Elevated Genetic Variation Within Virulence-Associated *Botrytis cinerea* Polygalacturonase Loci

Heather C. Rowe and Daniel J. Kliebenstein

Department of Plant Sciences, Mail Stop 3, University of California, One Shields Ave, Davis 95616, U.S.A.

Submitted 21 March 2007. Accepted 12 May 2007.

Botrytis cinerea, or gray mold, is a necrotrophic fungal pathogen of hundreds of plant species. The genetic diversity of *B. cinerea* may contribute to its broad host range; however, the level and structure of genetic variation at pathogenesis-associated loci has not been described. *B. cinerea* possesses six distinct cell-wall-degrading polygalacturonases (PGs), enzymes of demonstrated importance to pathogenesis and interaction with host plant defenses. Sequencing a collection of 34 *B. cinerea* isolates at three PG-encoding loci, *BcPG1*, *BcPG2*, and *BcPG3*, revealed limited evidence of host-mediated genetic subdivision within loci, yet suggested differences in the action of evolutionary forces among loci. *BcPG1* and *BcPG2* are highly polymorphic, particularly when compared with previously published data from nonpathogenicity loci, whereas *BcPG3* is relatively conserved. Sequence variation at *BcPG1* and *BcPG2* did not appear to be associated with virulence on *Arabidopsis* leaves; however, *BcPG2* variation showed a statistically significant association with growth rate on pectin. Rather than providing evidence for host-mediated genetic subdivision at individual PG loci, our data support specialization among PGs and the potential diversification of PGs interacting directly with host defenses.

Botrytis cinerea, the causal agent of both gray mold and noble rot, is a necrotrophic plant-pathogenic fungus. Gray mold infection causes large losses of agricultural and horticultural produce, both pre- and postharvest, with estimated control costs of US\$15 to 25 million per year (Elad et al. 2004). This pathogen is distinguished from closely related species by its extremely broad host range, including the major dicot families as well as pectinaceous monocots. Studies of *B. cinerea* biology are driven by both the practical desire to improve control of this pathogen and a broader interest in how this fungus can infect such a diversity of plants. *B. cinerea*'s broad host capacity could be explained by host specialization among isolates or by maintenance of high levels of intraspecific variation in pathogenesis mechanisms. Understanding the structure of pathogen genetic diversity may aid in identifying which of these models controls the genetic basis of host range diversity, with significant implications for agronomic control of this pathogen.

B. cinerea contains significant phenotypic variation (Beever and Weeds 2004) for form and growth rate (Lorbeer 1980), pathogenicity (Calpas et al. 2001; Chardonnet et al. 2000; Kerssies et al. 1997), and fungicide resistance (Moyano et al. 2004; Topolovec-Pintaric et al. 2004; Wedge et al. 2002). Analysis of nucleotide sequence data allows the generation of hypotheses regarding the functional and evolutionary significance of genetic variation and how this connects to phenotypic variation. To date, the analysis of genetic variation with molecular markers has not produced a cohesive picture of how genetic variation shapes phenotypic variation within this pathogen. Genetic polymorphism studies have shown high levels of intrapopulation variation, with little evidence of clonal lineages and strong evidence that recombination is an important factor in structuring genetic diversity within *B. cinerea*, despite a dearth of sexual morphs reported in the field (Alfonso et al. 2000; Baraldi et al. 2002; Giraud et al. 1997). Attempts at identifying population subdivision related to host plant have produced mixed results. Studies employing polymerase chain reaction (PCR) restriction fragment length polymorphisms showed significant genetic differentiation between grape and tomato isolates from Chile (Muñoz et al. 2002), and the presence of transposable elements has been associated with differences in host usage (Giraud et al. 1999). Yet other studies have found no evidence of genetic structure based on host (Ma and Michailides 2005; Martinez et al. 2003; Moyano et al. 2003).

A limitation of past attempts to associate genetic polymorphism with phenotypic variation controlling virulence may be that the *B. cinerea* loci examined to date are highly conserved (*β-tubulin*, *Bc-hch*), chosen as neutral markers (microsatellite locus 63R) (Fournier et al. 2005), or candidate mechanisms controlling fungicide resistance (*CYP51*) (Albertini et al. 2002). Therefore, these loci are unlikely to contribute directly to host–pathogen interactions. Measuring genetic variation at loci associated with pathogenesis may allow us to assess whether the structure of variation at these loci contributes to host specialization or host range breadth. Host specialization among isolates should be most detectable at loci contributing to pathogenesis through direct interaction of gene products with plant host cells, such as polygalacturonase (PG)-encoding loci.

B. cinerea possesses six PGs that are strong biological candidates for genes mediating host interaction and potentially host specialization (Wubben et al. 1999). These enzymes cleave pectin and thus play an important role in breakdown of the plant cell wall during infection. Additionally, the hydrolysis of polygalacturonic acid to pectin oligomers (Kars et al. 2005) by these enzymes can induce plant defense signaling responses (Pilling and Hofte 2003). PGs are directly inhibited by plant-produced PG-inhibiting proteins (PGIPs), which also contribute to plant defense signaling by modulating production of oligogalacturonides (Di Matteo et al. 2006). Therefore, PGs

Corresponding author: D. J. Kliebenstein; Telephone: 1-530-754-7775; Fax: 1-530-752-9659; E-mail: Kliebenstein@ucdavis.edu

Gene sequence data is available in the GenBank database under accession numbers EF195774 through EF195915.

*The e-Xtra logo stands for “electronic extra” and indicates supplemental material is available online. Figure 6 appears in color and one supplemental figure is published online.

and PGIPs potentially are co-evolving in a manner consistent with either an “arms race” or “trench warfare” model of plant–pathogen interactions (Stahl and Bishop 2000; Stahl et al. 1999).

