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Classical and molecular genetics of *Bremia lactucae*, cause of lettuce downy mildew

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Abstract Lettuce downy mildew caused by *Bremia lactucae* has long been a model for understanding biotrophic oomycete–plant interactions. Initial research involved physiological and cytological studies that have been reviewed earlier. This review provides an overview of the genetic and molecular analyses that have occurred in the past 25 years as well as perspectives on future directions. The interaction between *B. lactucae* and lettuce (*Lactuca sativa*) is determined by an extensively characterized gene-for-gene relationship. Resistance genes have been cloned from *L. sativa* that encode proteins similar to resistance proteins isolated from other plant species. Avirulence genes have yet to be cloned from *B. lactucae*, although candidate sequences have been identified on the basis of motifs present in secreted avirulence proteins characterized from other oomycetes. *Bremia lactucae* has a minimum of 7 or 8 chromosome pairs ranging in size from 3 to at least 8 Mb and a set of linear polymorphic molecules that range in size between 0.3 and 1.6 Mb and are inherited in a non-Mendelian manner. Several methods indicated the genome size of *B. lactucae* to be ca. 50 Mb, although this is probably an underestimate, comprising approximately equal fractions of highly repeated sequences, intermediate repeats, and low-copy sequences. The genome of *B. lactucae* still awaits sequencing. To date, several EST libraries have been sequenced to provide an incomplete view of the gene space. *Bremia lactucae* has yet to be transformed, but regulatory sequences from it form components of transformation vectors used for other oomycetes. Molecular technology has now advanced to the point where rapid progress is likely in determining the molecular basis of specificity, mating type, and fungicide insensitivity.

Keywords *Bremia lactucae* · Lettuce · Virulence · Resistance · Oomycete

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Introduction

Bremia lactucae is an obligate oomycete pathogen belonging to the Peronosporales. Members of the Peronosporales exhibit a gradient in modes of parasitism from saprotrophy through necrotrophy and varying degrees of biotrophy (Ingram 1981; Göker et al. 2007). *Bremia lactucae* represents one of the most highly specialized downy mildews at the biotrophic end of this spectrum. Like all members of the Peronosporaceae, it is an obligate biotroph (i.e. it can only currently be cultured in association with its host). However, the asexual spore germinates directly rather than via zoospores that are used by most other members of the Peronosporaceae. *Bremia lactucae* also

directly penetrates through the plant cuticle and epidermal cells rather than entering the leaf through stomata. Both of these attributes indicate that it is one of the most highly evolved downy mildews. Recent molecular phylogenetics supports the advanced taxonomic position of *B. lactucae* (Voglmayr et al. 2004).

Bremia lactucae has a long history as a model for understanding biotrophy in the Oomycetes (Maclean et al. 1974; Andrews 1975; Ingram et al. 1976; Maclean and Tommerup 1979; Ingram 1981; Woods et al. 1988). Its biotrophic mode of nutrition involves a close interaction with its host, in which the plant plasmalemma is invaginated around simply lobed haustoria. Compatible interactions result in minimal macroscopic disturbance until sporulation. Although it is an obligate pathogen, *B. lactucae* can readily be cultured in the laboratory on lettuce seedlings; it is a tractable genetic system and many of the necessary tools for manipulating it in the laboratory in conjunction with its host (*Lactuca* spp.) have been developed. The classical genetics of specificity in lettuce downy mildew is one of the best understood of any gene-for-gene plant–pathogen interaction. Simultaneous studies of host and pathogen showed that specificity is determined by numerous gene-for-gene interactions (Crute and Johnson 1976; Farrara and Michelmore 1987). The molecular determinants on the host side are becoming increasingly well worked out (Meyers et al. 1998a, b; Shen et al. 2002; Kuang et al. 2004). However, the molecular biology of *B. lactucae* has lagged behind.

