

A genomics perspective on cucurbit-oomycete interactions

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Abstract *Pseudoperonospora cubensis* and *Phytophthora capsici* are plant pathogenic oomycetes that are severe threats to cucurbit cultivation because of their global distribution, their broad host range among the *Cucurbitaceae* family, and their ability to overcome susceptibilities to host, environment, and chemical management. Historically, these pathogens have been extensively studied in terms of their life cycles and infection strategies in order to determine appropriate methods to manage disease. In recent years, the genomes of both pathogens have been sequenced, which will lead to greater opportunities for pathogen detection and will help researchers to better understand the host-pathogen interaction. In *Ps. cubensis*, the transcriptomes of both *Ps. cubensis* and *Cucumis sativus* (cucumber) have been sequenced, and this data is being analyzed to determine the function of *Ps. cubensis* effectors and the role of alternative splicing in the regulation of pathogen gene expression. Previous and ongoing work is being done to determine cucumber genes involved in resistance. In *P. capsici*, effectors have been identified in the genome sequence, and the genome being used to identify variation in different *P. capsici* isolates. Future work is needed to give biological meaning to genomics data and to determine mechanisms of pathogenicity in oomycetes and resistance in cucurbits. Herein, we will present an overview of the current and future objectives of genome-based research in this area, describing the molecular mechanisms of pathogen virulence and host response to infection.

Key words: *Pseudoperonospora cubensis*, *Phytophthora capsici*, cucurbit, RNA-Seq, genome.

Pseudoperonospora cubensis

History and taxonomy of *Pseudoperonospora cubensis*

Pseudoperonospora cubensis is an obligate biotroph and the causative oomycete pathogen of cucurbit downy mildew, a foliar disease characterized by the development of angular lesions on the leaf surface and by the production of sporangiophores on the underside of the leaf (Lebeda and Cohen 2011; Savory et al. 2011). *Ps. cubensis* was first discovered by Berkeley in 1868 from herbarium specimens originating in Cuba, thus the species name *cubensis*. However, it was not identified on live plants until 1903 in Moscow by Rostovzev (Lebeda and Cohen 2011). Although the taxonomy of this pathogen has varied, it is currently classified in Kingdom Straminipila, Phylum Oomycota, Class Oomycetes, Order Peronosporales, Family Peronosporaceae, Genus *Pseudoperonospora*, Species *Pseudoperonospora cubensis* (Göker et al. 2007; Savory et al. 2011). Like other oomycetes, *Ps. cubensis* is thiamine-dependent, forms haustoria, and is an obligate, meaning that it requires a host for survival (Göker et al. 2007). Similar to other

species in *Peronospora* and *Pseudoperonospora*, it has colored conidia, which may aid in the longevity of sporangia, as they are greater protected from solar radiation during wind dispersal (Göker et al. 2007). *Pseudoperonospora* is distinguished from *Peronospora* because it has porous spore walls, germinates to infective zoospores, and has sporangiophore branches that are at acute angles (Palti and Cohen 1980).

Host range and economic value of *Ps. cubensis*

Since the discovery of *Ps. cubensis*, several isolates have been identified, and recent data suggests that the pathogen is continually evolving; this is evidenced by different genotypes of *Ps. cubensis* observed in various geographic regions of the United States (Lebeda and Widrechner 2003; Quesada-Ocampo et al. 2012). At present, the host range for *Ps. cubensis* includes over 40 species within 20 genera of the *Cucurbitaceae* family, an important group of crops, which includes *Cucumis sativus* (cucumber), *Cucumis melo* (melon), *Cucurbita pepo* (squash), *Cucurbita maxima* (pumpkin), and *Citrullus lanatus* (watermelon) (Lebeda and Widrechner 2003). In the United States, for example, the cucurbit

industry produces \$1.45 billion through 109 million metric tons of cucurbits produced on 229,000 hectares (Cantliffe et al. 2007). Cucumber downy mildew is a major problem for cucumber growers in the United States and abroad. Similarly, in Europe, yield losses as high as 80% have been observed, and outbreaks since the 1980's have been managed by intensive fungicide treatments. In the United States, the threat of *Ps. cubensis* to cucumber production was successfully mitigated by host resistance until 2004; however, this pathogen has since overcome host resistance and is now a severe threat to growers, especially as it also evolves fungicide resistance (Holmes et al. 2006; Savory et al. 2011).

