Applied and Environmental Microbiology

Characterization of Single-Nucleotide-Polymorphism Markers for Plasmopara viticola, the Causal Agent of Grapevine Downy Mildew

F. Delmotte, V. Machefer, X. Giresse, S. Richard-Cervera, M. P. Latorse and R. Beffa

Appl. Environ. Microbiol. **2011, 77(21):7861. DOI: 10.1128/AEM.05782-11.**
Published Ahead of Print 16 September 2011.

Applied and Environmental Microbiology, Nov. 2011, p. 7861–7863 Vol. 77, No. 21
0099-2240/11/$12.00 doi:10.1128/AEM.05782-11

Characterization of Single-Nucleotide-Polymorphism Markers for *Plasmopara viticola*, the Causal Agent of Grapevine Downy Mildew▽

F. Delmotte,[1]* V. Machefer,[1] X. Giresse,[1] S. Richard-Cervera,[1] M. P. Latorse,[2] and R. Beffa[2]

INRA, UMR1065 SAVE, F-33883 Villenave d'Ornon Cedex, France,[1] and Bayer CropScience SA, La Dargoire Research Center, 17/20 Rue Pierre Baizet, BP 9163, F-69263 Lyon Cedex 09, France[2]

Received 9 June 2011/Accepted 4 September 2011

We report 34 new nuclear single-nucleotide-polymorphism (SNP) markers that have been developed from an expressed sequence tag library of *Plasmopara viticola*, the causal agent of grapevine downy mildew. This newly developed battery of markers will provide useful additional genetic tools for population genetic studies of this important agronomic species.

Grapevine downy mildew, a disease caused by the oomycete *Plasmopara viticola*, causes substantial losses of yields in vineyards worldwide. *P. viticola* is an invasive species accidentally introduced into Europe in the late 1870s, probably with American vine stocks used to graft the European varieties that were replenished after the phylloxeric crisis. Today, fungicide treatment is the only available method to control this biotrophic pathogen on *Vitis vinifera*. However, the systematic use of chemicals has led to fungicide resistance in *P. viticola* populations, thereby reducing the efficiency of a growing number of products. To establish long-term management of fungicide resistance in natural populations of *P. viticola*, the underlying evolutionary mechanisms that drive the appearance, propagation, and maintenance of resistance need to be elucidated (2). The use of population genetics allows evaluation of the major determinants of fungicide resistance, i.e., selection, mutation, recombination, genetic drift, and gene flow (7).

Conducting population genetic studies of obligate endoparasites, such as *P. viticola*, requires the development of specific and codominant markers. The species specificity of the marker is especially important, as it allows high-throughput genotyping of isolates directly from sporulating lesions collected from host leaves, avoiding the need for labor-intensive isolate subculture. Eleven microsatellite markers are available to assess the genetic structure within populations of *P. viticola* (3, 9). Among the 11 markers developed, 3 of them were difficult to score and the remaining 8 markers had a low number of alleles (mean number of alleles/locus of 3.7 ± 1.1). Expressed sequence tag (EST)-derived single-nucleotide-polymorphism (SNP) markers that have polymorphisms for point mutations and insertions or deletions provide promising molecular markers for species in which microsatellites are difficult to isolate and which have low levels of polymorphism (5). Confirming this view, SNP markers have already been successfully used to describe the genetic structure of other plant pathogens (6), including downy mildew species such as *Plasmopara halstedii* (4, 8). With the aim of increasing the available number of species-specific markers for *P. viticola*, we present the development of single-nucleotide polymorphisms (SNP) derived from an EST library from Bayer CropScience.

An EST library was constructed using material isolated from 5-day-old infected leaves of *Vitis vinifera* inoculated with a strain of *P. viticola* provided by Bayer CropScience. Total RNA was extracted from the leaf sample, cDNA was synthesized and cloned, and a total of 3,500 reads were generated by Sanger sequencing. Reads were trimmed and assembled using the Staden Package software program, resulting in 438 contigs and 1,887 singletons. Grapevine sequences were discarded by performing BLAST analyses on the *Vitis vinifera* genome (10), and the remaining cDNA sequences were compared to the transcripts of *P. sojae* and *P. ramorum* (12) using TBLASTX and to the Swiss-Prot amino acid database using BLASTX. This led to the unambiguous identification of 974 *P. viticola* sequences.

