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Identification of effector genes from the phytopathogenic Oomycete *Plasmopara viticola* through the analysis of gene expression in germinated zoospores

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ABSTRACT

Grapevine downy mildew caused by the Oomycete *Plasmopara viticola* is one of the most important diseases affecting Vitis spp. The current strategy of control relies on chemical fungicides. An alternative to the use of fungicides is using downy mildew resistant varieties, which is cost-effective and environmentally friendly. Knowledge about the genetic basis of the resistance to *P. viticola* has progressed in the recent years, but little data are available about *P. viticola* genetics, in particular concerning the nature of its avirulence genes. Identifying pathogen effectors as putative avirulence genes is a necessary step in order to understand the biology of the interaction. It is also important in order to select the most efficient combination of resistance genes in a strategy of pyramiding. On the basis of knowledge from other Oomycetes, *P. viticola* effectors can be identified by using a candidate gene strategy based on data mining of genomic resources. In this paper we describe the development of Expressed Sequence Tags (ESTs) from *P. viticola* by creating a cDNA library from *in vitro* germinated zoospores and the sequencing of 1543 clones. We present 563 putative nuclear *P. viticola* unigenes. Sequence analysis reveals 54 ESTs from putative secreted hydrolytic enzymes and effectors, showing the suitability of this material for the analysis of the *P. viticola* secretome and identification of effector genes. Next generation sequencing of cDNA from *in vitro* germinated zoospores should result in the identification of numerous candidate avirulence genes in the grapevine/downy mildew interaction.

Introduction

Grapevine downy mildew caused by the biotrophic Oomycete *Plasmopara viticola* (Berk. & Curt. *ex.* de Bary) is one of the most important diseases affecting *Vitis* spp. (Viennot-Bourgin 1949). *Plasmopara viticola* attacks all grapevine green tissues, including leaves, berries, tendrils, and shoots. In the absence of control it causes defoliation as well as drying of berries and stalk, leading to important losses of yield (Gessler *et al.* 2011). *Plasmopara viticola* is a heterothallic diploid whose life cycle consists of alternate sexual and asexual phases. The asexual phase is polycyclic and runs over the period of grapevine vegetative growth, while the sexual phase is responsible for the production of overwintering oospores, which represent the primary inoculum for the next season. A typical asexual cycle starts with a zoospore encysting next to stomata and then producing

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a germinative tube that penetrates inside the leaf through the stomata. After forming a vesicle in the substomatic cavity, the pathogen grows in the form of intercellular hyphae, producing haustoria to obtain nutrients from the plant. After a variable incubation time, which depends on environmental conditions, sporulation takes place on the abaxial side of the leaf *via* emission of sporangiophores through stomata. Release of sporangia, where zoospores are formed, marks the start of a new cycle.

Grapevine downy mildew is currently controlled by chemical fungicides. Using downy mildew resistant varieties is a cost-effective and environmentally friendly alternative (Bisson *et al.* 2002). However, since all *Vitis vinifera* cultivars producing quality wines are susceptible to downy mildew, the resistance needs to be introduced through breeding programs from *P. viticola*-resistant species found in the *Vitaceae* (Denzer *et al.* 1995; Staudt & Kassemeyer 1995; Brown *et al.* 1999; Kortekamp & Zyprian 2003; Cadle-Davidson 2008). As a consequence of several breeding efforts, knowledge about the genetic basis of the resistance to *P. viticola* has progressed in recent years (Marino *et al.* 2003; Merdinoglu *et al.* 2003; Welter *et al.* 2007; Bellin *et al.* 2009; Marguerit *et al.* 2009; Blasi *et al.* 2011).

Despite the advances in the characterisation of the resistance genes from the *Vitaceae*, knowledge about *P. viticola* genetics is scarce, and nothing is known about the nature of its avirulence genes. The identification of pathogen avirulence genes is a necessary step to understanding the biology of the interaction and has important implications in the breeding for disease resistance. In fact, identification of avirulence genes has proved useful for the discovery and functional profiling of disease resistance genes, allowing detection of functionally similar genes in different sources of resistance (Vleeshouwers *et al.* 2008). Furthermore, it has been proposed that studying the diversity and expression of putative avirulence genes in pathogen populations may provide valuable information when choosing the most efficient combination of resistance genes in order to achieve durable resistance (Michelmore 2003; Birch *et al.* 2008).