The specific roles of the different *B. cinerea* PGs are still being detailed; however, it has been experimentally demonstrated that *BcPG1* and *BcPG2* are required for full pathogenicity of the isolate BO5.10 (Kars et al. 2005; ten Have et al. 1998). Although *BcPG1* is constitutively expressed, *BcPG2* is strongly induced during the infection process and appears to be repressed by low pH (Wubben et al. 2000). *BcPG3* causes the breakdown of polygalacturonic acid to monomers and dimers rather than oligomers (Kars et al. 2005). Production of this enzyme is induced by low pH (ten Have et al. 2001). Gene expression data suggest that the six *B. cinerea* PGs exhibit differential host or tissue specificities (ten Have et al. 2001). Although *BcPG1*, *BcPG2*, and *BcPG4* are highly expressed when infecting leaf tissues, *BcPG3* and *BcPG5* are expressed primarily during infection of fruit. This diverse arsenal of PGs may facilitate *B. cinerea*’s broad host range by subfunctionalization during the infection process (Prins et al. 2000).

To better understand the level and structure of genetic variation in virulence-associated loci within *B. cinerea*, we analyzed nucleotide sequence variation at the *BcPG1*, *BcPG2*, *BcPG3*, and *CYP51* loci. These data were obtained from a collection of

primarily Californian isolates, with representation from Europe and Africa. The virulence-associated loci, *BcPG1* and *BcPG2*, showed a 5- to 10-fold increase in the level of genetic diversity in relation to a control set of neutral genes. Further, these two genes showed the influence of differing evolutionary forces compared with previously studied *B. cinerea* loci. We detected an association between variation at *BcPG2* and the ability to use apple pectin as a carbon source. However, we found little evidence of host-related genetic differentiation or genetic structure related to virulence. Comparison of *CYP51* sequence from this collection with previously published data from French isolates (Albertini et al. 2002) suggests similar polymorphism levels at this locus and some evidence of population structure at the continental level.

RESULTS

Isolate collection and sequencing.

The primary group of isolates examined in this study consists of 27 *B. cinerea* isolates from central California plus 7 additional isolates from Massachusetts (United States) ($n = 1$), South Africa ($n = 2$), Switzerland ($n = 1$), and the United Kingdom ($n = 3$) (Table 1). These isolates were sequenced at four loci, *CYP51*, *BcPG1*, *BcPG2*, and *BcPG3*, generating 1.4 to 2.2 kb of high-quality sequence data for each locus (Table 2). The

Table 1. *Botrytis* isolate information^a

Isolate	Host	Location	Source ^b	RV ^c
2004	Grape	California (U.S.A.)	D. Margosan, USDA, Parlier CA	55
83-2	Rose	California (U.S.A.)	D. Margosan, USDA, Parlier CA	70
94-1	Lemon	California (U.S.A.)	D. Margosan, USDA, Parlier CA	29
ACACIA	Acacia	South Africa	K. Denby, University of Warwick	5
A404	Apple	California (U.S.A.)	E. Mitcham, UC Davis, CA	92
A517	Apple	California (U.S.A.)	E. Mitcham, UC Davis, CA	90
AUS	Brassica	Massachusetts (U.S.A.)	F. Ausubel, Massachusetts General Hospital	25
DN	Orange	California (U.S.A.)	Isolated 2004–05, H. C. Rowe	91
EFR	Strawberry	California (U.S.A.)	Isolated 2004–05, H. C. Rowe	25
FEIJOA	Feijoa	California (U.S.A.)	E. Mitcham, UC Davis, CA	68
FDOR1	Raspberry	California (U.S.A.)	Isolated 2004–05, H. C. Rowe	17
FDOR2	Raspberry	California (U.S.A.)	Isolated 2004–05, H. C. Rowe	16
FR525	Strawberry	California (U.S.A.)	E. Mitcham, UC Davis, CA	24
GLO1	Grape	California (U.S.A.)	Isolated 2004–05, H. C. Rowe	39
GLO2	Grape	California (U.S.A.)	Isolated 2004–05, H. C. Rowe	25
GER	Geranium	California (U.S.A.)	Isolated 2004–05, H. C. Rowe	9
GRAPE	Grape	South Africa	M. Vivier, University of Stellenbosch	28
KT	Tomato	California (U.S.A.)	isolated 2004–2005, HCR	49
KA1	Grape	California (U.S.A.)	D. Gubler, UC Davis, CA	11
KA2	Grape	California (U.S.A.)	D. Gubler, UC Davis, CA	33
KB1	Grape	California (U.S.A.)	D. Gubler, UC Davis, CA	0
KB2	Grape	California (U.S.A.)	D. Gubler, UC Davis, CA	25
KGB1	Tomato	California (U.S.A.)	Isolated 2004–05, H. C. Rowe	80
KGB2	Tomato	California (U.S.A.)	Isolated 2004–05, H. C. Rowe	...
MEAP6G	Grape	Switzerland	M. Dewey, UC Davis, CA	54
MOLLY	Grape	California (U.S.A.)	M. Dewey, UC Davis, CA	34
NOBLE	Grape	California (U.S.A.)	G. Allen, Far Niente Winery, CA	61
PEPPER	Pepper	United Kingdom	G. Loake, University of Edinburgh	22
PHM	Grape	California (U.S.A.)	D. Gubler, UC Davis, CA	81
RASP	Raspberry	California (U.S.A.)	Isolated 2004–05, H. C. Rowe	45
SR4	Raspberry	United Kingdom	Isolated 2004–05, H. C. Rowe	26
T3	Tomato	California (U.S.A.)	Isolated 2004–05, H. C. Rowe	86
T7	Tomato	California (U.S.A.)	Isolated 2004–05, H. C. Rowe	100
UKR	Raspberry	United Kingdom	Isolated 2004–05, H. C. Rowe	60
94-4 ^d	Orange	California (U.S.A.)	D. Margosan, USDA, Parlier CA	...
Allii ^e	Onion	California (U.S.A.)	E. Mitcham, UC Davis, CA	0
ANCHO ^e	Pepper	California (U.S.A.)	Isolated 2004–05, H. C. Rowe	...

^a Plant host and collection location of all isolates used as sources of sequence data for this study.

^b USDA = United States Department of Agriculture and UC Davis = University of California, Davis.

^c RV = relative virulence; this value represents lesion growth rate on detached leaves of *Arabidopsis thaliana* as a percentage of lesion growth rate of the fastest-growing *Botrytis cinerea* isolate.