We screened these 974 *P. viticola* sequences for new specific markers, such as single-sequence repeats (SSRs) and SNPs. First, we "mined" the cDNA/expressed sequence tag (EST) database for potentially polymorphic SSRs by performing an *in silico* search for tandem repeat patterns of ≥10 bp using the sputnik program (http://espressosoftware.com/sputnik/index.html). SSRs of ≤10 bp are likely to be monomorphic in such a plant pathogen species; therefore, we did not include shorter SSRs in this analysis (5). Among the 974 cDNA clones, we found eight sequences that had an SSR of ≥10 bp. Of these, seven sequences were excluded because no primer pairs could be designed around the repeated motif (located at the extremities of the clone sequence). The other SSR was in a sequence that gave a significant BLAST hit with a *Phytophthora* species tRNA gene. This locus was not retained for further genetic analysis because of its mitochondrial origin. Second, we studied polymorphisms of 28 EST sequences with highly significant similarity (E values $< 10^{-20}$) to known protein sequences. We designed primers to screen the sequences for SNPs. We detected polymorphisms using a panel of 42 isolates collected in two different vineyards in France (Latresne in the Bordeaux vineyards, Nîmes in the Côte du Rhône vineyards).

We extracted the DNA from *P. viticola* isolates using freeze-dried infected plant tissue as previously described by Delmotte

* Corresponding author. Mailing address: INRA, UMR1065 SAVE, F-33883 Villenave d'Ornon Cedex, France. Phone: 33-557122642. Fax: 33-557 12 26 21. E-mail: delmotte@bordeaux.inra.fr.

▽ Published ahead of print on 16 September 2011.

TABLE 1. Localization (Loc.) and identity (ID) of the SNP within eight *P. viticola* EST-derived markers

Locus	SNP1		SNP2		SNP3		SNP4		SNP5		SNP6		SNP7	
	Loc.	ID	Loc.	ID	Loc.	ID	Loc.	ID	Loc.	ID	Loc.	ID	Loc.	ID
Pvi1	130	G/A	157	C/T	193	C/T	232	G/A	265	C/T	271	A/C	292	T/A
Pvi2	146	C/T												
Pvi3	32	C/T	131	C/G	150	G/A	173	A/G	269	A/G				
Pvi4	229	C/T	232	C/T	268	G/C	313	C/T	314	C/T	316	T/C	334	C/T
Pvi5	103	C/T	105	G/C	156	T/C	166	T/G	168	A/T	249	G/A	252	T/C
Pvi6	61	G/T	73	G/A										
Pvi12	149	A/G												
Pvi13	68	A/C	196	A/C	199	C/T	235	C/T						

et al. (3). All PCR amplification reactions were carried out in a final volume of 25 μl, containing 10 ng of genomic DNA, 2 mM $MgCl_2$, 150 μM each deoxynucleoside triphosphate (dNTP), 4 pmol of each primer, and 0.2U *Taq* Silverstar DNA polymerase (Eurogentec) in reaction buffer. Thermal cycling was performed under the following conditions: an initial denaturation step of 5 min at 94°C, followed by 38 cycles of 50 s denaturation at 94°C, 50 s annealing at 50°C, and 60 s elongation at 72°C, and a final elongation step of 10 min at 72°C. SNPs were detected by PCR-single-strand conformation polymorphism (PCR-SSCP): conformational differences due to the mutations were revealed on a 6% nondenaturing polyacrylamide gel with migration at 4°C at 10 W overnight. The polyacrylamide gels were silver stained as described by Benbouza et al. (1). Genepop version v4 (11) was used to calculate allelic frequencies, expected and observed heterozygosities, and fixation index (F_{ST}) and to perform exact tests for genotype linkage disequilibrium and deviation from Hardy-Weinberg equilibrium.

