (BLAST hit accession number)	Homologous species	Max score	*E* value	p*I*§
Defence-related								
EST01¶	432	1	GW397252	Class II chitinase (AB465728)	*Vaccinium corymbosum*	353	5.0×10^{-95}	8.82
EST08¶	159	1	GW397259	β-1,3-Glucanase (X75946)	*Beta vulgaris*	72	3.0×10^{-11}	9.37
EST10¶	239	3	GW397261	Pathogenesis-related protein 10 (AM489568)	*Actinidia deliciosa*	71	2.0×10^{-10}	5.36
Abiotic stimuli and development								
EST03¶	186	1	GW397254	Monodehydroascorbate reductase (EU327873)	*Vaccinium corymbosum*	127	7.0×10^{-28}	5.78
EST05¶	236	1	GW397256	Metallothionein-like (AY857933)	*Gossypium hirsutum*	100	3.0×10^{-19}	4.83
EST07	138	1	GW397258	Hydroxyproline-rich glycoprotein precursor (U18791)	*Phaseolus vulgaris*	41	6.0×10^{-5}	9.70
EST11¶	389	1	GW397262	Dihydroflavonol 4-reductase (AY221249)	*Allium cepa*	52	1.0×10^{-5}	5.86
Protein synthesis and processing								
EST06	223	1	GW397257	Small nuclear ribonucleoprotein E (EU974208)	*Zea mays*	101	1.0×10^{-19}	6.22
Signal transduction and post-translational regulation								
EST04¶	220	1	GW397255	Ubiquitin-conjugating enzyme (NM_104180)	*Arabidopsis thaliana*	69	4.0×10^{-21}	9.03

The hypothetical function is based on homology to sequences in translated nucleotide databases (DDBJ/EMBL/GenBank) using TBLASTX. This table only displays putative plant sequences with homologous *E* values lower than 1×10^{-5}.
*Identification code.
†bp, number of base pairs.
‡Number of times sequence was recovered.
§Theoretical isoelectric point (p*I*) of the most homologous protein.
¶ESTs selected for further study.

Table 2 Primer DNA sequences, guanine–cytosine (GC) percentages, calculated primer melting temperatures (T_m), expected product size, product melting temperature, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) standard curve plot slope, and calculated primer efficiency used in semi-quantitative and qRT-PCR expression analysis of differentially expressed sequence tags in blueberry fruit in response to *Colletotrichum acutatum* inoculation.

Primer name	Sequence (5′–3′)	Primer GC (%)	Primer T_m (°C)	Product size (number of base pairs)	Product T_m (°C)	qRT-PCR standard curve plot slope	qRT-PCR primer set efficiency (%)
EST01-F	ATCCCCGGTAGTTCCAAAAC	50.0	54.8	206	81.3	–3.30	100.9
EST01-R	TGAGGACTCTGGCACTCCTT	55.0	57.9				
EST03-F	CAGATTGGAGCTTTTGTTCTTATGG	40.0	59.0	98	74.1	–3.32	100.0
EST03-R	GGAAGAAATGCTTATTCAGCCTACA	40.0	59.0				
EST04-F	GGGCAGACCTTACCACAATCA	52.4	57.1	50	73.5	–3.53	91.9
EST04-R	TGGCGGTCTTCGAATAAACC	50.0	55.2				
EST05-F	GAGGTACCGCACTTGCACTT	55.0	57.7	225	83.2	–3.38	97.5
EST05-R	CCATTCACACCCAAGCTACA	50.0	55.0				
EST08-F	TCCAGTCAAGAAGCAGTTGACC	50.0	57.1	50	73.4	–3.21	104.9
EST08-R	CATCCTTCCAATGCCATTGG	50.0	54.3				
EST10-F	ACCCTACAATGTCTTTCACCAACA	41.7	56.5	50	74.0	–3.12	109.1
EST10-R	CGGCCGAGGTACTTTCCAC	63.2	58.2				
EST11-F	GTGTTCACATCGTCTGCTGGA	52.0	58.0	52	75.9	–3.24	103.5
EST11-R	TCGGTTGTTGGTGCTCTTGA	50.0	59.0				
EST13-F	ACCAGCCCGTCTTTAGTCCT	55.0	58.1	197	79.8	–2.95	118.1
EST13-R	TGAGCACGTTGCCTGTTACT	50.0	56.9				
ACT-F	TCAAGAGCCACGTATGCAAG	50.0	55.2	105	78.8	–3.47	94.3
ACT-R	TGCCCTCATGAAGATCCTTAC	47.6	54.2				

© 2011 THE AUTHORS

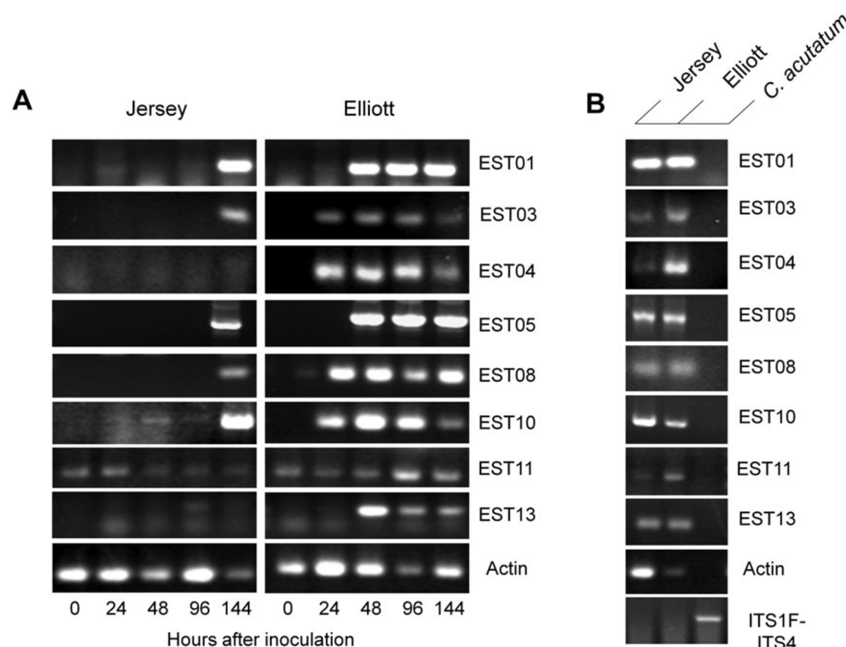

Fig. 5 Temporal pattern of transcript accumulation and origin determination of putative genes in blueberry fruit of the resistant cultivar Elliott and susceptible cultivar Jersey after inoculation with *Colletotrichum acutatum*. (A) Transcript accumulation of expressed sequence tags (ESTs) (Table 1) at 0, 24, 48, 96 and 144 h post-inoculation in semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). (B) The origin of ESTs as determined by PCR using genomic DNA from 'Elliott', 'Jersey' and *C. acutatum*. In (A) and (B), the experiments were performed with specific primers constructed for each individual EST of interest (Table 2). Controls were performed with primers specific for actin (Table 2) and the fungal internal transcribed spacer (ITS) region (ITS1F–ITS4).

to three times higher in 'Elliott' than in 'Jersey'. Even in the water control, the initial and maximum H_2O_2 concentrations were higher in 'Elliott' than in 'Jersey', particularly in green fruit.