^d Isolate assigned to “species” group *B. calthae* based on sequence similarity to reference specimens at the *RBP2* locus (Staats et al. 2005).

^e Isolate assigned to “species” group *B. allii* based on sequence similarity to reference specimens at the *RBP2* locus (Staats et al. 2005).

lack of congruence among phylograms generated for these four loci provided evidence of recombination and a lack of genome-level subdivision within this isolate collection (Figs. 1 to 4).

Comparison of isolate collections.

To ensure that our collection was not a significantly biased sample, we sequenced our *B. cinerea* isolates at the *CYP51* locus, allowing direct comparison with published *CYP51* data from isolates collected primarily in the Champagne region of France (Albertini et al. 2002; Fournier et al. 2005). Isolates belonging to the subspecies previously described as group I (Fournier et al. 2005) were excluded from statistical comparison of the two collections. Analysis of 1,559 bp of sequence from 9 French isolates (Albertini et al. 2002) and 33 isolates from this study revealed similar levels of polymorphism ($P_i = 0.0039$ and 0.0032 , respectively). DNA sequence polymorphism within *CYP51* for both collections was slightly higher than estimates for other published nonpathogenicity associated loci (*Bc-hch*: $P_i = 0.0012$, and *β -tubulin*: $P_i = 0.0013$), and similar to the microsatellite locus *63R* ($P_i = 0.0031$) (Table 3). Relationships among isolates at *CYP51* were poorly resolved by maximum parsimony analysis, yet isolates from both collections distribute among three clusters possessing >60% bootstrap support (Fig. 1). Population differentiation between French and Californian collections was estimated at $F_{ST} = 0.135$ and χ^2 tests showed significant genetic differentiation ($n = 41$, $\chi^2 = 30.9$, $P = 0.04$).

Comparison among loci.

Although the six known PGs of *B. cinerea* are structurally and functionally related, comparison with PGs from *Sclerotinia sclerotiorum* suggests that the origin of these different PG loci predates *Botrytis*–*Sclerotinia* separation (Kasza et al. 2004). These enzymes have evolved differing activity and tissue specificity, and may play differing roles during infection of plants.

Polymorphism. We analyzed the DNA sequence variation within *BcPG1*, -2, and -3 to test for patterns differing from previously described nonpathogenicity loci (*CYP51*, *Bc-hch*, *β -tubulin*, and *63R*), considered here as a “neutral” test group for comparison with pathogenicity-associated loci. *BcPG1* and *BcPG2* show dramatically elevated diversity within our *B. cinerea* sample, with estimates of P_i that are 5- to 10 fold higher than the neutral test group (Table 3; Figs. 2 and 3). In contrast, the sampled *B. cinerea* isolates possess levels of sequence diversity at the *BcPG3* locus similar to that for the neutral test group, with $P_i = 0.002$ (Table 3; Fig. 4). The pattern of polymorphism at the three PG-encoding loci, displayed as a sliding window plot of P_i , shows that *BcPG1* polymorphism is low in the 5′ untranslated or promoter region and increases throughout the sequenced portion of the coding region (Fig. 5A). In contrast, *BcPG2* and *BcPG3* polymorphism shows little spatial pattern within loci (Fig. 5B and C). Haplotype diversity (H_d), which takes into account both the number and frequency distribution of haplotypes, was higher in the three PG loci than

the comparison *CYP51* locus. Although the relatively high values of H_d at these loci are more consistent with diversifying selection or recent population expansion than population structure, estimates of H_d for these loci do not significantly exceed expected values (Depaulis and Veuille 1998).

Nucleotide substitution and selection. Measures used to detect the signature of natural selection within DNA sequence data include the ratio of nonsynonymous to synonymous nucleotide substitutions (D_N/D_S , K_A/K_S) (Kimura 1991; Li et al. 1985) and Tajima’s D test. Estimates of D_N/D_S show a pattern

Fig. 1. *CYP51* variation within *Botrytis cinerea*. *Botrytis* isolate relationships inferred by maximum parsimony analysis based on DNA sequence of 1,559 bp at the *CYP51* locus. Bootstrap significance values show nodes supported by >50% of 1,000 bootstrap resamplings. *B. calthae* serves as an outgroup (isolate “94-4”). Data for isolates marked with black circles (●) was published previously (Albertini et al. 2002). Sequence data for the isolate BO5.10 is from the Broad Institute *B. cinerea* sequencing project.

Table 2. Sequencing information^a

Locus	<i>n</i> ^b	Fragment length (bp) ^c	Coding (bp) ^d	Intron (bp) ^e	5′ UTR (bp) ^f	3′ UTR (bp) ^f	Outgroup ^g
CYP51	33	1,559	1,442	112	...	...	<i>Botrytis calthae</i>
PG1	35	1,865	1,128	...	723	...	<i>B. allii</i>
PG2	34	1,404	1,122	155	90	61	<i>B. calthae</i>
PG3	35	2,191	1,539	59	389	204	<i>B. calthae</i>

^a Results of sequencing, including number of isolates sequenced, length, and composition of sequence.

^b Number of *B. cinerea* isolates for which sequence was obtained.

^c Base pairs of sequence per locus.

^d Base pairs per locus encoding for the protein.

^e Base pairs per locus present in introns.

^f Base pairs per locus present in the 5′ and 3′ untranslated region (UTR).

^g Outgroup used to calculate K_A/K_S .

similar to the values of P_i , with *CYP51* and *BcPG3* having the lowest values (0.013 and 0.064, respectively) and *BcPG1* the highest (0.21) (Table 3). Analysis of variation along the genes for ratios of polymorphism and divergence at nonsynonymous sites versus synonymous sites shows that nonsynonymous substitutions and amino acid polymorphisms are distributed across the entire coding region for *BcPG1*, but are clustered in two peaks in the last exon of *BcPG2* (P_A/P_S and K_A/K_S) (Fig. 5D and E). *BcPG3*, as previously shown (Table 3; Figs. 4 and 5C and F), is highly conserved; a solitary amino acid polymorphism was present among *B. cinerea* isolates and no obvious hotspots for amino acid substitution were present.