Most Oomycete avirulence proteins known to date belong to the RXLR family of effector proteins. They are small secreted proteins containing a signal peptide and an RXLR (Arg-X-Leu-Arg) motif (Kamoun 2006; Stassen & van den Ackerveken 2011). Based on this information, *P. viticola* avirulence genes can be identified using a candidate gene strategy aimed at finding RXLR effectors by data mining of genomic resources. Unfortunately, public genomic resources of *P. viticola* are very limited. A recent search at EMBL/Genbank databases produced 83 *P. viticola* entries (ten ESTs and 73 core nucleotides), the majority corresponding to sequences of mitochondrial or ribosomal origin. Otherwise, cDNA-AFLP analysis of grapevine infected leaves produced 96 *P. viticola* sequences with an average size of 221 nucleotides (Polesani *et al.* 2008), and Solexa sequencing of cDNA derived from infected grapevine leaves attributed 251 short reads to *P. viticola* (Wu *et al.* 2010). The preliminary step of creation of *P. viticola* genomic resources is therefore required in order to identify putative avirulence genes in the interaction between grapevine and downy mildew.

Expression of Oomycete RXLR effectors is induced upon infection (Schornack *et al.* 2009, and references therein), so effector genes can be found in cDNA libraries from infected tissue at early stages of the interaction. Accordingly, identification of pathogen effectors by data mining of Expressed Sequence Tags (ESTs) derived from infected tissue has been reported for several pathosystems (Bittner-Eddy *et al.* 2003; Catanzariti *et al.* 2006; Cramer *et al.* 2006; Torto-Alalibo *et al.* 2007; Bowen *et al.* 2009; As-sadi *et al.* 2011; Cabral *et al.* 2011). However, this strategy involves sequencing a high number of cDNA clones since, despite the enrichment in putative effector genes, the pathogen biomass in the early stages of infection is still low compared to the plant biomass. An alternative to the use of infected tissues as the source of ESTs is using pathogen zoospores, which can be obtained in considerable amounts without difficulty, thus solving the problem of limited pathogen biomass. *Plasmopara viticola* zoospores are easily obtained by washing off sporangia from infected leaves and the first stages of pathogen development (growth of germinative tubes and vesicle formation) can be reproduced *in vitro* (Riemann *et al.* 2002). Interestingly, the expression of genes putatively involved in pathogenicity has been observed in zoospores from Oomycetes (Judelson & Blanco 2005), opening the possibility of using this material to search for putative effectors.

In this paper we report on the development of ESTs resources for *P. viticola*. We obtained cDNA libraries from both *P. viticola* infected grapevine leaves and *in vitro* germinated zoospores, and evaluated the amount of pathogen sequences present in each library. To explore the suitability of *in vitro* germinated zoospores for the identification of pathogen effectors, we sequenced 1920 cDNA clones and identified 827 *P. viticola* nuclear ESTs. Sequence analysis revealed the presence of 54 ESTs from genes putatively involved in pathogenicity, together with other putative secreted proteins. Based on our results, in-depth analysis of the *P. viticola* transcriptome using next generation sequencing of cDNA from *in vitro* germinated zoospores should result in the identification of numerous candidate avirulence genes in the grapevine/downy mildew interaction.

Materials and methods

Plant and pathogen materials

Seedlings of *Vitis vinifera* cv. Muscat Ottonel were used in this study. Seedlings were grown on stone wool in a greenhouse at 22–19 °C (day–night) and a photoperiod of 16 h of light.

Plasmopara viticola isolate SC was collected from *V. vinifera* Chardonnay in the experimental field of INRA in Colmar (France) and maintained on detached leaves from seedlings of *V. vinifera* cv. Muscat Ottonel.

Sample preparation

Plasmopara viticola-infected material for cDNA library construction was obtained by inoculating detached leaves as described in Peressotti *et al.* (2010). Briefly, leaves were surface-sterilized with bleach, followed by three washes in sterile water. Leaves were inoculated all through their surface with 10 μl-drops of a suspension of 50 000 sporangia/ml, kept in Petri dishes on wet filter paper and incubated in a growing chamber at 21 °C