DISCUSSION

Following direct penetration of host tissues, *Colletotrichum* species have two different host colonization strategies depending on the host or tissue being colonized: intracellular hemibiotrophy or subcuticular intramural necrotrophy (Curry *et al*., 2002; Dieguez-Uribeondo *et al*., 2005; Gomes *et al*., 2009; O'Connell *et al*., 1985; Pring *et al*., 1995). Wharton and Schilder (2008) linked the strategy of subcuticular intramural necrotrophy by *C. acutatum* on blueberry to host plant resistance and noted defence responses, such as the production of amorphous phenolic globules around intracellular hyphae. They also observed a lower rate of conidium germination and appressorium formation on the resistant cultivar Elliott compared with the susceptible cultivar Jersey. However, they did not investigate the infection process on immature fruit. Our results suggest that differences in pre-penetration activities of *C. acutatum* are confined to ripe fruit, as no differences were observed on immature fruit of the two cultivars. The difference appears to be the result of a relative increase in the rate of appressorium formation on 'Jersey' as the fruit ripens, whereas the rate on 'Elliott' remains steady across fruit development stages. A possible reason for this phenomenon could be a change in the structure or composition of the waxy cuticle in 'Jersey' fruit that stimulates conidium germination and appressorium formation. In avocado, cuticular wax has been shown to trigger conidium germination and appressorium formation of *C. gloeosporioides* (Podila *et al*., 1993). More research is needed to determine the cause of the observed differences.

This study represents the first investigation of gene expression in a *Vaccinium–Colletotrichum* interaction at different stages of the infection process, and provides additional evidence for an active resistance response in the blueberry cultivar Elliott. It appears that this cultivar recognizes the pathogen at an earlier time point during infection than the susceptible cultivar Jersey, as expression of defence-related and other genes was detected earlier in 'Elliott' than in 'Jersey', often within 24 hpi. Given the scarcity of DNA sequence information for blueberry and *Vaccinium* spp. in general, the most limiting constraint on these experiments was the identification of homologous sequences. Many of the differentially expressed DNA sequences (41%) found in this study had no homology to known sequences. In addition, pooling of cDNA from multiple time points after inoculation into a single cDNA library for each cultivar for cost-efficient SSH screening may have reduced our ability to detect certain genes that were differentially expressed at specific time points. For example, defence genes expressed late in the infection process in the susceptible cultivar could have masked early overexpression in the resistant cultivar. In addition, cloning the cDNA library of 'Elliott' only could have limited our ability to detect specific plant genes that were upregulated in 'Jersey', as well as fungal genes that were upregulated in the compatible host–pathogen interaction.

Nonetheless, this study provides valuable new insight into the molecular mechanisms underpinning fruit rot resistance in blueberry, and also forms a basis for comparing defence-associated genes of blueberry fruit with those of other plant species. The

© 2011 THE AUTHORS
MOLECULAR PLANT PATHOLOGY © 2011 BSPP AND BLACKWELL PUBLISHING LTD

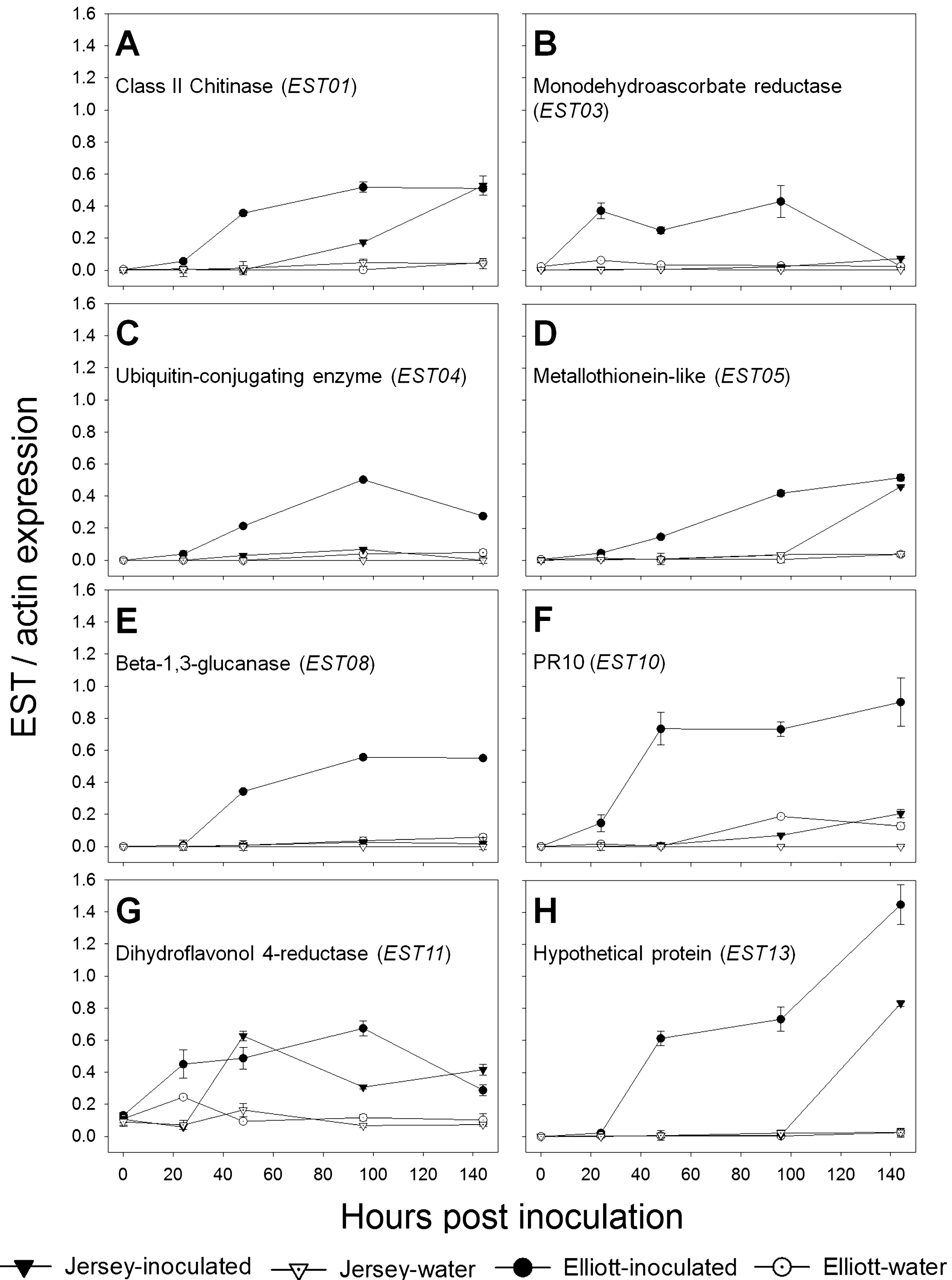

Fig. 6 Ratio of transcript accumulation of expressed sequence tags (ESTs) relative to actin in quantitative reverse transcription-polymerase chain reaction (RT-PCR) at 0, 24, 48, 96 and 144 h post-inoculation of ripe blueberry fruit of the susceptible cultivar Jersey and resistant cultivar Elliott with *Colletotrichum acutatum* or water (control). Mean expression levels were normalized with mean endogenous actin levels, and an induced sample was used to generate the standard curve plots for each individual EST. ΔR_n thresholds varied between 0.15 and 0.75. Error bars denote the standard error of the means of the replicates within a representative experiment ($n = 3$).