Life cycle and infection stages

To evaluate the threat of *Ps. cubensis* and to develop prevention and treatment plans, it is important to study its life cycle. *Ps. cubensis* is a threatening pathogen because its polycyclic nature—the ability to complete its life cycle multiple times in a growing season—as well as the rapidity of its life cycle, which can be completed in less than a week (Savory et al. 2011). The cycle begins when an airborne sporangia lands on the surface of a leaf and germinates into zoospores (Savory et al. 2011). Several studies have investigated the effects of temperature and leaf wetness on germination and infection, and have found that *Ps. cubensis* prefers slightly cool temperatures (20°C) and higher humidity (Arauz et al. 2010; Neufeld and Ojiambo 2011). After germination, a zoospore encysts on stoma and forms a germ tube which develops into an appressorium, followed by a penetration hyphae that begins colonization of the intercellular spaces of the mesophyll and palisade tissues (Savory et al. 2011). Hyphae are formed throughout infection and are accompanied by haustoria, which are specialized structures at the plant-pathogen interface that allow for the exchange of materials (Savory et al. 2011). Haustoria are the secretion sites for pathogen-associated proteins called effectors, which are involved in manipulating the plant-pathogen interaction in both the apoplast and the plant cytoplasm (Kamoun 2006). Once *Ps. cubensis* has colonized the plant, sporangiophores are formed, and sporangia develop at the end of the branches. These sporangia are then released into the wind, and the cycle of infection continues.

Ps. cubensis* transcriptome and effector analyses in *C. sativus

To better understand the pathogen and its life cycle at a molecular level, previous research has characterized the infection stages using and combination of microscopy and next-generation sequencing (Adhikari et al. 2012; Savory et al. 2012b). To this end, the transcriptomes of *Ps. cubensis* and 'Vlaspik,' a susceptible *C. sativus* cultivar, have been sequenced at 1, 2, 3, 4, 6, and 8 days

post inoculation (dpi) using next generation Illumina technology (Adhikari et al. 2012; Savory et al. 2012b). These 35–42 bp single end reads from the Illumina Genome Analyzer II platform were then mapped to the *Ps. cubensis* genome using Bowtie and TopHat and were processed and quantified using Cufflinks (Adhikari et al. 2012; Savory et al. 2012b). Out of the approximately 23,000 genes in the 67.9 Mb genome of *Ps. cubensis*, 7,821 genes were expressed within the time course (Adhikari et al. 2012; Savory et al. 2012b). The expression of differentially expressed genes was evaluated using the Pearson correlation coefficient, and early, middle and late phases of infection were determined (Adhikari et al. 2012; Savory et al. 2012b). These phases correlated well with the expression of orthologous genes in another oomycete pathogen, *Phytophthora infestans*, especially at 4 dpi during which haustoria formation and pathogen colonization is occurring in both oomycetes (Adhikari et al. 2012; Savory et al. 2012b). In addition to using the Pearson correlation coefficient, the data was also analyzed using the Weighted Gene Correlation Network Analysis in which genes with similar expression patterns over the time course were grouped into representative gene models called eigengenes (Adhikari et al. 2012; Savory et al. 2012b).

Within this set, multiple effectors have been predicted in *Ps. cubensis*; Figure 1 diagrams a mechanism by which these effectors, both apoplastic and cytoplasmic, could be expressed and utilized to the benefit of the pathogen. For example, genes related to the virulence and pathogenicity of *Ps. cubensis* include enzymes that damage the host, including proteases, lipases, and carbohydrate active enzymes (Adhikari et al. 2012; Savory et al. 2012b). In addition, 271 putative pathogen effector genes were identified, many of which were found to have an RXLR-type motif with a varied R1 position (Adhikari et al. 2012; Savory et al. 2012b). The number of effectors predicted in *Ps. cubensis* is within the range of predicted effectors in other oomycetes, including the obligate *Arabidopsis thaliana* oomycete, with 134 RXLR-like effectors and the well-characterized potato late blight pathogen, *Phytophthora infestans*, with 563 predicted RXLR-like effectors (Coates and Beynon 2010; Stassen and Van den Ackerveken 2011). The RXLR motif, an important component of cytoplasmic effectors, has been demonstrated to facilitate effector entry through interaction with phosphatidylinositol-3-phosphate (PI3P) on the plant cell (Jiang et al. 2008; Kale et al. 2010; Stassen and Van den Ackerveken 2011; Whisson et al. 2007). Once delivered into the host cell, the pathogen molecule functions in large part to abrogate host defenses and/or facilitate pathogen survival. While the mechanism(s) and targeting of host defenses by pathogen effectors has made significant strides over the past decade, one area of research whose mechanism remains