Bremia lactucae causes lettuce downy mildew, the most important disease affecting lettuce worldwide. Lettuce ranks as one of the top ten most valuable crops in the USA with an annual value of over $2.26 billion (US Department of Agriculture 2003, 2006). Lettuce is grown as extensive monocultures, often with several crops per year. Such intensive production makes the crop susceptible to major epidemics and lettuce suffers from several economically important pests and diseases, particularly downy mildew. These are currently controlled by a combination of genetic resistance, cultural practices, and chemical protection including the application of over 1.5 million pounds of insecticides and fungicides per year (US Department of Agriculture 2003). Several of these compounds are being withdrawn from agricultural use due to environmental concerns over their safety or have been rendered ineffective by changes in *B. lactucae* (Crute et al. 1987; Schettini et al. 1991; Brown et al. 2004). Breeding for resistance to *B. lactucae* is a major activity of most lettuce improvement programmes, and there is an increasing need for information and methods to accelerate the development of new disease-resistant cultivars. Downy mildew resistance (*Dm*) genes provide high levels of resistance but have only remained effective for limited periods of time due to changes in pathogen virulence. Much of the breeding effort is currently focused on introgressing new genes from wild species in response to pathogen changes. New strategies are needed to provide more durable forms of resistance.

The purpose of this review is to summarize what is now known of the classical and molecular genetics of *B. lactucae* and its interaction with lettuce, as well as to consider future developments that are imminent due to the application of genomics approaches.

Classical genetics of resistance

The interaction between lettuce and *B. lactucae* is one of the most extensively characterized gene-for-gene plant–pathogen relationships (Crute and Johnson 1976; Farrara et al. 1987; Hulbert and Michelmore 1985; Michelmore et al. 1984; Norwood and Crute 1984; Norwood et al. 1983; Ilott et al. 1987, 1989). The genetics of resistance has been facilitated by simultaneous studies of avirulence. At least 27 major *Dm* genes or resistance (R) factors are now known that provide resistance against specific isolates of *B. lactucae* in a gene-for-gene manner (Farrara et al. 1987; Bonnier et al. 1994; Maisonneuve et al. 1994; Jeuken and Lindhout 2002). Many other sources of resistance have been identified but have not yet been extensively characterized genetically (e.g. Farrara and Michelmore 1987; Gustafsson 1989; Bonnier et al. 1994; Lebeda and Zinkernagel 2003; Beharav et al. 2006). As more *Dm* genes are characterized from these and other sources, it is likely that many hundred *Dm* genes with specificity to *B. lactucae* will be identified.

Most of the currently identified *Dm* genes confer high levels of resistance. This may be a consequence of these genes being the ones identified and used by breeders. Some *Dm* genes, e.g. *Dm6*, confer incomplete resistance phenotypes (Crute and Norwood 1978). Partial phenotypes do not necessarily imply

quantitative inheritance or more durable resistance. The phenotype of the interaction depends on the gene and environment. Heterozygotes of some *Dm* genes, e.g. *Dm18*, also confer incomplete resistance (Maisonneuve et al. 1994). In addition, different isolates of *B. lactucae* can exhibit different levels of incompatibility to the same *Dm* gene (Ilott et al. 1989). At lower temperatures, resistance conferred by several *Dm* genes becomes less effective; temperature shift experiments suggested that the determinants of specificity are present in most host cells and expressed throughout pathogen development (Judelson and Michelmore 1992). There are also resistance genes of minor effect that confer incomplete or field resistance (Eenink et al. 1983; Jeuken and Lindhout 2002). Many genes of minor effect will probably be identified in the future by quantitative trait locus (QTL) analysis using molecular markers.

The known *Dm* resistance phenotypes are located in at least five clusters in the lettuce genome (Hulbert and Michelmore 1985; Farrara et al. 1987; Bonnier et al. 1994). The major cluster contains over nine genetically separable *Dm* specificities, as well as resistance to root aphid. Another large cluster contains several *Dm* genes, resistance to the root-infecting downy mildew *Plasmopara lactucae-radicis*, and the hypersensitive reaction to *Turnip mosaic virus* (Witsenboer et al. 1995).