The use of D_N/D_S ratios to detect selection assumes that nucleotide variation at synonymous sites is neutral. However, codon usage patterns frequently differ from random expectation and codon usage may face selection for translational efficiency. Codon bias can be measured as the effective number of codons (ENC), which is compared with the theoretical maxi-

mum number of codons, 61 (Fuglsang 2004). *BcPG1* shows the most dramatic codon bias (ENC = 32.5). *BcPG3* is intermediate (ENC = 40.2), whereas *BcPG2* and *CYP51* have higher ENC (Table 3).

The Tajima's D test compares the number of segregating sites in a sequence dataset with the average number of pairwise differences; in the absence of selection, these parameters should be equal and Tajima's D statistic will be zero (Tajima 1989). Positive values of Tajima's D are associated with balancing selection or population bottlenecks, whereas negative values are associated with purifying selection or population expansion. *BcPG1* showed a value of Tajima's D significantly greater than 1 ($P = 0.012$) (Table 3). Tajima's D for *BcPG1* exceeds a positive value of 2 when only the coding region is considered, and a sliding window analysis indicates that Tajima's D increases toward the 3' end of the single exon. Values of Tajima's D calculated for the entire available sequence length did not significantly differ from zero for *CYP51*, *BcPG2*, or

Fig. 2. *BcPG1* variation within *Botrytis cinerea*. *B. cinerea* isolate relationships inferred by maximum parsimony analysis of DNA sequence at the *BcPG1* locus. Bootstrap significance values show nodes supported by >50% of 1,000 bootstrap resamplings. *B. allii* serves as an outgroup (isolate "Allii"). Host origins are abbreviated in parenthesis following the isolate name: (V) = *Vitis*; (R) = Rosaceae (including strawberry, raspberry, rose, and apple); (S) = Solanaceae (tomato and pepper); and (O) = other. Sequence for isolate SAS56 was published previously (ten Have et al. 1998).

Fig. 3. *BcPG2* variation within *Botrytis cinerea*. *B. cinerea* isolate relationships inferred by maximum parsimony analysis of DNA sequence at the *BcPG2* locus. Bootstrap significance values are shown adjacent to nodes supported by >50% of 1,000 bootstrap resamplings. *B. calthae* serves as an outgroup (isolate "94-4"). Host origin abbreviations follow the isolate name: (V) = *Vitis*; (R) = Rosaceae (including strawberry, raspberry, rose, and apple); (S) = Solanaceae (tomato and pepper); and (O) = other. Sequence for isolate SAS56 was published previously (Wubben et al. 1999).

BcPG3. Calculating Tajima's D for individual exons and for sliding windows across the sequence length suggested regions of purifying selection in the third exon of *BcPG2* and the first exon of *BcPG3*. Demographic influences on Tajima's D (population bottlenecks or expansion) should affect all loci equally; therefore, differences in estimates of Tajima's D among these four loci suggests that values of Tajima's D differing significantly from zero are attributable to selective forces. Although this data is suggestive of positive selection at *BcPG1*, a broader analysis of Tajima's D is required to ensure that these observations do not result from a genome-wide bias in Tajima's D in *B. cinerea*, as has been detected previously in *Arabidopsis thaliana* (Schmid et al. 2005).

Subdivision by host.

To test for host-based subdivision at PG loci, we divided isolates into four groups based on collection host: Rosid (including strawberry, raspberry, apple, and rose; $n = 10$), *Vitis* (grape; $n = 13$), Solanaceous (tomato and pepper; $n = 6$), and Other ($n = 6$) (Table 1). Overall F_{ST} among host groups for each locus and pairwise F_{ST} between host groups were calculated. Genetic subdivision among host groups was greatest at *BcPG1* ($F_{ST} = 0.269$), with Solanaceous isolates showing the greatest divergence. Host group differentiation at this locus was not statistically significant ($\chi^2 = 50.4$, $P = 0.17$). Because five of six Solanaceous isolates were isolated from the same greenhouse complex, we recalculated F_{ST} comparing only the two largest isolate groups (*Vitis* and Rosid). This did not dramatically alter estimates of host group divergence ($F_{ST} = 0.21$, $\chi^2 = 15.4$, $P = 0.22$), particularly when compared with the low subdivision between *Vitis* and Rosid isolates for *BcPG2* and *BcPG3* ($F_{ST} = 0.038$ and 0.064 , respectively).

Virulence polymorphism.

We proceeded to test whether either host origin or genetic polymorphism was correlated with virulence. Several studies have suggested that variation in *B. cinerea* pathogenicity and genetic markers is associated with plant host (Calpas et al. 2001; Martinez et al. 2003; Munoz et al. 2002; Vallejo et al. 2003). To provide a measurement of relative virulence for comparison of our *B. cinerea* isolates, isolates were tested on mature detached leaves of Col-0, a common reference accession of *A. thaliana*. Under the tested conditions, virulence of these isolates on *A. thaliana* was highly variable, with several isolates unable to form secondary lesions (Table 1). The host origin of *B. cinerea* isolates collected from members of Vitaceae ($n = 11$) or Rosaceae ($n = 10$) was not correlated with virulence on *Arabidopsis* leaves ($t = -0.866$, $P = 0.398$). Mapping relative pathogenicity of isolates on *A. thaliana* accession Col-0 onto the *BcPG1* phylogram (Fig. 6A) reveals no pattern relating pathogenicity on this *Arabidopsis* accession to sequence variation at this locus. Mapping relative pathogenicity of isolates on *A. thaliana* accession Col-0 onto

the *BcPG2* phylogram (Fig. 6B) shows some clustering of highly virulent isolates by haplotype. Because the haplotype group containing the most virulent isolates also contains the largest number of isolates, this pattern may not be robust.

Fig. 4. *BcPG3* variation within *Botrytis cinerea*. *B. cinerea* isolate relationships inferred by maximum parsimony analysis of DNA sequence at the PG3 locus. Bootstrap significance values are shown adjacent to nodes supported by >50% of 1,000 bootstrap re-samplings. *Botrytis calthae* serves as an outgroup (isolate "94-4"). Host origins are abbreviated in parenthesis following the isolate name: (V) = *Vitis*; (R) = Rosaceae (including strawberry, raspberry, rose, and apple); (S) = Solanaceous (tomato and pepper); and (O) = other. Sequence for isolate SAS56 was published previously (Wubben et al. 1999).