© 2011 THE AUTHORS

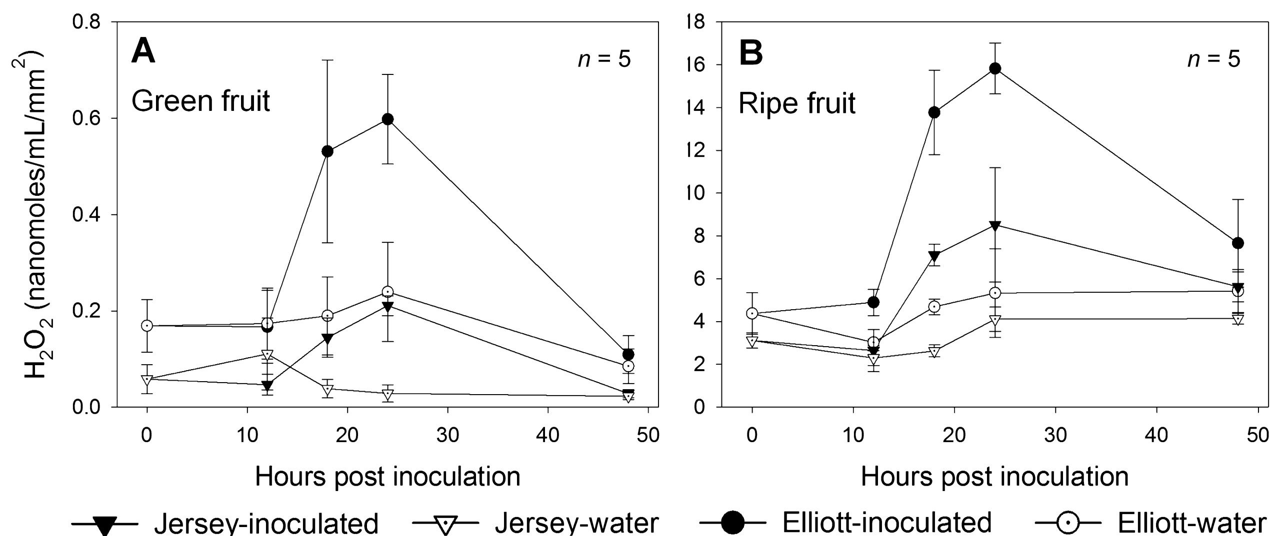

Fig. 7 H_2O_2 accumulation in epidermal peels of green (A) and ripe (B) fruit of the susceptible blueberry cultivar Jersey and resistant cultivar Elliott inoculated with *Colletotrichum acutatum* or water (control) at 0, 12, 18, 24 and 48 h post-inoculation ($n = 5$). H_2O_2 was detected by the Amplex® Red Hydrogen Peroxide/Peroxidase Kit. Results from a representative experiment of three independent experiments are displayed.

selected ESTs may be useful in the development of markers for breeding new disease-resistant cultivars and as possible markers for the expression of systemic acquired resistance (SAR).

Limited research has been carried out on the molecular basis of host plant resistance to the fruit rot pathogen *C. acutatum*. In strawberry, researchers have found that several defence-related proteins, such as peroxidases and chitinases, are induced in infected fruit. Interestingly, they also noted reduced expression of defence-related genes in the susceptible cultivar as opposed to induction in the resistant cultivar (Casado-Diaz *et al*., 2006). In addition, expression was found to be tissue dependent when comparing infected fruits and crowns. We investigated the expression profiles of three putative defence-related genes: a class II chitinase (EST01), β-1,3-glucanase (EST08) and PR10 (EST10). These genes have been classically identified as PR proteins, i.e. they are induced upon pathogen inoculation and are also known to be important in the hypersensitive response (van Loon and van Strien, 1999). Furthermore, given the homology of EST08 and EST01 with other known proteins and their induction upon pathogen inoculation, they could also be referenced as PR2 and PR3, respectively (van Loon and van Strien, 1999). When these genes are overexpressed in plant tissue, the result is often increased disease resistance. For example, in transgenic tobacco plants, the overexpression of a chitinase gene led to broad resistance against the fungal pathogen *Rhizoctonia solani* and the bacterial pathogen *Pseudomonas syringae* pv. *tabaci* (Dana *et al*., 2006). Interestingly, the class II chitinase (EST01) that we identified has also been shown to accumulate in stems of blueberry at low temperatures and appears to be important in cold hardiness (Kikuchi and Masuda, 2009). Both EST01 and EST08 are homologous to genes coding for basic proteins, i.e. their expression is most probably linked to the ethylene/jasmonic acid pathway, and the proteins are probably not secreted into the apoplast. However, the most homologous protein to EST10 was acidic, suggesting that it may be linked to the salicylic acid signalling pathway, and could be secreted into the plant apoplast during infection (van Loon and van Strien, 1999).

In the group of ESTs related to abiotic stimuli and development, the dihydroflavonol 4-reductase gene (EST11) was expressed constitutively, but expression was boosted by inoculation with *C. acutatum* in both cultivars, albeit earlier in the infection process in 'Elliott' than in 'Jersey'. This enzyme typically catalyses the reduction of leucoanthocyanidins to anthocyanidins (Nakajima *et al*., 2001). Previous research has shown an accumulation of anthocyanins in 'Elliott' in response to infection by *C. acutatum* (Miles *et al*., 2009). This gene possibly plays a role in the production of anthocyanins and may be an antifungal compound detected in 'Elliott' fruit and capable of inhibiting the growth of *C. acutatum in vitro* (T. D. Miles and A. M. C. Schilder, unpublished data).

Other identified genes, such as monodehydroascorbate reductase (EST03) and a metallothionein-like protein (EST05), are often associated with oxidative stress and protection of the plant from oxidative damage (Kumari *et al*., 1998; Leterrier *et al*., 2005). In the *C. acutatum*–blueberry fruit pathosystem, the timing of the increase in H_2O_2 is similar to that published previously in the *C. coccodes*–tomato fruit interaction (Mellersh *et al*., 2002) and seems to correlate well with the formation of melanized appressoria (Wharton and Schilder, 2008), indicating attempted penetration. However, reactive oxygen species may

© 2011 THE AUTHORS
MOLECULAR PLANT PATHOLOGY © 2011 BSPP AND BLACKWELL PUBLISHING LTD

be plant or pathogen derived. If it is plant derived, H_2O_2 may be important in the resistance response in 'Elliott' by preventing fungal penetration. However, if H_2O_2 is pathogen derived, this could indicate preferential necrotrophy of *C. acutatum* on 'Elliott' fruit. As H_2O_2 can be a pathogenicity factor for necrotrophic pathogens, it may be important in the initial colonization of 'Elliott' fruit. However, because of the relatively short duration of the H_2O_2 boost and coincident timing with peak appressorium formation, it seems likely that it serves to prevent pathogen ingress. In either case, the above-mentioned genes are probably upregulated in 'Elliott' fruit to prevent oxidative damage to plant tissues.

The ubiquitin-conjugating enzyme or E2 ligase gene identified (EST04) was present in both 'Elliott' and 'Jersey', but appears to be expressed only in 'Elliott'. The ubiquitin–proteasome system is important in signal transduction in cellular processes, including defence responses (Sullivan *et al*., 2003). E2 ligases enzymatically transfer ubiquitin and bind with E3 ligases, which target proteins for proteasomal degradation by ubiquitination. However, there is also evidence that E2 ligase genes can be upregulated in response to elicitors (Takai *et al*., 2002), ultraviolet light (Ri-He *et al*., 2003) and heat shock (Feussner *et al*., 1997). The identified E2 ligase may play a role in the resistance response in 'Elliott' fruit.

Our results clearly show a time delay between inoculation and measurable gene expression. Consequently, there was a significant lag period before a defence response was detectable, which would correlate well with previous microscopy studies outlined in Wharton and Schilder (2008). This could be a result of the amount of time required by the host for measurable expression of defence-related genes, or delayed recognition of the invading pathogen by the host. In support of the latter hypothesis, a similar result was observed in sorghum mesocotyls when inoculated with *C. sublineolum* (Lo *et al*., 1999). Although the expression of PR10 was detected as early as 4 h after inoculation with the fungus *Cochliobolus heterostrophus*, the authors did not record significant expression of PR10 until 36 h and peak expression at 48 h after inoculation with *C. sublineolum* (Lo *et al*., 1999). Therefore, the lag period in the *C. acutatum*–blueberry pathosystem is most probably a result of the amount of time required by the fungus to gain entry and be recognized by the host.

Our results add new insight into the host responses of blueberry fruit to infection by *C. acutatum* at the molecular level, and suggest that pathogen ingress into the host is required for the activation of resistance. A more detailed investigation of gene expression during the early stages of infection, including pre-penetration events, will help to pinpoint when the host first recognizes that it is being attacked by *C. acutatum* and initiates the resistance response. Furthermore, chemical analysis and studies on the genetic inheritance of resistance will complement molecular research in elucidating the basis of anthracnose fruit rot resistance in blueberry.