Molecular genetics of resistance

One downy mildew resistance gene, *Dm3*, has been cloned through a combination of map-based cloning and candidate gene approaches (Shen et al. 1998, 2002; Meyers et al. 1998a). *Dm3* encodes a nucleotide binding site and leucine-rich repeat (NBS-LRR) protein, similar to genes cloned from other species for resistance to downy mildews and other pathogens (McHale et al. 2006). *Dm3* is large, containing nearly double the number of LRRs compared to proteins characterized in other species. *Dm3* is a member of the large *RGC2* (*Resistance Gene Candidate2*) multigene family that can vary in copy number from 12 to over 30 (Meyers et al. 1998a, b; Kuang et al. 2004). Sequence analysis of paralogues from several species indicated that this large cluster evolves by a birth-and-death mechanism (Michelmore and Meyers 1998; Kuang et al. 2004). Genes in the *RGC2* family exhibit two distinct patterns of evolution. Type I genes are extensive chimeras resulting from frequent sequence exchange between paralogues, and individual genes are rare in nature. *Dm3* is a Type I gene and only rarely present in nature (Kuang et al. 2006). Type II genes occur more frequently in nature, and sequence exchanges only rarely occur between individual lineages (Kuang et al. 2004). Trans-specific polymorphism was observed for different groups of Type II orthologues, suggesting balancing selection. Different evolutionary forces have impacted different parts of *RGC2* genes. The *RGC2* cluster is not highly recombinogenic; it exhibits a recombination frequency 18 times lower than the genome-wide average (Chin et al. 2001). This is consistent with reduced pairing during meiosis between haplotypes due to structural heterozygosity.

The meiotic spontaneous mutation rates differ between the *Dm* genes (Chin et al. 2001). Spontaneous mutations in *Dm1*, *Dm3* and *Dm7* occurred at the rate of 10^{-3} to 10^{-4} per generation. No spontaneous mutations were detected for *Dm5/8*. Spontaneous mutations at the *Dm3* locus but not the *Dm7* locus were frequently associated with large deletions resulting from unequal crossing-over. One spontaneous loss of *Dm3* resistance was observed to be the result of a gene conversion event between the LRR-encoding regions of similar paralogues (Chin et al. 2001). Given that a lettuce plant is capable of producing several thousand seeds per generation, such mutation rates suggest that in every generation an average of one progeny with a novel haplotype at a resistance locus is produced per plant.

PCR using degenerate oligonucleotides designed to sequences encoding conserved NBS domains has resulted in the identification of over 20 distinct families of resistance gene candidates (*RGCs*; Shen et al. 1998; McHale and Michelmore, unpublished). These are being mapped relative to phenotypic resistances to provide a comprehensive view of the genomic distribution of resistance genes, including many *Dm* genes.

The clustered genomic distribution of *Dm* genes suggests that they are similar genes. This has been confirmed for the major cluster of *Dm* genes. An interfering hairpin RNA (ihpRNA) construct containing fragments encoding the LRR of *Dm3* was used to induce post-transcriptional gene silencing of the

RGC2 family (Wroblewski et al. 2007). This showed that the resistance specificity encoded by the genetically defined *Dm18* locus is the combination of two resistance specificities, only one of which was silenced by ihpRNA derived from *Dm3*. Analysis of progeny from crosses between transgenic, silenced tester stocks and lettuce accessions carrying other resistance genes previously mapped to the *RGC2* locus indicated that two additional resistance specificities *to B. lactucae*, *Dm14* and *Dm16*, as well as resistance to lettuce root aphid (*Pemphigus bursarius*), *Ra*, are encoded by *RGC2* family members. This strategy is now being extended to other clusters of resistance genes for which *RGC* sequences and phenotypic resistances co-segregate.