Table 3. Population genetic parameters as calculated for *CYP51*, *BcPG1*, *BcPG2*, and *BcPG3*

Locus	Pi^a						D_N/D_S^b	Tajima's D	ENC ^c
	Total	NC	Pi_A	Pi_S	Pi_A/Pi_S	H_d			
CYP51	0.003	0.007	0.0006	0.015	0.009	0.788	0.013	-0.43	48.1
BcPG1	0.021	0.008	0.0183	0.062	0.299	0.908	0.213	1.741	32.5
BcPG2	0.010	0.014	0.0053	0.018	0.283	0.825	0.177	0.004	44.5
BcPG3	0.002	0.004	0.0002	0.004	0.046	0.884	0.064	0.084	40.2

^a Polymorphism per polymorphic site (Pi) is presented for the entire sequenced region (total) and separately for noncoding (intron and untranslated regions) (NC). Pi_A , Pi_S (the ratio of Pi calculated for nonsynonymous versus synonymous sites), haplotype diversity (H_d) (Nei 1987), and Tajima's D (Tajima 1989) for the entire sequence length were calculated using DnaSP.

^b D_N/D_S and codon usage frequencies were calculated with PAML.

^c Effective number of codons (ENC) was calculated using codon usage frequencies via the method of Fuglsang (2004).

Carbon source utilization.

Pectin structure varies among plant species, and variation in PG amino acid sequence or structure may relate to substrate utilization rather than interaction with plant defenses. Therefore, we tested for an association between sequence variation at the PG loci and the isolates' ability to utilize apple pectin as a growth substrate. *B. cinerea* isolates showed significant differences in growth on pectin-based versus sucrose-based solid media. On average, isolates grew more quickly on pectin than

sucrose. However, several isolates were unable to utilize pectin as a carbon source, despite showing growth on sucrose comparable with that of other isolates. Maximum parsimony analysis of deduced amino acid sequences at *BcPG2* supported four major isolate clusters (Fig. 7). We tested for an association between these haplotypes and growth on pectin or the ratio of growth on pectin to growth on sucrose using analysis of variance (ANOVA). This showed a significant association, with group D possessing enhanced growth on pectin versus sucrose

Fig. 5. Distribution of polygalacturonase (PG) polymorphism. Sliding window graphs showing polymorphism along the length of the sequence obtained for loci **A**, *BcPG1*, **B**, *BcPG2*, and **C**, *BcPG3*. Sliding window graphs depicting variation in the ratio of nonsynonymous to synonymous nucleotide substitutions (K_A/K_S) and the ratio of polymorphism at nonsynonymous versus synonymous sites (Pi_A/Pi_S) along the length of the sequenced regions of **D**, *BcPG1*, **E**, *BcPG2* (inset shows first 1,000 bp), and **F**, *BcPG3*. Plots were generated using DnaSP (Rozas and Rozas 1999), with window-length of 50 bp and a step size of 10 bp. Black bars above graphs indicate the location of coding regions.

when compared with groups A or B, and group C being intermediate (Fig. 7). Group D is particularly distinguished by two nonconserved changes at positions 317 to 318, which are responsible for the second spike in K_A/K_S and Pi_A/Pi_S seen in the fourth exon of *BcPG2* (Fig. 5E). Group C possesses four amino acid polymorphisms between position 277 and 283 that account for the first spike in K_A/K_S and Pi_A/Pi_S . Similar analyses of *BcPG1* and *BcPG3* showed no significant relationship between growth on pectin and deduced amino acid sequence at these loci. This suggests that genetic variation in *BcPG2* influences *B. cinerea*'s ability to derive energy from pectin and potentially alters virulence.

DISCUSSION

Efforts to control the destructive agricultural pathogen *B. cinerea* are hampered by its diversity and an incomplete understanding of how this diversity relates to host range. *B. cinerea*'s broad host capacity could be explained by the maintenance of high levels of variation in mechanisms of pathogenesis within

the species or by cryptic genetic subdivision based on host specialization. Two virulence loci, *BcPG1* and *BcPG2*, are approximately 10-fold more polymorphic than three previously characterized *B. cinerea* loci (*Bc-hch*: $Pi = 0.0012$, β -*tubulin*: $Pi = 0.0013$, and *63R*: $Pi = 0.0031$) and two additional loci characterized in this study (*CYP51*: $Pi = 0.003$ and *BcPG3*: $Pi = 0.002$) (Table 3) (Fournier et al. 2003, 2005). This result suggests that *B. cinerea*'s broad host range may be enabled by elevated variation within some pathogenesis loci (Figs. 2 and 3; Table 3).

BcPG1 and -2: Balancing selection.

Increased diversity at the *BcPG1* and *BcPG2* loci supports the direct interaction of these proteins with plant defense proteins, which include PGIPs. PGIPs also show high levels of intra and interspecific variation, and may co-evolve in an antagonistic manner with pathogen-produced PGs (Di Matteo et al. 2006). Sites showing elevated K_A/K_S (*BcPG2*) (Figs. 5E and 7C) or significantly positive Tajima's D (*BcPG1*) detected in this study have not been previously implicated in PG-PGIP interactions (Federici et al. 2001; Sella et al. 2004); however,

Fig. 6. Relation of *Botrytis cinerea* virulence to polygalacturonase (PG) diversity. Relative virulence of *B. cinerea* isolates on *Arabidopsis thaliana* Col-0 in relation to DNA sequence divergence at pathogenicity-associated loci. Phylograms were generated by maximum parsimony and visualized using UNROOTED (Perrière and Gouy 1996); scale bar indicates number of nucleotide substitutions per nucleotide site. Relative virulence is scored as percent of lesion growth rate exhibited by the most aggressive isolate. **A**, *BcPG1* and **B**, *BcPG2*.

PGIPs have been shown to be capable of diverse modes of interaction, both competitive and noncompetitive (Federici et al. 2006). Putative positively selected sites in *BcPG1* and *BcPG2* are located in the c-terminal portion of the protein and are predicted by homology modeling to be exposed residues lying outside the active site cleft. Amino acid position 277 of *BcPG2* may be of particular interest, because three variants were detected in our sample (D, G, and N) (Fig. 7C) and this is also the site of the sole amino acid substitution (G—D) detected for the highly conserved *BcPG3* locus.