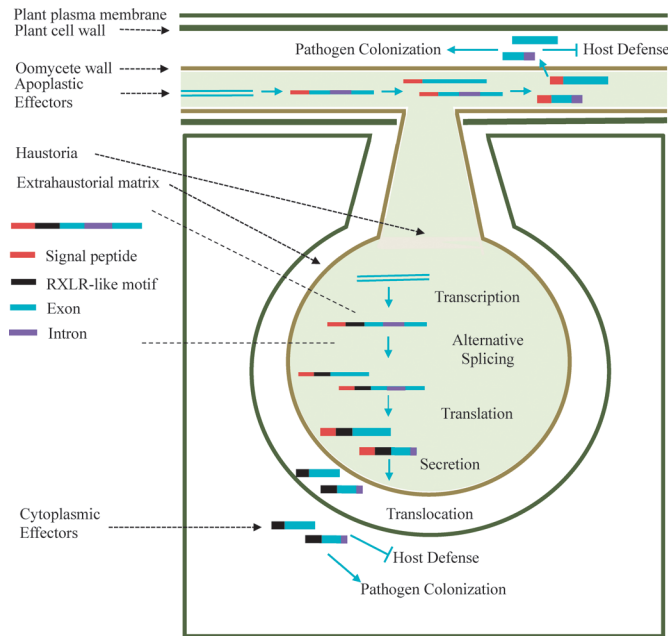


Figure 1. Diagrammatic representation of oomycete infection at site of haustoria invasion of host cell. The molecular mechanism by which oomycete genes are expressed, spliced, translated, and secreted (as denoted by the red signal peptide box) is highlighted. Cytoplasmic effectors, which enter the plant cell, are shown to have an RXLR-like motif, as denoted by the black box. Apoplastic effectors, which are secreted from the oomycete but do not enter the plant cell, are also shown and follow the same pattern of transcription, splicing, translation, and secretion. Both types of effectors function to abrogate host defense responses and promote pathogen colonization.

largely undefined is the process of host entry (Jiang and Tyler 2012). In this regard, recent debate has challenged the role of the RXLR/PI3P-mediated effector entry, and specifically, has proposed the importance of a positively charged patch over the canonical RXLR (Ellis and Dodds 2011; Yaeno et al. 2011). In support of this, research from our group has demonstrated that variation of the translocation-associated RXLR motif, in association with the previously characterized dEER motif, is important for effector translocation into the host (Tian et al. 2011). Mechanistically, it has been determined that the RXLR motif and surrounding amino acids are important in effector dimerization (Wawra et al. 2012). The same study also indicated that the C-terminus, not the RXLR, is able to bind to PI3P; however, this binding was only observed when the protein was denatured, suggesting that this observation is likely not biologically relevant (Wawra et al. 2012). Moving forward, a complete analysis and understanding of the role of the RXLR-motif in effector entry is needed to resolve current debate.

Alternative splicing of *Ps. cubensis* effectors

The transcriptome of *Ps. cubensis* (Savory et al. 2012b) provides insight into the potential roles of identified effectors, given their patterns and levels of gene expression. Models of effectors have gained additional complexity through a recent study showing that effector genes from *Ps. cubensis* can be alternatively spliced, which would hypothetically broaden the potential effector proteome (Savory et al. 2012a). Savory et al.

2012a demonstrated that a gene predicted to be a non-effector multi-drug transporter when the full-length gene was expressed resulted in a functional effector as a result of an alternative splicing mechanism called intron retention, in which a premature stop codon was introduced (Savory et al. 2012a). Previous and ongoing work involves the characterization of alternatively spliced effectors (Savory et al. 2012a). Currently, alternatively spliced isoforms are being cloned, and differences in splicing between *Ps. cubensis* sporangia and *Ps. cubensis* over a time course of infection are being studied using both real-time PCR and Illumina Hi-Seq. In this current research, the phenomenon of alternative splicing in *Ps. cubensis* is being examined on a genome-wide scale and both effector and non-effector transcripts are being analyzed. Thus far, preliminary data indicated that intron retention is the primary mechanism of alternative splicing in *Ps. cubensis*, as suggested in Figure 1.