Numerous haplotypes and homologues at the major cluster of resistance genes that contains *Dm3* have been identified. Fifty-one different haplotypes were identified in 74 accessions studied using molecular markers diagnostic of the *RGC2* cluster (Sicard et al. 1999). The copy number of *RGC2* paralogues at a locus can vary from 12 to >30 (Kuang et al. 2004). No accessions have been observed that completely lack *RGC2* genes even though they do not carry detectable *Dm* specificities. The large number of different haplotypes is consistent with there being a minimum of several hundred distinct *Dm* genes in *Lactuca* species and indicates that wild germplasm will be a rich source of new resistance genes that can be introgressed and pyramided using molecular markers.

There is also a growing understanding of the signalling pathways and downstream genes and proteins that are involved in plant resistance (Jones and Dangl 2006). However, there are little specific data on genes involved downstream of *Dm* genes in the interaction with *B. lactucae*. Homologues of genes from other species known to be involved in pathogen interactions are present in ESTs from *Lactuca* spp. (http://compgenomics.ucdavis.edu), and therefore it is likely that similar processes are involved in lettuce as in other plants. Ultrastructural and biochemical studies indicate that the hypersensitive response is typical but includes the induction of phytoalexins characteristic of the Compositae (Maclean and Tommerup 1979; Bennett et al. 1996; Bestwick et al. 1998; Lebeda et al. 2008). As the molecular understanding of *B. lactucae* develops, it will be interesting to determine how the pathogen has adapted to deal with these defences.

Mating system

Bremia lactucae is diploid for the majority of its life-cycle and predominantly heterothallic (Michelmore and Ingram 1980; Michelmore and Sansome 1982). Both the asexual life-cycle of 1 to 3 weeks and the sexual cycle of several months' to many years' duration can be readily induced in the laboratory. The asexual cycle allows the facile clonal propagation of individual phenotypes. Its heterothallic nature allows controlled crosses between isolates of known phenotypes for the investigation of the genetics of (a)virulence.

When hyphae of opposite mating type come into physical contact, asexual sporulation is suppressed, clusters of gametangia are elaborated at the point of contact, synchronous meioses occur in the oogonium and periclinal antheridium, and haploid gametes are transferred from the antheridium to the oogonium to effect fertilization (Michelmore and Ingram 1981; Michelmore and Sansome 1982). Each mating type can probably produce both oogonia and antheridia, as do *Phytophthora* species. Differences in maleness and femaleness have not been investigated.

Heterothallism seems to be determined by two haplotypes at a single locus, with the B_1 compatibility type being conferred by a homozygous recessive condition and the B_2 mating type by a heterozygous condition. The two mating types segregate in approximately 1:1 ratios in sexual progeny (Michelmore and Ingram 1981; Norwood et al. 1983; Michelmore et al. 1984; Sicard et al. 2003). However, the current data do not preclude a more complicated situation such as double heterozygotes and balanced lethals, as has been proposed for *Phytophthora infestans* (Fabritius and Judelson 1997). The molecular determinants of mating type for *B. lactucae* await characterization as they do for all oomycetes.

Some isolates exhibit secondary homothallism (Michelmore and Ingram 1982). These isolates behave predominantly as B_2 types in that they usually reproduce asexually except when cultured in combination with B_1 isolates, whereupon they produce abundant oospores. However, they also produce oospores at low frequency when cultured alone, particularly at high inoculum densities. This is due to the generation of B_1 components at low frequency, as shown by the isolation of stable B_1 and B_2 as well as self-fertile derivatives by single–spore analysis (Michelmore and Ingram 1982). This self-fertility

may be due to trisomy of the determinants of mating type (Michelmore and Sansome 1982). Somatic segregation of self-sterile lines from self-fertile progenitors involves at least transitory heterokaryosis.

The prevalence of each mating type varies in nature. Isolates of both mating types have been frequently identified in Europe and New York State, although the B_2 type sometimes predominated (Michelmore and Ingram 1980; Lebeda and Blok 1990; Gustafsson et al. 1985; Yuen and Lorbeer 1987; Petrželová and Lebeda 2003). This is consistent with a sexually reproducing population and the high diversity of virulence phenotypes observed. In contrast, the B_2 mating type predominates in isolates from cultivated lettuce in California; B_1 isolates are identified extremely rarely. In addition, the one B_1 isolate analyzed from California had reduced fertility (Ilott et al. 1987). The data for California isolates are indicative of an asexual population that propagates clonally. This is consistent with the more restricted spectrum of virulence phenotypes observed and widespread pathotypes that are stable from year to year. However, even in the apparent absence of the sexual cycle and the oospore as a survival stage, *B. lactucae* has been able to change virulence phenotype in response to the deployment of new *Dm* genes and it is unclear how the pathogen survives crop-free periods in California.