EXPERIMENTAL PROCEDURES

Fungal cultures and media

A single-conidium isolate of *C. acutatum* from blueberry fruit collected at Grand Junction, MI, USA in August 2006 was used for all experiments. The isolate was stored as conidia in a nutrient solution (20% glycerol, 0.04% yeast extract, 0.1% malt extract, 0.04% glucose, 0.02% K_2HPO_4) at −80 °C. Cultures were grown on one-quarter-strength potato dextrose agar (PDA) for a period of 14 days, after which conidia were harvested, re-cultured on one-quarter-strength PDA and allowed to microconidiate for 3–4 days. This procedure was used to prepare conidial suspensions for storage and inoculum production, and has been employed in previous studies (Smith *et al*., 1996; Wharton and Schilder, 2008; Wharton *et al*., 2003). For inoculum production, sporulating cultures were flooded with 3 mL of sterile deionized water (SDW), and microconidia were dislodged using a sterilized L-shaped glass rod. Conidia were counted using a haemocytometer, and the appropriate concentration was achieved via dilution with SDW.

Plant material and inoculation procedures

To confirm the virulence of the pathogen and the host resistance phenotype observed in Wharton and Schilder (2008), ripe fruit were collected on 6 August 2006 from mature 'Elliott' and 'Jersey' bushes at the Southwest Michigan Research and Extension Center, Benton Harbor, MI, USA. Ten fruit of each cultivar were spray inoculated until run-off with a conidial suspension containing 10^6 conidia/mL, placed equidistantly on wire mesh over a layer of water in covered aluminium pans that acted as humidity chambers, and incubated for 8 days at 22–24 °C. After 5 days, fruits were macroscopically examined daily for disease symptoms and signs. For scanning electron micrographs, 10 fruit epidermal peels (1 mm in diameter) of each cultivar were dehydrated with ethanol, critical point dried, mounted on aluminium mounting stubs and coated with gold using a gold sputter coater (EMSCOPE SC500 sputter coater, Ashford, Kent, UK). Images were captured on a scanning electron microscope (JEOL 6400V, Japan Electron Optics Laboratories, Tokyo, Japan). Micrographs were used to measure the diameter (in two perpendicular directions) of 50 randomly selected acervuli per cultivar. To quantify sporulation, inoculated fruit were incubated at 100% humidity on wire mesh screens in aluminium pans for 8 days (10 fruit per replicate with five replicates). After incubation, 10 inoculated fruits per replicate were shaken in 10 mL of sterile water for 5 min, and conidium concentration was determined using a haemocytometer.

© 2011 THE AUTHORS
 MOLECULAR PLANT PATHOLOGY © 2011 BSPP AND BLACKWELL PUBLISHING LTD

In order to monitor the pre-penetration activities of *C. acutatum* on fruit at different stages of development, 10-cm-long twigs with attached fruit clusters were collected from mature 'Elliott' and 'Jersey' bushes at Benton Harbor, MI, USA, in 2008. The bushes had not been treated with fungicides for at least 2 months before removing the fruit clusters. Fruit clusters were collected at the following growth stages: petal fall (1 June 2008), green fruit (16 June 2008), fruit colouring (14 July 2008 for 'Jersey' and 28 July 2008 for 'Elliott') and ripe fruit (11 August 2008). Fruiting twigs were placed with the cut ends in wet Oasis® Floral foam (Smithers-Oasis, Kent, OH, USA) and fruit were inoculated with 10-μL droplets of *C. acutatum* (1×10^5 conidia/mL) and incubated in round plastic containers (~950 mL) for 24, 48, 96 and 144 hpi at 22–24 °C and 100% relative humidity under continuous fluorescent light. After incubation, epidermal peels were collected from the inoculation sites, and were fixed and stained in accordance with the procedures used by Wharton and Schilder (2008). One hundred conidia per replicate for five replicates were examined for germination and formation of unmelanized and melanized appressoria using light microscopy.

All statistical analyses of the data were performed using a paired Student's *t*-test ($\alpha = 0.05$) employing the StatGraphics statistical computer program (StatPoint Inc., Warrenton, VA, USA) after checking for equality of variance.

For the molecular experiments, ripe fruit of 'Elliott' and 'Jersey' (not treated with fungicides for at least 3 weeks prior to harvest) were provided by Hortifrut Chile S.A. (Santiago, Chile), and were equidistantly placed on wire mesh grates in humidity chambers constructed from two Petri dish bottoms (diameter, 15 cm; height, 2.5 cm) with moist filter paper on the bottom. Fruit were inoculated with 10-μL droplets of an aqueous suspension of *C. acutatum* (10^6 conidia/mL) or SDW (control) and incubated at 25 °C in the dark. Fruit epidermal samples were collected prior to inoculation and at 24, 48, 96 and 144 hpi using a size 2 cork borer (5.5 mm in diameter) and forceps to remove the inoculated sections of the peel. The epidermal peels were placed in 100-mg aliquots (approximately five to six peels) in microcentrifuge tubes and were flash frozen in liquid nitrogen. Ten aliquots were collected for each combination of cultivar, time interval after inoculation and treatment (inoculation or water control). Samples were then stored at −80 °C until RNA extraction was performed.

RNA extraction and cDNA library synthesis

RNA was extracted from 100 mg of epidermal peels (five to six fruits) per cultivar (Elliott and Jersey) and time point (0, 24, 48, 96 and 144 hpi) combination using a phenol–chloroform-based protocol that was adapted for use in a refrigerated microcentrifuge (Salzman *et al*., 1999). The RNA extracts were then treated with a DNA-free kit (Applied Biosystems/Ambion, Austin, TX, USA) to remove residual genomic DNA. Extracted total RNA was denatured in glyoxal-containing sample-loading dye (Ambion, Austin, TX, USA) at 50 °C and visualized on a 1.0% agarose Tris–acetate–ethylenediaminetetraacetic acid (TAE) gel.

Total RNA in each sample was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). For construction of the cDNA libraries for SSH, one compound sample per cultivar was created by combining 0.2 μg RNA from each time point after inoculation with *C. acutatum*. cDNA synthesis was performed using 1.0 μg of RNA from each composite library in the Super SMART cDNA synthesis kit (Clontech Laboratories, Inc., Palo Alto, CA, USA), in accordance with the manufacturer's protocols.

SSH, differential screening and sequencing

SSH was performed with cDNA from the Super SMART cDNA synthesis kit using 21 cycles to enrich the initial cDNA (Fig. S1, see Supporting Information) and the SSH Kit (Clontech Laboratories, Inc.) in accordance with the manufacturer's protocols (Figs S2 and S3, see Supporting Information). A forward-subtracted 'Elliott' library (where 'Elliott' served as the tester and 'Jersey' as the driver) and a reverse-subtracted 'Jersey' library (where 'Jersey' served as the tester and 'Elliott' as the driver) were constructed. The forward-subtracted cDNA library of 'Elliott' was cloned using the pGEM-T Easy Kit (Promega, Madison, WI, USA). Then, 5 μL of the ligation reaction was used to transform DH5α ElectroMAX competent cells (Invitrogen, Carlsbad, CA, USA) by electroporation with the following parameters: 2.0 kV, 200 Ω, 25 μF.