Transcriptomic changes in response *Ps. cubensis* infection and susceptibility

RNA-Seq data from the previously described experiment (Savory et al. 2012) also has yielded valuable information on the cucumber transcriptome response during infection, in which 14,476 genes were expressed, with 3,286 genes being differentially expressed between the time points collected 1–8 dpi (Adhikari et al. 2012). These genes were grouped into modules using the Weighted Gene Correlation Network Analysis (WGCNA), and groups of genes with similar expression patterns were

identified and represented as eigengenes (Adhikari et al. 2012). This type of analysis allows genes that are highly coexpressed at specific time points to be quickly identified. For example, a group of defense-related cucumber genes, including lipoxygenases, cationic peroxidases, and cinnamate 4-hydrolases were down-regulated at 2 dpi in this susceptible interaction (Adhikari et al. 2012). This is very interesting because it could indicate that the pathogen is actively mitigating host defense responses through diminishing the expression of host defense genes. Other genes involved in defense and stress signaling were expressed throughout the time course—including peroxidases, protease inhibitors, catalases, lipoxygenases, and galactinol synthase—indicating that a susceptible cucumber is capable of eliciting a response to the pathogen, but is likely not sufficient to stop or prevent infection (Adhikari et al. 2012). Present and ongoing work in our lab is currently focused on determining the transcriptome of a resistant *C. sativus* cultivar, PI 197088, over the time course of infection with *Ps. cubensis* in order to determine which genes are differentially expressed at which time points when compared to the susceptible Vlaspiik-*Ps. cubensis* interaction.

Downy mildew resistance in cucurbits

Previous research investigated a susceptible interaction between *Ps. cubensis* and *C. sativus*; however, the genetic interactions between *Ps. cubensis* and resistant *C. sativus* are still unknown. Before 2004, host resistance to downy mildew was conferred by the *dm-1* allele, which is no longer effective (Call et al. 2012; Holmes et al. 2006). After 2004, resistance was investigated in plant introduction (PI) lines and bred varieties which display resistance or reduced sporulation, respectively (Call et al. 2012). Resistance is now hypothesized to be associated with multiple genes in the PI 197088 line, but the identity of these genes remains unknown (Call et al. 2012; Holmes et al. 2006; personal communication, Dr. Todd Wehner, NC State).

In addition, a suppression subtractive hybridization study was done in China using inoculated and non-inoculated samples from the resistant IL-57 line to identify genes up-regulated during infection (Li et al. 2010). This method identified multiple genes, some of which were transcription factors or were related to reactive oxygen species (ROS), but not all of which were involved in defense-associated responses (Li et al. 2010). The same group has identified a heat shock protein that may be involved in downy mildew resistance as well as abiotic stresses, including increased temperature (Li et al. 2012). Recently, a different Chinese group, Zhang et al. 2012, has completed a QTL map for downy mildew resistance from two Chinese inbred cucumber lines and has found 5 QTLs associated with resistance to

downy mildew using simple sequence repeat (SSR) markers (Zhang et al. 2012). These QTLs are located on chromosomes 1, 6, and 5 and provide broad regions associated with downy mildew resistance, leaving further work to determine the specific genes conferring resistance (Zhang et al. 2012).

In contrast to our sparse understanding of DM resistance in cucumber, specific genes conferring downy mildew resistance in melon are known. Taler et al. (2004) found that glyoxylate aminotransferases *At1* and *At2* confer enzymatic resistance genes to downy mildew. These genes were identified in the wild melon PI 124111 and resistance is observed as chlorotic lesions, a massive amount of callose, and phenolics and lignin-like substances in the cytoplasm (Taler et al. 2004). When the *At1* and *At2* genes are transformed into a susceptible melon, they are able to confer resistance and no *Ps. cubensis* sporulation is observed (Taler et al. 2004). The type of resistance conferred by these two genes is unique in that the resistance-associated genes are not the typical R genes, but instead are constitutively highly expressed enzymes (Taler et al. 2004).