Genetics of avirulence

Several initial studies established that avirulence to specific *Dm* genes was inherited as single dominant unlinked loci (Michelmore and Ingram 1981; Norwood et al. 1983; Norwood and Crute 1984; Michelmore et al. 1984; Ilott et al. 1987). The gene-for-gene interaction between lettuce and *B. lactucae* was subsequently analyzed critically, involving extensive crosses between 20 isolates of diverse worldwide geographical origins to complement the simultaneous genetic analysis of resistance (Farrara et al. 1987; Ilott et al. 1989). The majority of the data were consistent with the underlying tenets of a gene-for-gene interaction. Avirulence was usually determined by dominant alleles at unlinked loci, although their expression could be modified depending on the genetic background of the host and pathogen. Some segregation anomalies could be explained by hyperploidy and gene dosage effects. In order to test for complementation between *Avr* loci, 125 tests involving 19 crosses were analyzed. In no case were all progeny avirulent to a specific *Dm* gene when both parental isolates had been virulent; therefore, there was no evidence for complementation, indicating that avirulence to individual *Dm* genes was conferred at the same locus. To investigate the presence of dominant inhibitors of avirulence, crosses were made between avirulent and virulent isolates. The data for an inhibitor locus epistatic to *Avr5/8* were good but not unequivocal; there was no evidence for inhibitors of other *Avr* loci (Ilott et al. 1989). Therefore, unlike the situation in phytopathogenic bacteria (Abramovitch et al. 2003; Espinosa et al. 2003; Jamir et al. 2004; Fu et al. 2007), inhibitor loci do not seem to be common in *B. lactucae*.

Genetic mapping

A preliminary genetic linkage map of *B. lactucae* was constructed using the segregation of 53 RFLP loci, 8 *Avr* loci, and the mating type locus in a total of 70 F_1 individuals from two crosses (Hulbert et al. 1988). This map consisted of 13 small linkage groups, including 35 RFLP loci and one *Avr* gene. However, construction of a more detailed genetic map was hindered by the ambiguous phase of the alleles in the parents and an insufficient number of markers due to the type of marker technology available at the time.

A more comprehensive genetic map of *B. lactucae* was subsequently constructed using PCR-based markers as well as additional RFLP loci (Sicard et al. 2003). The more heterozygous of the two crosses that had been used previously was expanded to 97 F_1 progeny to facilitate the identification of the phase of the parental alleles and to improve the detection of linkage. Two parental maps and a consensus map were constructed using a total of 347 AFLP and 83 RFLP markers, six *Avr* genes, and the mating-type locus. One parental map contained 24 linkage groups distributed over 835 cM; the second map contained 21 linkage groups distributed over 606 cM. The consensus map contained 12 linkage groups with markers from both maps and 24 parent-specific groups.

There was no evidence for clustering of *Avr* genes. All six mapped to different linkage groups. This is consistent with the lack of linkage observed in

classical segregation analysis of 12 *Avr* loci (Ilott et al. 1989). Also, the genetic data provided no evidence for pathogenicity islands that have been identified in bacteria (Alfano et al. 2000; Guttman et al. 2002; Jackson et al. 1999; Sugio et al. 2005). Four *Avr* loci were located at the ends of linkage groups. Telomeric locations of *Avr* genes would be consistent with the high instability of the avirulence phenotype in *B. lactucae*. In the fungal pathogen *Magnaporthe grisea*, four out of eight known *Avr* genes are close to a telomere, and losses in avirulence were associated with deletions (Mandel et al. 1997; Dioh et al. 2000). Linkage of three *Avr* genes with distorted markers in *B. lactucae* may be indicative of other mechanisms of instability of *Avr* genes, such as high frequencies of mitotic gene conversion as observed in *P. sojae* (Chamnanpunt et al. 2001).