'Elliott' cDNA clones were screened using a Southern dot blot technique according to Takahara *et al*. (2005). To construct probes for each library, the PCR DIG Probe Synthesis Kit (Roche Applied Science, Mannheim, Germany) and 1 μL of the primary PCR product from the forward- and reverse-subtracted libraries (derived from the SSH kit) were used as template. The primers T7 (5′-AATACGACTCACTATAG-3′) and Sp6 (5′-ATTTAGGTGACACTATAG-3′) were used in a PCR to amplify the insert from each of the clones (Fig. 4A). For the blots, the DIG Luminescent Detection Kit for Nucleic Acids (Roche Applied Science) was used and a 1-μL PCR product was loaded onto two separate 0.45-μm MAGNACHARGE nylon transfer membranes (GE Water and Process Technologies, Trevose, PA, USA) in a high-throughput, 96-well system. One membrane was probed using 15 μL of the forward 'Elliott' library DIG PCR product and the other with 15 μL of the reverse 'Jersey' library DIG PCR product. Clones were considered to be differentially expressed if the diameter of the spot was more than 50% of that in the 'Jersey' reverse blot. Differentially expressed clones

© 2011 THE AUTHORS
MOLECULAR PLANT PATHOLOGY © 2011 BSPP AND BLACKWELL PUBLISHING LTD

were independently screened three times, and a putative actin gene of *Vaccinium corymbosum* with significant homology (*E* value, 7.0×10^{-31} using BLASTN) to a *Prunus avium* actin gene (GenBank accession no. FJ560908) served as an internal standard (Fig. 4).

The differentially expressed clones were sequenced in both directions using the PCR product from the blot screens and the primers T7 and Sp6. The sequences were combined to form contigs using Lasergene software (DNAStar, Madison, WI, USA) and analysed using BLASTX and TBLASTX (Atschul *et al*., 1990). For each contig, the nucleotide sequence with highest homology that had an annotated physiological role and an *E* value of less than 0.01 using TBLASTX was chosen (Table 1). All DNA sequences were submitted to GenBank at NCBI (http://blast.ncbi.nlm.nih.gov/), and grouped into categories according to their putative physiological function, such as protein synthesis and processing, signal transduction and post-translational regulation, defence-related, abiotic stimuli and development, hypothetical proteins and no homology to known proteins (Die *et al*., 2007). The isoelectric point (p*I*) was calculated for the homologous protein sequence of each putative plant EST using the ProtParam tool at the ExPASy Proteomic Server (http://ca.expasy.org/tools/protparam.html).

The nucleotide sequence data reported here are available in the GenBank database under accession numbers GW397252 (*EST01*), GW397253 (*EST02*), GW397254 (*EST03*), GW397255 (*EST04*), GW397256 (*EST05*), GW397257 (*EST06*), GW397258 (*EST07*), GW397259 (*EST08*), GW397260 (*EST09*), GW397261 (*EST10*), GW397262 (*EST11*), GW397263 (*EST12*), GW397264 (*EST13*), GW397265 (*EST14*), GW397266 (*EST15*), GW397267 (*EST16*), GW397268 (*EST17*), GW397269 (*EST18*), HO762505 (*EST19*), HO762506 (*EST20*), HO762507 (*EST21*), HO762508 (*EST22*), HO762509 (*EST23*), HO762510 (*EST24*), HO762511 (*EST25*), HO762512 (*EST26*), HO762513 (*EST27*), HO762514 (*EST28*), HO762515 (*EST29*), HO762516 (*EST30*), HO762517 (*EST31*), HO762518 (*EST32*), HO762519 (*EST33*) and GW397251 (*actin*).

Semi-quantitative RT-PCR and origin determination

Total RNA was extracted from epidermal peels at each of the time points, as described above, and treated with the DNA-free kit. cDNA was then synthesized using SuperScript III Reverse Transcriptase (Invitrogen) and an oligo DT primer (20 bp). Two independent cDNA synthesis reactions were performed per sample. Eight ESTs were chosen on the basis of their sequence similarities with genes or proteins related to disease resistance (EST01, EST03, EST04, EST05, EST08, EST10, EST11 and EST13), and primers were constructed for each EST using Primer Express Software (Applied Biosystems, Foster City, CA, USA) (Table 2). Primers were optimized by varying the primer concentration and annealing temperature. A protocol using 25 cycles, a 58 °C annealing temperature and 300 nM of each primer was found to be consistently optimal across the different primer sets. Semi-quantitative RT-PCR was carried out to determine the presence or absence of gene transcripts in both cultivars following inoculation. PCRs always contained cDNA reverse transcribed from 1.0 μg of total RNA as the template, which was extracted from 100-mg tissue aliquots (approximately five to six epidermal peels). The amplification protocol included an initial denaturation step at 94 °C for 2 min, followed by 25 cycles at 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min and 30 s. The reaction was completed by a 10-min extension at 72 °C. PCR products were separated on a 1.0% agarose TAE gel. Experiments were repeated with two independent biological samples. For all time points, the putative actin protein described above was used as an internal loading standard. Semi-quantitative RT-PCR products were sequenced to ensure that the correct gene was being amplified from the template. In addition, the DNAse-treated total RNA of each sample was tested for evidence of contaminating genomic DNA by using it as a template in a PCR with the putative actin gene primers.

To confirm the plant or fungal origin of each EST, DNA was extracted from fungal mycelium according to Lee *et al*. (1988) and from young leaves of 'Elliott' and 'Jersey' blueberry plants according to Doyle and Doyle (1990). To confirm the presence of genomic DNA, the putative actin gene and the fungal ITS region were amplified using PCR with the primers ACT-F/ACT-R (Table 2) and ITS1F/ITS4 (Gardes and Bruns, 1993), respectively. Finally, PCR was performed as described above using extracted genomic DNA (using 35 instead of 25 cycles) and appropriate primers for semi-quantitative RT-PCR (Table 2). PCR products were visualized on a 1.0% agarose TAE gel.

Quantitative RT-PCR

For quantitative RT-PCR, the same primers were used as described above in order to validate the previous techniques employing two independent biological samples (Table 2). Quantitative RT-PCR was performed to detect differences in transcript levels of the selected ESTs and to look for an induction at particular time points following infection in both cultivars. For each sample, 1.0 μg of treated RNA was converted to cDNA using the protocol described above. Amplification was determined using SYBR Green PCR Master Mix (Applied Biosystems) in the Applied Biosystems 7900HT sequence detection system (Genomics Technology Support Facility, Michigan State University, East Lansing, MI, USA). The appropriate primers were used at a concentration of 300 nM under the following conditions: 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 95 °C for 15 s followed by 60 °C for 1 min (Table 2). PCRs were carried out in

triplicate and were conducted at least twice, and the mean of the three replicates was used to generate an average concentration. Using a dilution series, cDNA from a representative sample ('Elliott' inoculated, 96 hpi) was used to generate standard curves and threshold values (C_T) for each EST of interest. Standard curves were considered to be reliable if slopes were between –2.9 and –3.5. The quality of the amplification was assessed by dissociation curves, and the product size was verified by gel electrophoresis. The concentration mean of each EST was then normalized to the endogenous putative actin control. Samples were also tested for the presence of contaminating genomic DNA in the treated RNA samples prior to cDNA synthesis using the DNA-free kit mentioned above.

H_2O_2 detection

Green and ripe fruit were collected from the same location as described above on 2 August 2010 and stored at 4 °C. No fungicides were applied in the last 3 weeks prior to harvest. Fruit were inoculated with a 10-μL drop of an aqueous suspension containing 10^6 conidia/mL or water (untreated control), and then placed equidistantly on wire mesh grates in Petri dishes at 100% relative humidity. After 0, 12, 18, 24 and 48 hpi, inoculated peel sections were excised with a cork borer (size 1, 4.5 mm in diameter). H_2O_2 was detected using the Amplex® Red Hydrogen Peroxide/Peroxidase Kit (Invitrogen) as described in Alkan *et al*. (2009) with the following exceptions: epidermal peels of approximately 4.5 mm in diameter were used instead of a fruit disc (diameter, 10 mm; depth, 10 mm), and the reaction volume was 1 mL of phosphate-buffered saline as opposed to 5 mL. A standard curve plot was used to calculate the H_2O_2 concentration employing a 10-fold dilution series from 1 mmol to 1 nmol of H_2O_2 ($R^2 = 0.95$). Based on this curve, values in the 1-mL reaction ranged between 1 and 754 nmol, and these values were divided by the total area of the peels (3 peels $\times$ 15.9 mm^2 per peel) to calculate the concentration (nmol/mL/mm^2). Three independent experiments were conducted with five replicates per treatment.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge funding from Michigan State University Project GREEEN (Generating Research and Extension to meet Economic and Environmental Needs). The authors would also like to thank Dr James Hancock and Hortifrut Chile S.A. (Santiago, Chile) for providing fruit, Dr Miaoying Tian for technical training, Christine Bates for technical assistance and Dr Paul Goodwin (University of Guelph, Guelph, ON, Canada) and Dr Raymond Hammerschmidt for critical review of the manuscript.