Phytophthora capsici

Host range, taxonomy, and symptoms of Phytophthora capsici

Like *Ps. cubensis*, *P. capsici* is a diploid oomycete that infects a wide range of cucurbits, including cucumbers, but its host range also extends to pepper, tomato, snap and lima beans, eggplant, and many others (Granke et al. 2012; Lamour et al. 2011; Quesada-Ocampo et al. 2011). *P. capsici* is compared to *Ps. cubensis* in Table 1. *P. capsici* is classified in the Kingdom Chromista, Phylum Oomycota, Class Oomycetes, Order Peronosporales, Family Peronosporaceae, Genus *Phytophthora*, Species *capsici* (Lamour et al. 2011). The pathogen was first described in 1922 on chili pepper collected in 1918 from a New Mexico Agriculture Experiment Station (Lamour et al. 2011; Leonian 1922). Since then, *P. capsici* has been identified on several continents including North and South America, Asia, Europe, and Africa (Granke et al. 2012). In parts of the United States and Africa, sexual reproduction and oospore formation has been observed; however asexual reproduction is far more common in South America (Lamour et al. 2011). The different climate of the continents on which *P. capsici* is found also affects the symptoms and severity of the disease, in which a greater range of symptoms affecting the whole plant, including leaf infection, damping off of the root, and stunted plant growth are observed in moister areas (Granke et al. 2012; Lamour et al. 2011). In addition, symptoms of *P. capsici* include infected fruit, identified by the appearance of sporangia in late infection stages, and black/brown lesions on the plant (Lamour et al. 2011).

Table 1. Lifecycle, host range, and genomics of cucurbit oomycete pathogens.

	<i>Ps. cubensis</i>	<i>P. capsici</i>
Plant families infected	Cucurbitaceae	Cucurbitaceae, Solanaceae, Fabaceae, and many others
Part of plant affected	Leaves only	Leaves, fruit, stem, root
Dispersal	Wind	Water, soil
Overwintering method	Greenhouses/warm climates in USA; oospores in other countries	Warm climates; irrigation water; oospores in soil are common in USA
Life style	Obligate biotroph	Hemibiotroph
Time to complete asexual life cycle	6–10 days	2–3 days
Infection mechanism	Germinated zoospores encyst on stomata and form appressorium	Germinated oospore, germinated sporangia, or zoospore penetrates plant cuticle
Genome	67.9 Mb; 23,000 predicted genes	64 Mb; 17,123 predicted genes

Life cycle and infection stages

Unlike the obligate biotroph *Ps. cubensis*, *P. capsici* is a hemibiotroph, meaning that it has both a biotrophic and a necrotrophic phase and can also be cultured on artificial media in a laboratory. In addition, unlike *Ps. cubensis*, *P. capsici* regularly forms oospores in the United States, which are a result of sexual reproduction (Lamour et al. 2011). Oospores are formed when two different mating types of *P. capsici* are stimulated to mate and form male and female gametangia, which form haploid gametes that fuse and develop into an oospore (Lamour et al. 2011). These oospores are able to overwinter and allow this pathogen to be a persistent, difficult problem to manage. Asexually, *P. capsici* reproduces through the formation and propagation of sporangia. The infection cycle begins with penetration of the plant cuticle via a germinated oospore, directly germinated sporangia, or a zoospore resulting from a germinated sporangia (Lamour et al. 2011). Once inside the plant, *P. capsici* enters a biotrophic phase in which it colonizes with hyphae and forms haustoria, or feeding structures, but it does not kill the plant tissue (Lamour et al. 2011). Later, the pathogen switches to a necrotrophic phase in which it does kill the plant tissue, which results in tissue collapse (Lamour et al. 2011). Under optimal conditions, these two stages will be completed and the pathogen will sporulate in 2–3 days, and the cycle will continue (Lamour et al. 2011).

P. capsici genome

Previously, genetic data from *P. capsici* has been used to evaluate population structure using amplified fragment length polymorphisms (AFLP) or single-nucleotide polymorphism (SNP) markers of a select group of common genes (Lamour and Hausbeck 2001; Quesada-Ocampo et al. 2011). This type of work allowed for regions of the *P. capsici* genome to be sequenced; however, the full genome was only recently published in 2012 by Lamour et al. In order to sequence this genetically diverse pathogen, a partial inbred line, LT 1534 was developed (Lamour et al. 2012). Arachne was used to assemble the 64 Mb genome using reads from both the 454 and Sanger platforms (Lamour et al.