The current genetic map of *B. lactucae* is far from saturated. Over 20% of the markers remain unlinked. It is difficult to estimate the total number of chromosomal groups and genetic genome size because of the possible redundancy between the parent-specific linkage groups. The mating type locus and two *Avr* loci are flanked by molecular markers; however, no close linkages have been identified. The closest marker is 1 cM, and only loose linkages have been identified for the majority of *Avr* genes. Whether this represents a dearth of polymorphic low-copy sequences or high rates of recombination close to avirulence genes is unknown. We attempted bulked segregant analysis (Michelmore et al. 1991) to identify markers closely linked to several avirulence genes; however, this was unsuccessful (Zungri and Michelmore, unpublished).

Karyotype and chromosomal assignment of markers

Cytological analysis of *B. lactucae* resolved at least 7 or 8 chromosome pairs at meiosis (Michelmore and Sansome 1982). However, these chromosomes are too small to be resolved clearly using conventional light microscopy. Examination of isolates of diverse geographical origins as well as progeny from sexual crosses by pulsed-field gel electrophoresis (PFGE) revealed a minimum of seven chromosomes ranging in size from 3 to at least 8 Mb and a set of linear polymorphic molecules from 0.3 to 1.6 Mb (Francis and Michelmore 1993). Genetic and hybridization analyses confirmed the existence of two classes of molecules.

The class of smaller molecules is sequence-related, non-ribosomal, nuclear, highly polymorphic, variable in number, and inherited in a non-Mendelian manner. These small polymorphic molecules are therefore B chromosomes or large liner plasmids. No RFLP markers, and consequently none of the *Avr* genes, were assigned to the small polymorphic 0.3–1.6 Mb molecules. Therefore, there was no evidence that these small variable molecules are involved in variation in specificity of *B. lactucae*.

The second class of molecules is larger than 2 Mb, is more constant in size and number and represents the true chromosomes. A total of 25 probes were successfully hybridized to these chromosomes (Sicard et al. 2003). Of these, 23 had been mapped and represented 16 of the linkage groups in the consensus map; two were unlinked. This resulted in two consensus linkage groups and seven parent-specific linkage groups being assigned to chromosomes. Linkage to RFLP markers allowed three *Avr* loci also to be assigned to chromosomes. The mating–type locus could not be assigned to any chromosome-sized molecule. Together the genetic and physical data suggest that there are at least 10 chromosomes in *B. lactucae*.

Somatic variation

Bremia lactucae can exhibit somatic variation in addition to the segregation of phenotypes following sexual reproduction. RFLP analysis of 25 isolates from diverse worldwide geographical origins revealed different ploidy levels and somatic variants (Hulbert and Michelmore 1987). Most European isolates were clearly diploid. They were heterozygous at approximately 44% of their loci and had highly variable genotypes consistent with the frequent occurrence of the sexual cycle. In contrast, many of the isolates from Australia, Japan, Wisconsin and Australia had more than two alleles at multiple loci, indicating that they were either polyploids or stable heterokaryons (hyperploid). Variation between similar sympatric isolates indicated that they had arisen by the somatic loss of alleles. One hyperploid California isolate had resulted from the fusion of

two diploid California isolates of the same mating type, providing the first evidence for natural somatic fusion in the Oomycetes.

Several phenotypic changes in *B. lactucae* seem to have resulted from somatic changes. The segregation of self-sterile lines in secondary homothallic isolates is one example (Michelmore and Ingram 1982). Fungicide insensitivity seems to have arisen in the most common virulence phenotype, rather than involving sexual progeny (Crute et al. 1987; Schettini et al. 1991; Brown et al. 2004). Recent changes in virulence phenotype in California seem also to be somatic (Ilott et al. 1987; Ochoa and Michelmore, unpublished). The molecular genetic changes underlying these changes are unknown, but they are becoming amenable to analysis with the advent of technologies for whole genome analysis.