REFERENCES

Adaskaveg, J. and Hartin, R. (1997) Characterization of *Colletotrichum acutatum* isolates causing anthracnose of almond and peach in California. *Phytopathology*, **87**, 979–987.

Afanador-Kafuri, L., Minz, D., Maymon, M. and Freeman, S. (2003) Characterization of *Colletotrichum* isolates from tamarillo, passiflora, and mango in Colombia and identification of a unique species from the genus. *Phytopathology*, **93**, 579–587.

Alkan, N., Davydov, O., Sagi, M., Fluhr, R. and Prusky, D. (2009) Ammonium secretion by *Colletotrichum coccodes* activates host NADPH oxidase activity enhancing host cell death and fungal virulence in tomato fruits. *Mol. Plant–Microbe Interact.* **22**, 1484–1491.

Anand, T., Bhaskaran, R., Raguchander, T., Samiyappan, R., Prakasam, V. and Gopalakrishnan, C. (2009) Defense responses of chili fruits to *Colletotrichum capsici* and *Alternaria alternata*. *Biol. Plant.* **53**, 553–559.

Arroyo, F.T., Moreno, J., García-Herdugo, G., Santos, B.D., Barrau, C., Porras, M., Blanco, C. and Romero, F. (2005) Ultrastructure of the early stages of *Colletotrichum acutatum* infection of strawberry tissues. *Can. J. Bot.* **83**, 491–500.

Arroyo, F.T., Moreno, J., Daza, P., Boianova, L. and Romero, F. (2007) Antifungal activity of strawberry fruit volatile compounds against *Colletotrichum acutatum*. *J. Agric. Food Chem.* **55**, 5701–5707.

Atschul, S., Gish, W., Miller, W., Myers, E. and Lipman, D. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410.

Batson, W. and Roy, K. (1982) Species of *Colletotrichum* and *Glomerella* pathogenic to tomato fruit. *Plant Dis.* **66**, 1153–1155.

Bergstrom, G. and Nicholson, R. (2000) The biology of *Colletotrichum graminicola* and maize anthracnose. In: *Colletotrichum: Host Specificity, Pathology, and Host–Pathogen Interaction* (Prusky, D., Freeman, S. and Dickman, M.B., eds), pp. 374–394. St. Paul, MN: APS Press.

Bernstein, B., Zehr, E., Dean, R. and Shabi, E. (1995) Characteristics of *Colletotrichum* from peach, apple, pecan, and other hosts. *Plant Dis.* **79**, 478–482.

Brown, G.E. (1977) Ultrastructure of penetration of ethylene-degreened Robinson tangerines by *Colletotrichum gloeosporioides* after ethylene treatment. *Phytopathology*, **67**, 700–706.

Brown, S.H., Yarden, O., Gollop, N., Chen, S., Zveibil, A., Belausov, E. and Freeman, S. (2008) Differential protein expression in *Colletotrichum acutatum*: changes associated with reactive oxygen species and nitrogen starvation implicated in pathogenicity on strawberry. *Mol. Plant Pathol.* **9**, 171–190.

Campos, Â., Ferreira, A., Hampe, M., Antunes, I., Brancão, N., Silveira, E., Silva, J. and Osório, V. (2003) Induction of chalcone synthase and phenylalanine ammonia-lyase by salicylic acid and *Colletotrichum lindemuthianum* in common bean. *Braz. J. Plant Physiol.* **15**, 129–134.

Casado-Diaz, A., Encinas-Villarejo, S., Santos, B., Schiliro, E., Yubero-Serrano, E.M., Amil-Ruiz, F., Pocovi, M.I., Pliego-Alfaro, F., Dorado, G. and Rey, M. (2006) Analysis of strawberry genes differentially expressed in response to *Colletotrichum* infection. *Physiol. Plant* **128**, 633–650.

Curry, K., Abril, M., Avant, J. and Smith, B. (2002) Strawberry anthracnose: histopathology of *Colletotrichum acutatum* and *C. fragariae*. *Phytopathology*, **92**, 1055–1063.

Dana, M.M., Pintor-Toro, J.A. and Cubero, B. (2006) Transgenic tobacco plants overexpressing chitinases of fungal origin show enhanced resistance to biotic and abiotic stress agents. *Plant Physiol.* **142**, 722.

Daykin, M. and Milholland, R. (1984) Infection of blueberry fruit by *Colletotrichum gloeosporioides*. *Plant Dis.* **68**, 948–950.

Die, J.V., Dita, M.A., Krajinski, F., González-Verdejo, C.I., Rubiales, D., Moreno, M.T., Román, B. (2007) Identification by suppression subtractive hybridization and expression analysis of *Medicago truncatula* putative defense genes in response to *Orobanche crenata* parasitization. *Physiol. Mol. Plant Pathol.* **70**, 49–59.

Dieguez-Uribeondo, J., Forster, H., Soto-Estrada, A. and Adaskaveg, J.E. (2005) Subcuticular-intracellular hemibiotrophic and intercellular

© 2011 THE AUTHORS
MOLECULAR PLANT PATHOLOGY © 2011 BSPP AND BLACKWELL PUBLISHING LTD

necrotrophic development of *Colletotrichum acutatum* on almond. *Phytopathology*, **95**, 751–758.

Doyle, J.J. and Doyle, J.L. (1990) Isolation of plant DNA from fresh tissue. *Focus*, **12**, 13–15.

Droby, S., Prusky, D., Jacoby, B. and Goldman, A. (1986) Presence of antifungal compounds in the peel of mango fruits and their relation to latent infections of *Alternaria alternata*. *Physiol. Mol. Plant Pathol.* **29**, 173–183.

Ehlenfeldt, M. (2003) 'Elliott' highbush blueberry. *J. Am. Pomol. Soc.* **57**, 2–6.

Ehlenfeldt, M.K., Polashock, J.J., Stretch, A.W. and Kramer, M. (2006) Leaf disk infection by *Colletotrichum acutatum* and its relation to fruit rot in diverse blueberry germplasm. *HortScience*, **41**, 270–271.

Feussner, K., Feussner, I., Leopold, I. and Wasternack, C. (1997) Isolation of a cDNA coding for an ubiquitin-conjugating enzyme UBC1 of tomato—the first stress-induced UBC of higher plants. *FEBS Lett.* **409**, 211.

Freeman, S., Katan, T. and Shabi, E. (1998) Characterization of *Colletotrichum* species responsible for anthracnose diseases of various fruits. *Plant Dis.* **82**, 596–605.

Freeman, S., Minz, D., Jurkevitch, E., Maymon, M. and Shabi, E. (2000) Molecular analyses of *Colletotrichum* species from almond and other fruits. *Phytopathology*, **90**, 608–614.

Gardes, M. and Bruns, T.D. (1993) ITS primers with enhanced specificity for basidiomycetes—application to the identification of mycorrhizae and rusts. *Mol. Ecol.* **2**, 113–118.

Gomes, S., Prieto, P., Martins-Lopes, P., Carvalho, T., Martin, A. and Guedes-Pinto, H. (2009) Development of *Colletotrichum acutatum* on tolerant and susceptible *Olea europaea* L. cultivars: a microscopic analysis. *Mycopathologia*, **168**, 203–211.