The *BcPG1* locus may be subject to conflicting evolutionary forces: purifying selection acting to maximize translational efficiency and diversifying selection on amino acid sequence, potentially to counteract plant PGIPs. Selection for maintenance of transcriptional and translational efficiency at this locus is manifested by low polymorphism in the 5' untranslated

region (Fig. 5A) and strong codon bias (Table 3). Detection of selection via the ratio of nonsynonymous to synonymous substitutions relies on the assumption that variation at synonymous sites is neutral (Kimura 1991; Li et al. 1985). This assumption is violated when codon usage is under selection (Table 3). *BcPG1* transcripts are strongly induced during infection of leaf tissue (ten Have et al. 2001) and fruit (Mehli et al. 2005). *BcPG1* is also the only PG found at the leading edge of developing lesions (Kamoen and Van der Cruyssen 1996). Early and rapid production of this enzyme may be important for successful infection and, thus, a likely target of purifying selection to maintain this capacity. Additionally, codon usage and subsequent effects on translational kinetics recently have been demonstrated to affect not only the rate of protein synthesis but also protein folding and activity (Kimchi-Sarfaty et al. 2007). Thus, hypotheses regarding the interaction of particular amino

Fig 7. Relation of carbon source utilization to polygalacturonase (PG) diversity. Growth of *Botrytis cinerea* isolates on solid media containing either pectin or sucrose as the sole carbon source. **A**, Phylogram shows relationships among isolates as inferred by maximum parsimony from *BcPG2* deduced amino acid sequences. Boxes to the right of isolate clusters correspond to statistically-supported clusters of four or more isolates, labeled A to D. **B**, Bars display the mean (± standard error) observed ratio of growth on pectin-based media to growth on sucrose-based media for each isolate cluster at 72 h postinoculation. Lowercase letters (x–z) indicate statistically supported separation of means; isolate groups sharing letters were not significantly different ($P > 0.05$, Tukey's highly significant difference). **C**, Amino acid differences among isolate clusters, with amino acid position in relation to the start codon shown along the horizontal axis. The sequence of group A, the most common haplotype, is used as the consensus; a dot indicates a match with the consensus at this position. Boxes highlight nonconservative amino acid substitutions.

acid sites with PGIPs based on homology modeling should be approached with caution.

BcPG3 Conservation.

In contrast to *BcPG1* and *BcPG2*, the DNA sequence of *BcPG3* is highly conserved (Fig. 4). The majority of nucleotide polymorphism occurs at synonymous sites, suggesting the action of purifying selection at this locus; however, the negative values estimated for Tajima's D were not statistically significant when the entire locus was considered. Significant negative estimates of Tajima's D for the first exon of *BcPG3* ($D = -1.9$, $P < 0.05$) are consistent with purifying selection in this region. Interestingly, this region contains 120 amino acid residues that do not align with the other five known *B. cinerea* PGs, nor do they contain any identified conserved domains. Because the protein with highest similarity to this region of *BcPG3* is an acidic endopolygalacturonase from *S. sclerotiorum*, it is possible that this portion of the *BcPG3* protein is essential for its function at low pH. *BcPG3* is induced by low pH and, therefore, may play a greater role in the infection of acidic fruits (ten Have et al. 2001; Wubben et al. 2000). Digestion of polygalacturonic acid by *BcPG3* leads to the accumulation of pectin monomers and dimers, rather than the oligomers produced by the action of *BcPG1* and *BcPG2* (Kars et al. 2005). These monomers and dimers then can be used as direct energy sources by the pathogen. A primarily catabolic role of *BcPG3* also is supported by evidence that this gene is induced in intermediate to late infection stages (Mehli et al. 2005; ten Have et al. 2001), after the infected plant tissue has died. These observations suggest that *BcPG3* has little direct interaction with plant defenses, but plays an important and conserved role in providing energy for established infections. The alignment of the single detected amino acid polymorphism (419G/D) detected at this locus with a putatively selected region of *BcPG2* (discussed above) presents an intriguing counterpoint to the overall conclusions regarding this locus.

Relationship of PG sequence to host specialization and carbon source utilization.

In this study, we identified an association between amino acid sequence polymorphism in *BcPG2* and the ability of iso-

lates to utilize pectin. An association of *BcPG2* variation with pathogenicity was suggested by differences in virulence on *A. thaliana* leaves, where highly virulent isolates were unequally distributed across the *BcPG2* phylogram. This suggests that variation within *BcPG2* impacts *B. cinerea*'s ability to grow on and infect *Arabidopsis* and, potentially, other plant species. Direct tests of the effects of PG variation on pectin utilization and pathogenicity will require construction of allele-replacement strains of *B. cinerea* to reduce the influence of other genetic elements.

In contrast to the relationship with general virulence, we could find little evidence for host specialization at the three PG-encoding loci examined in this study. *B. cinerea* isolates did not form exclusive clusters of DNA sequence similarity defined by host from which the isolate was obtained (Figs. 2–4). Although statistically significant genetic differentiation was not observed between host-based isolate groups, elevated F_{ST} at *BcPG1* compared with other loci suggests that differentiation could be detected in a larger sample. In particular, divergence of Solanaceous isolates from other groups supports studies showing differentiation between tomato and grape isolates (Munoz et al. 2002); however, more extensive sampling will be required to validate these results.

Future work.