Genome size and complexity

The physical genome size of *B. lactucae* has been estimated using several methods: comparisons of hybridizations between cloned DNA fragments and genomic DNA in dot blot reconstructions, DNA–DNA reassociation kinetics assayed by hydroxyapatite chromatography, and summation of chromosomal sizes determined by CHEF gel electrophoresis (Francis et al. 1990; Francis and Michelmore 1993). All three methods gave similar estimates of 50 Mb; however, this may be an underestimate. *Aspergillus nidulans* and *Arabidopsis thaliana* were used as controls in the genomic reconstruction experiments and their sizes were estimated to be 17 and 52 Mb, respectively; genomic sequencing has now shown their genome sizes to be 30 and 125 Mb, respectively (Galagan et al. 2005; The *Arabidopsis* Genome Initiative 2000). Therefore the estimate for the genome size of *B. lactucae* should probably be revised upward to approximately 100 Mb. This is consistent with estimates of 70 to 144 Mb, depending on the isolate, measured by Feulgen absorbance cytophotometry (Voglmayr and Greilhuber 1998). This size is comparable to that of *Phytophthora* species that range from 65 Mb for *P. capsici* to 240 Mb for *P. infestans* as well as similar to *Hyaloperonospora parasitica* (75 Mb; Govers and Gijzen 2006). Only sequencing the entire genome of *B. lactucae* will provide an accurate determination of its genome size.

DNA reassociation kinetics indicated that the nuclear DNA of *B. lactucae* is comprised of approximately 65% repeated sequences and 35% low-copy sequences (Francis et al. 1990). The repeat fraction is made up of approximately 21% high-copy sequences and 38% intermediate-copy sequences. Hybridization analysis of random genomic λ clones demonstrated that the low-copy-number sequences are interspersed with repeated sequences.

Regulatory sequences for transformation of *B. lactucae* and other oomycetes

Transformation of *B. lactucae* has yet to be achieved. Early work towards this goal involved the isolation of regulatory sequences from *B. lactucae*. These included the promoters and terminators from *Hsp70* and a constitutively highly expressed single-copy gene, *HAM34* (Judelson and Michelmore 1989, 1990). Although there was evidence for transient expression, no stable transformants of *B. lactucae* were obtained. Efforts were therefore directed towards transformation of culturable oomycetes including *P. infestans* (Judelson and Michelmore 1991). These studies ultimately resulted in the stable transformation of several *Phytophthora* species using vectors originally developed for *B. lactucae* (Judelson et al. 1991, 1993). The function of *HAM34* is still unknown; it is present in *P. infestans* (Win et al. 2005) but not yet evident in the sequence of *H. parasitica*.

These experiments indicated that the transcriptional machinery of oomycetes differs significantly from that of higher fungi but that sufficient similarity exists so vectors developed using regulatory sequences from one oomycete will likely function in other oomycetes (Judelson et al. 1992). It is now time to reinitiate experiments on the transformation of *B. lactucae* using better selectable markers and reporter genes that have become available, as well as novel methods for introducing the transgenes.

(A)virulence effectors

Pathogens have evolved sophisticated mechanisms to alter their hosts' metabolism and interfere with host defences (Jones and Dangl 2006). This is best

understood for Gram-negative bacteria that secrete virulence effector proteins into host cells and the extracellular space (Nomura et al. 2005). Some effectors can trigger defences dependent on specific resistance genes. Some can also block the resistance response elicited by the activities of other effectors (Abramovitch et al. 2003; Espinosa et al. 2003; Jamir et al. 2004; Fu et al. 2007). Such effectors exhibit a dominant inhibitor of avirulence phenotype. The recent availabilities of sequenced genomes of phytopathogenic bacteria, bioinformatic tools, and efficient functional screens have resulted in the identification of numerous genes encoding candidate effectors (e.g. Guttman et al. 2002; Petnicki-Ocwieja et al. 2002; Greenberg and Vinatzer 2003; Chang et al. 2005). It is now recognized that individual strains of phytopathogenic bacteria secrete ~40 effectors into their hosts. Functional studies and the sequences of several effectors suggest that they alter plant defence signalling (reviewed in Grant et al. 2006).