2012). Following the removal of genes with homology to transposable elements, 17,123 genes were predicted, which is similar to the number of predicted genes in other sequenced oomycete genomes (Lamour et al. 2012). In addition, the structure of the *P. capsici* genome was similar to other oomycete genomes in that gene-rich regions were conserved in blocks, while gene-poor regions contained mostly repetitive sequences and unique sequences which are potentially related to pathogenesis (Jiang and Tyler 2012; Lamour et al. 2012). Among these genes related to pathogenesis are effectors, including the well-characterized RXLR and crinklers (CRN) (Lamour et al. 2012). Extensive effector characterization has not been completed in *P. capsici*; however, a recent study has identified nine pectin methyltransferases (Li et al. 2011).

In addition, restriction-site-associated DNA (RAD) sequencing and single nucleotide variant (SNV) density were used to genotype 65 different isolates of *P. capsici* to generate a genetic map and examine the genotype of each isolate (Lamour et al. 2012). Through this work, a loss of heterozygosity (LOH) was observed in many of the isolates in at least one of the SNV markers used (Lamour et al. 2012). LOH is hypothesized to have significant consequences in *P. capsici*, including changing mating types of an isolate and losing pathogenicity (Lamour et al. 2012).

Resistance to *P. capsici*

Like *Ps. cubensis*, host resistance to *P. capsici* is not common in cucurbits, and the pathogen is largely managed through cultural practices, like proper irrigation and sanitation, and fungicide usage (Granke et al. 2012). In addition, rapid evolution to fungicides and a great diversity of *P. capsici* populations make managing and breeding for resistance to this pathogen difficult (Granke et al. 2012). Some sources of complete resistance have been identified in the Solanaceae family in peppers and tomatoes, but thus far the only complete resistance in the Cucurbitaceae family has been identified in squash (Padley et al. 2009; Quesada-Ocampo and Hausbeck 2010; Thabuis et al. 2003; Thabuis et al. 2004). In squash,

Padley et al. 2009. identified 3 dominant alleles through classical breeding that were found to be involved in conferring resistance to *P. capsici* as observed by an absence of the crown rot syndrome.

In other cucurbits, including cucumber, quantitative, but not qualitative resistance has been observed (Ando et al. 2009; Gevens et al. 2006; Lee et al. 2001). Gevens et al. 2006 developed a detached fruit assay to survey the susceptibility of 480 cucumber cultigens and found that while no cultigens displayed a complete absence of infection, some did show a reduced level of sporulation, supporting the concept of quantitative resistance. Lee et al. 2001 also observed quantitative resistance to nine isolates of *P. capsici* when tested on a wide range of pumpkin cultivars from Korea and Japan. In cucumber and other cucurbits, Ando et al. 2009 demonstrated that the age of the fruit has a role in determining its susceptibility to *P. capsici*, with younger fruits, especially cucumbers, being the most susceptible until they reach full fruit length at about 10–12 days after pollination. In addition, Ando et al. 2009 demonstrated that other cucurbits, zucchini and summer squash, were the most susceptible fruits to *P. capsici* and that cucumbers also displayed differences in susceptibility along the length of the fruit in addition to the age-related variation in susceptibility. Further studies are needed to determine the exact genes involved in qualitative resistance in squash and in quantitative resistance in the other cucurbits, including cucumber and pumpkin.

Oomycete genomics: Moving forward

Given the persistent threat of oomycetes to the production of cucurbits and other crops, more research is needed to better evaluate host resistance and to identify the genetic and molecular mechanisms of resistance. Although cultural practices and fungicides are able to manage *P. capsici* and *Ps. cubensis*, these methods are not completely effective and can still result in yield losses, especially when conditions are favorable for disease. Given the increased instability of the environmental conditions due to climate change, developing crops resistance to oomycetes will be even more important to future cucurbit production. There is a great need for growers, seed companies, applied researchers, and molecular-focused researchers to work together in order to identify sources of resistance in cucurbits and to develop resistant crops. Molecular-based research is important in order to identify the specific genes involved in resistance and the mechanism by which resistance is conferred. This will allow for perfect genetic markers and will enable the future development of genetically modified crops with full resistance to oomycetes.

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