Goodwin, P.H., Oliver, R.P. and Hsiang, T. (2004) Comparative analysis of expressed sequence tags from *Malva pusilla*, *Sorghum bicolor*, and *Medicago truncatula* infected with *Colletotrichum* species. *Plant Sci.* **167**, 481–489.

Hartung, J., Burton, C. and Ramsdell, D. (1981) Epidemiological studies of blueberry anthracnose disease caused by *Colletotrichum gloeosporioides*. *Phytopathology*, **71**, 449–453.

Hassan, M.K., Dann, E.K., Irving, D.E. and Coates, L.M. (2007) Concentrations of constitutive alk(en)ylresorcinols in peel of commercial mango varieties and resistance to postharvest anthracnose. *Physiol. Mol. Plant Pathol.* **71**, 158–165.

Kikuchi, T. and Masuda, K. (2009) Class II chitinase accumulated in the bark tissue involved with the cold hardiness of shoot stems in highbush blueberry (*Vaccinium corymbosum* L.). *Sci. Hortic.* **120**, 230–236.

Kim, K.-H., Yoon, J.-B., Park, H.-G., Park, E.W. and Kim, Y.H. (2004) Structural modifications and programmed cell death of chili pepper fruit related to resistance responses to *Colletotrichum gloeosporioides* infection. *Phytopathology*, **94**, 1295–1304.

Kim, S., Yoon, J., Do, J. and Park, H. (2008) A major recessive gene associated with anthracnose resistance to *Colletotrichum capsici* in chili pepper (*Capsicum annuum* L.). *Breed. Sci.* **58**, 137–141.

Kim, Y.S., Park, J.Y., Kim, K.S., Ko, M.K., Cheong, S.J. and Oh, B.J. (2002) A thaumatin-like gene in nonclimacteric pepper fruits used as molecular marker in probing disease resistance, ripening, and sugar accumulation. *Plant Mol. Biol.* **49**, 125–135.

Ko, M.K., Jeon, W.B., Kim, K.S., Lee, H.H., Seo, H.H., Kim, Y.S., and Oh, B.J. (2005) A *Colletotrichum gloeosporioides*-induced esterase gene of nonclimacteric pepper (*Capsicum annuum*) fruit during ripening plays a role in resistance against fungal infection. *Plant Mol. Biol.* **58**, 529–541.

Kumari, M.V.R., Hiramatsu, M. and Ebadi, M. (1998) Free radical scavenging actions of metallothionein isoforms I and II. *Free Radic. Res.* **29**, 93–101.

Lafitte, C., Barthe, J., Gansel, X., Dechamp-Guillaume, G., Faucher, C., Mazau, D. and Esquerré-Tugayé, M. (1993) Differential induction by endopolygalacturonase of β-1,3-glucanases in *Phaseolus vulgaris* isolines susceptible and resistant to *Colletotrichum lindemuthianum* race β. *Mol. Plant–Microbe Interact.* **6**, 628–634.

Lahey, K.A., Yuan, R., Burns, J.K., Ueng, P.P., Timmer, L.W. and Chung, K.R. (2004) Induction of phytohormones and differential gene expression in citrus flowers infected by the fungus *Colletotrichum acutatum*. *Mol. Plant–Microbe Interact.* **17**, 1394–1401.

Lee, S., Milgroom, M. and Taylor, J. (1988) A rapid, high yield mini-prep method for isolation of total genomic DNA from fungi. *Fungal Genet. Newsl.* **35**, 23–24.

Leterrier, M., Corpas, F.J., Barroso, J.B., Sandalio, L.M. and Del Río, L.A. (2005) Peroxisomal monodehydroascorbate reductase. Genomic clone characterization and functional analysis under environmental stress conditions. *Plant Physiol.* **138**, 2111.

Lo, S.C., Hipskind, J.D. and Nicholson, R.L. (1999) cDNA cloning of a sorghum pathogenesis-related protein (PR-10) and differential expression of defense-related genes following inoculation with *Cochliobolus heterostrophus* or *Colletotrichum sublineolum*. *Mol. Plant–Microbe Interact.* **12**, 479–489.

van Loon, L.C. and van Strien, E.A. (1999) The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiol. Mol. Plant Pathol.* **55**, 85–97.

Mahasuk, P., Taylor, P.W.J. and Mongkolporn, O. (2009) Identification of two new genes conferring resistance to *Colletotrichum acutatum* in *Capsicum baccatum*. *Phytopathology*, **99**, 1100–1104.

Mellersh, D., Foulds, I., Higgins, V. and Heath, M. (2002) H_2O_2 plays different roles in determining penetration failure in three diverse plant–fungal interactions. *Plant J.* **29**, 257–268.

Miles, T.D., Wharton, P.S. and Schilder, A.C. (2009) Cytological and chemical evidence for an active resistance response to infection by *Colletotrichum acutatum* in 'Elliott' blueberries. *Acta Hortic.* **810**, 361–368.

Milholland, R.D. (1995) Anthracnose fruit rot (ripe rot). In: *Compendium of Blueberry and Cranberry Diseases* (Caruso, F.L. and Ramsdell, D.C., eds), p. 17. St. Paul, MN: APS Press.

Moral, J., Bouhmidi, K. and Trapero, A. (2008) Influence of fruit maturity, cultivar susceptibility, and inoculation method on infection of olive fruit by *Colletotrichum acutatum*. *Plant Dis.* **92**, 1421–1426.

Nakajima, J., Tanaka, Y., Yamazaki, M. and Saito, K. (2001) Reaction mechanism from leucoanthocyanidin to anthocyanidin 3-glucoside, a key reaction for coloring in anthocyanin biosynthesis. *J. Biol. Chem.* **276**, 25 797–25 803.

O'Connell, R., Bailey, J. and Richmond, D. (1985) Cytology and physiology of infection of *Phaseolus vulgaris* by *Colletotrichum lindemuthianum*. *Physiol. Plant Pathol.* **27**, 75–98.

O'Connell, R.J., Uronu, A.B., Waksman, G., Nash, C., Keon, J.P.R. and Bailey, J.A. (1993) Hemibiotrophic infection of *Pisum sativum* by *Colletotrichum truncatum*. *Plant Pathol.* **42**, 774–783.

Oh, B.J., Ko, M.K., Kim, Y.S., Kim, K.S., Kostenyuk, I. and Kee, H.K. (1999a) A cytochrome P450 gene is differentially expressed in compatible and incompatible interactions between pepper (*Capsicum annuum*) and the anthracnose fungus, *Colletotrichum gloeosporioides*. *Mol. Plant–Microbe Interact.* **12**, 1044–1052.

Oh, B.J., Ko, M.K., Kostenyuk, I., Shin, B. and Kim, K.S. (1999b) Coexpression of a defensin gene and a thionin-like gene via different signal transduction pathways in pepper and *Colletotrichum gloeosporioides* interactions. *Plant Mol. Biol.* **41**, 313–319.

Orengo, C., Michie, A., Jones, S., Jones, D., Swindells, M. and Thornton, J. (1997) CATH—a hierarchic classification of protein domain structures. *Structure*, **5**, 1093–1109.

Peres, N.A., Timmer, L.W., Adaskaveg, J.E. and Correll, J.C. (2005) Lifestyles of *Colletotrichum acutatum*. *Plant Dis.* **89**, 784–796.

Podila, G.K., Rogers, L.M. and Kolattukudy, P.E. (1993) Chemical signals from avocado surface wax trigger germination and appressorium formation in *Colletotrichum gloeosporioides*. *Plant Physiol.* **103**, 267–272.

Polashock, J., Saftner, R. and Kramer, M. (2007) Postharvest highbush blueberry fruit antimicrobial volatile profiles in relation to anthracnose fruit rot resistance. *J. Am. Soc. Hortic. Sci.* **132**, 859–868.

Polashock, J.J., Ehlenfeldt, M.K., Stretch, A.W. and Kramer, M. (2005) Anthracnose fruit rot resistance in blueberry cultivars. *Plant Dis.* **89**, 33–38.