There is increasing evidence that fungi and oomycete pathogens also secrete diverse effector proteins into their hosts (Torto et al. 2003; Birch et al. 2006; Kamoun 2006). Initially avirulence genes have been cloned from *Phytophthora* spp. and *H. parasitica* on a gene-by-gene basis (Tyler 2002; MacGregor et al. 2002; Shan et al. 2004; Allen et al. 2004; Rehmany et al. 2005; Armstrong et al. 2005). Recent studies of avirulence and secreted proteins from *H. parasitica* and *Phytophthora* spp. revealed a novel, highly conserved RXLR amino acid motif (Rehmany et al. 2005). This motif is predicted to be required for translocation from the pathogen to the host (Bhattacharjee et al. 2006) and it was recently shown to be required for translocation of the avirulence protein *Avr3a* by *P. infestans* (Whisson et al. 2007). Bioinformatic analyses have identified hundreds of genes encoding other potentially secreted proteins in the genome sequences of *Phytophthora* spp. (Birch et al. 2006; Tyler et al. 2006).

In order to identify (a)virulence effector proteins in *B. lactucae*, we have generated several cDNA libraries of *B. lactucae* from a variety of sources including conidia, germlings and infected tissue. One subtraction library was made by subtracting mock-inoculated leaf material against heavily infected leaf material. The resulting sequences had a bimodal distribution of GC contents. On the basis of GC content and (dis)similarity to plant or oomycete sequences, sequences were categorized as most likely to be of *B. lactucae* origin (38%), lettuce origin (35%), or uncertain origin (27%). Many of the putative *B. lactucae* unigenes had an average GC content of 50%. In order to obtain more full-length clones, we generated and sequenced a new library that was enriched for *B. lactucae* sequences by hybridizing cDNA from heavily infected leaves to *B. lactucae* genomic DNA using a protocol developed by J. Jones (Sainsbury Laboratory, Norwich, UK). Sequences from all libraries are being analyzed for candidate effectors using several bioinformatics approaches. We are searching for sequence similarity to genes encoding known avirulence proteins and putative secreted proteins from other oomycetes. Candidate effector sequences have yet to be identified; however, this is not surprising as effector proteins may be evolving rapidly. We are also searching for the presence of a secretion signal peptide and the RXLR amino acid motif. These analyses have so far yielded over 15 candidate sequences that satisfied one or more of these criteria. These are currently being assayed for function in lettuce using *Agrobacterium*-mediated transient assays (Wroblewski et al. 2005).

The impact of genomic sequencing

Although *B. lactucae* was ranked as one of the high-priority plant pathogens targeted for sequencing since 2002 (American Phytopathological Society 2006), this has yet to occur. The latest generation of sequencing technologies combined with conventional Sanger sequencing will provide large amounts of sequence information for *B. lactucae*. Sequencing the whole genome will provide an expedient and cost-efficient approach to the identification of effector proteins and other types of molecules involved in determining specificity and mating type. It will also provide targets for disease control strategies as well as provide an important reference genome.

Sequencing of multiple isolates will provide large numbers of single nucleotide polymorphisms that, combined with the new generation of marker technologies, will allow large-scale population analyses for variation in both effector genes and genes involved in other aspects of the pathogen's biology. It is likely that these whole-genome analyses will reveal a variety of mechanisms of variation. It will be particularly interesting to determine the basis of

insensitivity to the fungicides metalaxyl (Ridomil) and fosetyl A (Alliette), as well as the bases for changes in virulence phenotype.

Sequencing the genome will also provide insights into what extent the genome of *B. lactucae* has become streamlined in parallel with its total dependence on its host. Also, it will facilitate the identification of which biosynthetic capabilities appear to be lacking and therefore can be supplemented in media for axenic culture.

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