Study of nucleotide sequence variation in *B. cinerea* PGs has demonstrated that, in the collection of isolates studied, different evolutionary pressures have influenced the structure of genetic diversity at these loci. These observations suggest several testable predictions. The high levels of nucleotide polymorphism found in *BcPG1* and *BcPG2* suggest a co-evolutionary interaction with PGIPs. In particular, highly polymorphic amino acid residues may be important to PGIP binding and thus present intriguing targets for site-directed mutagenesis and functional binding studies with diverse PGIPs. Compared with *BcPG1* and *BcPG2*, *BcPG3* shows low levels of intraspecific variation. An analysis of pathogenicity and pectin-degradation capacity of knock-out mutants at this locus may help to determine whether this lack of variation is due to purifying selection. The role of three additional PGs, *BcPG4*, *BcPG5*, and

Table 4. Primer information

Primer name	Reference ^a	Primer sequence (5'–3')	Start ^b	Product (bp)
PG1o-F	U68715	CACTCGCAAAACCATCAGTTAG	77	1,241
PG1o-R	...	CGTAGAAGAAGCTTTGGCTTGGT	1,317	...
PG1a-F	...	CCATTCAACAAGAAAGTGGTGTA	471	1,415
PG1a-R	...	GTACCCTTGACGTTGGAGAAAG	1,885	...
PG1y-F	...	CCCATCCGGTACTACTCTTGAC	1,088	1,149
PG1z-F	...	CTTCGGTCTTACTCTCTCCAA	1,399	...
PG1z-R	...	CTGGAGAGTTCACTGAAGAGCA	2,236	...
PG1fix-F	...	GTCAGAATGCGGACCTTGAT	566	889
PG1fix-R	...	ACCAGCATCTCCAGCAGAGT	1,454	...
PG2-1-F	U68716	TCCGAATGCGAAATACCTCTAT	171	943
PG2-1-R	...	GGATAAACTTACGGTGCCAGAG	1,113	...
PG2-2-F	...	GAAAAGGCAAAGTCAAGCCTAA	821	908
PG2-2-R	...	TCAAAACGTTCTCTCAAAAACCT	1,728	...
PG3-1-F	U68717	TGGGAATCAGGGGTATATTGTC	31	908
PG3-1-R	...	GACGTAGGAGCTTCGAACGTAT	938	...
PG3-2-F	...	ATACCCGTGTCTTGAAATTTGG	789	900
PG3-2-R	...	TGAACAGGTCAATCCTGTAAACG	1,688	...
PG3-3-F	...	ATTACTGGTGCCCAAGGTCTTA	1,461	994
PG3-3-R	...	CTGAGCATCAAACAGGATTTTG	2,454	...
Bo-CYP51-F ^c	AF279912	TGCGATGGGGATTCTTGAA	110	1,081
BcSP1up-R ^c	...	GCATAATCCACGAGATAGAAGATGAGGAAG	1,190	...
CYP51a-F	...	TGGGCTTCAGTCTATCAACTT	906	912
CYP51a-R	...	CTCCATTAATCCATCCGTTTCAT	1,817	...

^a Reference indicates the GenBank identification number of the sequence used to design primers.

^b Start refers to the position of the first base complementary to the 5' end of the primer with respect to the reference sequence.

^c As previously published (Albertini et al. 2002).

BcPG6, in pathogenicity and host selection has been relatively unexamined, and analyses of variation at these loci may contribute to a broader understanding of how PG specialization contributes to pathogenicity or virulence. The recent sequencing of the *B. cinerea* genome should accelerate identification of additional genes contributing to pathogenicity and provide new candidates for host determining loci.

MATERIALS AND METHODS

Isolate collection, culture, and storage.

B. cinerea isolates were obtained directly from infected plant material or from other researchers. The plant host and geographic origin of isolates used as sources of sequence data for this study are given in Table 1.

Potato dextrose agar (Gibco/Invitrogen, Carlsbad, CA, U.S.A.) was inoculated using swabs from infected plant material. Cultures were purified by successive conidial isolation. For inoculum and storage, conidia were collected by rinsing sporulating cultures with distilled water. Conidial suspensions were filtered through glass wool to remove mycelial fragments, quantified using a hemacytometer, and stored at -80°C in 30% glycerol until further use.

Cultures were confirmed as *Botrytis* spp. by hybridization with a genus-specific monoclonal antibody BC-12.CA4 (Dewey et al. 2000). Of 37 fungal isolates identified as *Botrytis* spp. by immunoassay, 34 were confirmed as *B. cinerea* by DNA sequence at the *CYP51* locus. Three divergent isolates (94-4, Allii, and Ancho) were sequenced at a more conserved locus, *BcRBP2*, for which interspecific data was available (Staats et al. 2005). Comparison with a phylogeny for the genus *Botrytis* based on data from *BcRBP2* identified Ancho and Allii as *B. allii*, whereas 94-4 is most closely related to *B. calthae* (data not shown).

DNA extraction and sequencing.

Conidia of *Botrytis* isolates were inoculated into 30-ml volumes of liquid Czapek-Dox media (3 g of NaNO_3 , 1 g of K_2HPO_4 , 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g of KCl, 55 mg of FeSO_4 , and 30 g of sucrose per liter) supplemented with 1% yeast extract. These cultures were incubated on a rotary shaker for 2 to 3 days at 200 rpm and 25°C . Freshly grown mycelia were removed, rinsed with distilled water, and frozen in liquid nitrogen prior to tissue disruption. DNA was isolated using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's protocol.

PCR primers and reaction conditions used to amplify a 1,559-bp fragment at the *CYP51* locus are as previously described (Albertini et al. 2002). Primers for amplification of PG loci were based on published sequence from *B. cinerea* isolate SAS56 (ten Have et al. 1998; Wubben et al. 1999) (Table 4). Gene fragments were amplified by PCR in 50- μl reaction mixtures containing 1.2 mM MgCl_2 , 0.2 mM dNTP, 0.2 μM each primer, and 0.5 to 1 μg of genomic DNA, thermocycled for 35 cycles of 94°C (30 s), 56°C (60 s), and 72°C (90 s). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and concentration of the purified product was determined using a Nanodrop Spectrophotometer (Wilmington, DE, U.S.A.). PCR products were sequenced via BigDye Terminator v3.1 Cycle Sequencing chemistry with an ABI 3730 Capillary Electrophoresis Genetic Analyzer at the University of California–Davis Department of Biological Sciences Sequencing Facility.

Analysis of DNA sequence data.