Pring, R., Nash, C., Zakaria, M. and Bailey, J. (1995) Infection process and host range of *Colletotrichum capsici*. *Physiol. Mol. Plant Pathol.* **46**, 137–152.

© 2011 THE AUTHORS

Prusky, D., Plumbley, R.A. and Kobiler, I. (1991) The relationship between antifungal diene levels and fungal inhibition during quiescent infection of unripe avocado fruits by *Colletotrichum gloeosporioides*. *Plant Pathol.* **40**, 45–52.

Prusky, D., Koblier, I., Ardi, R., Beno-Moalem, D., Yakoby, N. and Keen, N. (2000) Resistance mechanisms of subtropical fruits to *Colletotrichum gloeosporioides*. In: *Colletotrichum: Host Specificity, Pathology, and Host–Pathogen Interaction* (Prusky, D., Freeman, S. and Dickman, M.B., eds), pp. 232–244. St. Paul, MN: APS Press.

Ri-He, P., Quan-Hong, Y., Ai-Sheng, X., Hui-Qin, F., Xian, L. and You-Liang, P. (2003) Ubiquitin-conjugating enzyme (E2) confers rice UV protection through phenylalanine ammonia-lyase gene promoter unit. *Acta Bot. Sin.* **45**, 1351–1358.

Roberts, R. and Snow, J. (1984) Histopathology of cotton boll rot caused by *Colletotrichum capsici*. *Phytopathology*, **74**, 390–397.

Salzman, R.A., Fujita, T., Zhu-Salzman, K., Hasegawa, P.M. and Bressan, R.A. (1999) An improved RNA isolation method for plant tissues containing high levels of phenolic compounds or carbohydrates. *Plant Mol. Biol. Rep.* **17**, 11–17.

Schilder, A., Gillett, J. and Woodworth, J. (2002) The kaleidoscopic nature of blueberry fruit rots. *Acta Hortic.* **574**, 81–83.

Shen, S., Goodwin, P. and Hsiang, T. (2001) Hemibiotrophic infection and identity of the fungus, *Colletotrichum destructivum*, causing anthracnose of tobacco. *Mycol. Res.* **105**, 1340–1347.

Simmonds, J. (1965) A study of the species of *Colletotrichum* causing ripe fruit rots in Queensland. *Qld. J. Agric. Sci.* **22**, 437–459.

Smith, B.J., Magee, J.B. and Gupton, C.L. (1996) Susceptibility of rabbiteye blueberry cultivars to postharvest diseases. *Plant Dis.* **80**, 215–218.

Stanghellini, M. and Aragaki, M. (1966) Relation of periderm formation and callose deposition to anthracnose resistance in papaya fruit. *Phytopathology*, **56**, 444–450.

Sullivan, J., Shirasu, K. and Deng, X. (2003) The diverse roles of ubiquitin and the 26S proteasome in the life of plants. *Nat. Rev. Genet.* **4**, 948–958.

Takahara, H., Toyoda, K., Tsuji, G., Kubo, Y., Inagaki, Y., Ichinose, Y. and Shiraishi, T. (2005) Identification of genes expressed during spore germination of *Mycosphaerella pinodes*. *J. Gen. Plant Pathol.* **71**, 190–195.

Takai, R., Matsuda, N., Nakano, A., Hasegawa, K., Akimoto, C., Shibuya, N. and Minami, E. (2002) EL5, a rice N-acetylchitooligosaccharide elicitor responsive RING H2 finger protein, is a ubiquitin ligase which functions in vitro in co-operation with an elicitor responsive ubiquitin conjugating enzyme, OsUBC5b. *Plant J.* **30**, 447–455.

Timmer, L. and Brown, G. (2000) Biology and control of anthracnose diseases of citrus. In: *Colletotrichum: Host Specificity, Pathology, and Host–Pathogen Interaction* (Prusky, D., Freeman, S. and Dickman, M.B., eds), pp. 300–316. St. Paul, MN: APS Press.

Verma, N., MacDonald, L. and Punja, Z.K. (2006) Inoculum prevalence, host infection and biological control of *Colletotrichum acutatum*: causal agent of blueberry anthracnose in British Columbia. *Plant Pathol.* **55**, 442–450.

Wharton, P.S. and Julian, A.M. (1996) A cytological study of compatible and incompatible interactions between *Sorghum bicolor* and *Colletotrichum sublineolum*. *New Phytol.* **134**, 25–34.

Wharton, P.S. and Schilder, A.M.C. (2008) Novel infection strategies of *Colletotrichum acutatum* on ripe blueberry fruit. *Plant Pathol.* **57**, 122–134.

Wharton, P.S., Iezzoni, A. and Jones, A.L. (2003) Screening cherry germ plasm for resistance to leaf spot. *Plant Dis.* **87**, 471–477.

Wijesundera, R., Bailey, J., Byrde, R. and Fielding, A. (1989) Cell wall degrading enzymes of *Colletotrichum lindemuthianum*: their role in the development of bean anthracnose. *Physiol. Mol. Plant Pathol.* **34**, 403–413.

Yoshida, S. and Tsukiboshi, T. (2002) Shoot blight and leaf spot of blueberry anthracnose caused by *Colletotrichum acutatum*. *J. Gen. Plant Pathol.* **68**, 246–248.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Optimization of cDNA amplification using various polymerase chain reaction (PCR) amplification cycles (15–30) for *Colletotrichum acutatum*-inoculated fruit of blueberry cultivars Jersey (lanes 2–7) and Elliott (lanes 9–14) (5 μL loaded per well). Lanes 1 and 8, 1-kb+ DNA ladder; lanes 2 and 9, 15 cycles; lanes 3 and 10, 18 cycles; lanes 4 and 11, 21 cycles; lanes 5 and 12, 24 cycles; lanes 6 and 13, 27 cycles; lanes 7 and 14, 30 cycles. Twenty-one cycles were found to be optimal for both libraries and these products were used for digestion and adaptor ligation.

Fig. S2 Restriction enzyme digestion of pooled cDNA libraries of the blueberry cultivars Jersey and Elliott after inoculation with *Colletotrichum acutatum* for the generation of shorter, blunt-end fragments (necessary for adaptor ligation and subtraction). Lanes 1 and 4, 1-kb+ DNA ladder; lanes 2 and 5, samples from 'Jersey' and 'Elliott', respectively, before digestion; lanes 3 and 6, samples from 'Jersey' and 'Elliott', respectively, after digestion.

Fig. S3 Primary (lanes 2–5) and secondary (lanes 7–10) polymerase chain reaction (PCR) products of pooled cDNA libraries in subtracted and nonsubtracted samples of blueberry cultivars Elliott and Jersey after inoculation with *Colletotrichum acutatum*. These products were used for the construction of DIG probes and for cloning of the subtractive libraries for differential screening. Lanes 1 and 6, 1-kb+ DNA ladder; lanes 2 and 3, 'Elliott' and 'Jersey' subtracted primary PCR products, respectively; lanes 4 and 5, 'Elliott' and 'Jersey' nonsubtracted primary PCR products, respectively; lanes 7 and 8, 'Elliott' and 'Jersey' subtracted secondary PCR products, respectively; lanes 9 and 10, 'Elliott' and 'Jersey' nonsubtracted secondary PCR products, respectively.

Table S1 Characteristics and predicted physiological function of differentially expressed sequence tags (ESTs) from ripe fruit of highbush blueberry cultivar Elliott versus cultivar Jersey after inoculation with *Colletotrichum acutatum*. The hypothetical function is based on homology to sequences in translated nucleotide databases (DDBJ/EMBL/GenBank) using TBLASTX. Some ESTs are probably of fungal origin based on homology with fungal genes.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

© 2011 THE AUTHORS
MOLECULAR PLANT PATHOLOGY © 2011 BSPP AND BLACKWELL PUBLISHING LTD