Raw DNA sequence data in FASTA format was trimmed to remove low-quality sequence and assembled into contigs using

Vector NTI Contig Express (Informax, Bethesda, MD, U.S.A.). Consensus sequences for each isolate were obtained for each region with at least twofold high-quality coverage. No heterozygosity was detected in the sequence data. Alignment of isolate consensus sequences and generation of neighbor-joining phylograms for each locus was performed using ClustalX 1.81 (Thompson et al. 1997). Aligned sequence data was transferred into NBS/PIR format for further analysis using DnaSP 4.0 (Rozas and Rozas 1999), into Phylip format for analyses employing PAML3.15 (Yang 1997), or into MEGA format for construction of additional phylograms (Kumar et al. 2004). Phylogenies were reconstructed via maximum parsimony, using Close Neighbor Interchange with random addition trees for tree selection and 1,000 bootstrap replications to assess statistical support for branches. Trees constructed via neighbor-joining and maximum parsimony did not have significant topological differences. Dendrograms displaying interisolate relationships based on deduced amino acid sequences were constructed by similar methods.

Population genetic analyses, including estimation of P_i (polymorphism per polymorphic site), haplotype diversity (H_d), and Tajima's D (Tajima 1989) were performed using DnaSP, using settings for haploid, autosomal data. Tajima's D was estimated based on segregating sites, with statistical significance of the estimates determined by generation of 95% confidence intervals derived from 1,000 replicate coalescent simulations allowing intermediate levels of recombination. Recombination levels were based on empirically derived estimates of R (Hudson 1987). Sliding window analyses of polymorphism (P_i , P_A/P_S , K_A/K_S , and Tajima's D) within each *BcPG* locus included both coding and noncoding sites. All sliding window analyses (P_i , P_A/P_S , K_A/K_S , and Tajima's D) used a step size of 10 sites and a window length of 50 sites. Expected values of H_d were estimated using the sample size and number of polymorphic sites (Depaulis and Veuille 1998). Nucleotide sequence divergence among subpopulations (either geographic or host-based) was examined via calculated values of F_{ST} (Wright 1978) and χ^2 tests for population differentiation. The ratio of nonsynonymous to synonymous nucleotide substitutions (D_N/D_S) was calculated using the CODEML module of PAML 3.15 (Yang 1997). Outgroup sequences used for K_A/K_S and D_N/D_S estimates are listed in Table 2. Codon usage bias was assessed by calculating the ENC (Fuglsang 2004), using codon usage frequencies for each locus generated by the CODEML module of PAML 3.15.

We used *CYP51* as a reference locus for comparison with published data from isolates collected in the Champagne region of France (Albertini et al. 2002). Our analysis excludes isolates belonging to the divergent subspecies identified as group I (Fournier et al. 2005). Although that study contained sequence data from 35 isolates, for 21 of these, sequence data was available from only two regions of approximately 350 bp each. We performed comparisons using only the smaller subset of French isolates for which full-length sequence was available ($n = 9$). DNA sequence analyses were as described above. Estimates of P_i from β -tubulin, *Bc-hch* (vegetative incompatibility locus), and *63R* (microsatellite sequence) were obtained via reanalysis of published sequence data using DnaSP (Fournier et al. 2005).

Virulence testing.

Isolates were tested for virulence on detached leaves of *A. thaliana* as previously described (Kliebenstein et al. 2005). *Arabidopsis* (Col-0 ecotype) was grown in environmental chambers at 22°C , 50% humidity, with a photoperiod of 12 h of light and 12 h of dark to promote vegetative growth. Leaves excised from 4- to 5-week-old rosette plants were placed in

trays of 1% phytagar and inoculated with single 4- μ l droplets of *B. cinerea* conidial suspension (5,000 spores/ml in 50% organic grape juice). Trays were fitted with transparent plastic covers to retain humidity. Following lesion initiation, digital photographs of leaves were taken every 8 to 12 h and analyzed using Image J (Abramoff et al. 2004). Mean values for fungal lesion diameter and lesion growth rate were estimated using SAS (SAS Institute Inc., Cary, NC, U.S.A.). Relative virulence was calculated by dividing the lesion growth rate of each isolate by the highest lesion growth rate measured. Thus, the most aggressive isolate had a relative virulence of 100. A two-sample *t* test was used to test the null hypothesis that no difference in relative virulence existed between isolates from the two most represented host plant families, Vitaceae (grape, *n* = 11) and Rosaceae (strawberry, raspberry, rose, and apple, *n* = 10).

Carbon-source utilization.

To test for a potential association between sequence variation at PG-encoding loci and ability to use pectin as a carbon source, isolates were grown on Czapek Synthetic Agar media with either apple pectin (1%; MP Biomedicals, Solon, OH, U.S.A.) or sucrose (3%) as the sole carbon source. Replicate pairs of pectin or sucrose-based solid media plates were inoculated with 10- μ l droplets of *B. cinerea* spores (10⁵ spores/ml in 50% organic grape juice) placed in the center of the plate. Plates were incubated at 25°C, 50% relative humidity. The diameter of the plate area covered by radial outgrowth of mycelia from the inoculation point was measured every 24 h until growth reached the plate edge.

To improve comparison among isolates with different overall mycelial growth rates, the ratio of growth diameter on pectin to growth diameter on sucrose was calculated for each pair of plates. Isolates were grouped by deduced amino acid sequence of *BcPG1*, *BcPG2*, and *BcPG3*. Both primary growth measurements on pectin and sucrose, as well as their ratio, were analyzed using SAS PROC GLM with a model that nests the effect of individual isolates within PG amino acid groups.

ACKNOWLEDGMENTS

We would like to thank D. Margosan (United States Department of Agriculture, Parlier, CA, U.S.A.), K. Denby (University of Warwick), M. Vivier (University of Stellenbosch), E. Mitcham (University of California, Davis), and D. Gubler (University of California, Davis) for sharing *Botrytis* isolates with us. We are grateful to M. Dewey (University of California, Davis) for her generosity with antibodies and *B. cinerea* isolates. D. Rualizo and K. Lyons provided valuable laboratory assistance, and K. Schuller-Smith assisted in isolate collection. We additionally thank C. Willet, M. Clauss, C. Hagen, and J. Whittall for critical reviews of this manuscript prior to submission. This research was partially funded by a Henry A. Jastro and Peter J. Shields Graduate Research Award granted to H. C. Rowe.

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