Among the 28 sequences evaluated by PCR-SSCP, 17 were polymorphic (61%). However, 9 of these 17 DNA sequences were excluded from further population genetic analysis because they had limited genetic diversity (i.e., an expected heterozygosity lower than 0.1). For each of the remaining markers ($n = 8$), all of the alleles detected by PCR-SSCP were sequenced to determine the exact position of the mutation responsible for the observed polymorphism. The markers gave a total of 34 SNPs, including 24 transitions and 10 transversions (Table 1). The mean (± standard error [SE]) number of alleles per marker was 2.4 (± 0.18), and the frequency of the rarest allele ranged from 0.06 to 0.35, with a mean (± SE) frequency of 0.21 (± 0.1) (Table 2). The expected heterozygosity for each locus ranged between 0.20 and 0.66, demonstrating the presence of genetic diversity in these markers. Pairwise tests revealed that these EST-derived markers were not in linkage disequilibrium. Significant deviation from the Hardy-Weinberg equilibrium was observed for Pvi2, Pvi3, and Pvi5, due to a deficit in expected heterozygotes (Table 2). This might result from the Wahlund effect, i.e., reduction of heterozygosity caused by subpopulation structure. On this basis, the estimated genetic differentiation between the two *P. viticola* populations analyzed was significantly different from zero ($F_{ST} = 0.01$, $P < 0.01$).

Finally, we evaluated the potential use of primers for these

TABLE 2. Characterization of the *P. viticola* markers obtained from an expressed sequence tag library[a]

Locus	Accession no.	Homology	Primers (5′–3′)	Ta (°C)	Size (bp)	n_a	Hetero-zygosity		Hardy-Weinberg		Allelic frequency		
							H_E	H_O	Deficit	Excess	Allele 1	Allele 2	Allele 3
Pvi1	JF897856	Hypothetical protein	F: CCGTGACTCCCTTGTATTCC R: AACGAATAGGGTGCGTAGGA	50	494	2	0.45	0.34	0.17	0.96	0.66	0.35	
Pvi2	JF897857	Ribosomal protein	F: TAAAGGAGGGCAAGATCAGC R: CGATACCAGCCATACCCAAC	50	450	3	0.66	0.25	0	1	0.40	0.33	0.27
Pvi3	JF897858	Hsp 60	F: CTCAGGGCGCAGATCAAT R: CAAATCCGTAGGGTTCATGC	50	299	3	0.54	0.32	0	1	0.63	0.23	0.14
Pvi4	JF897859	Manganese superoxide dismutase	F: CTACATCTCGTCCGAGAAAGG R: ATAGGAATGAGCGGCTGGT	50	366	2	0.20	0.15	0.27	0.98	0.89	0.11	
Pvi5	JF897860	Annexin	F: GAGCATTTGCGCGTTGTG R: CGCAGCTCCTTTCCATATTT	50	278	3	0.55	0.14	0	1	0.61	0.21	0.18
Pvi6	JF897861	HSP 90	F: GGAAGTATTGGACGACAAGGTC R: TAATAGGGTGAAGCGGGTTG	50	200	2	0.45	0.48	0.77	0.52	0.66	0.34	
Pvi12	JF897862	Ubiquitin	F: CTGACGGGCAAGACCATTAC R: GAACACACCAGCACCACACT	50	372	2	0.55	0.42	0.15	0.88	0.40	0.54	0.06
Pvi13	JF897863	Peptidyl-prolyl isomerase	F: CCAAGTCGCAAGCAAGTAAA R: GCGAAAAAGGAAAAATAAGCA	50	638	2	0.33	0.28	0.32	0.93	0.79	0.21	

[a] Polymorphisms were detected using a panel of 42 isolates collected in two different vineyards in France. Summary statistics for the number of alleles (n_a), expected and observed heterozygosities (H_E and H_O, respectively), the probability of heterozygote deficit or heterozygote excess (compared with Hardy-Weinberg proportions), and the frequency of alleles are given for each locus.

SNPs in three closely related oomycetes species. We did not detect cross-amplification of the EST-derived markers in *Bremia lactucae* (lettuce downy mildew), *Phytophthora infestans* (potato late blight), or *Plasmopara halstedii* (sunflower downy mildew).

So far, no nuclear SNPs in *Plasmopara viticola* have been reported in the literature (but see reference 2 for the characterization of mitochondrial SNPs). The EST-derived markers described here, combined with the previously described SSRs, will increase our capacity to study the fine-scale spatial genetic structure of *P. viticola* populations.

This work was supported by Bayer CropScience under research program no. 22000150.

We thank I. Haeuser-Hahn (BCS) for providing the EST library, C. Dutech for valuable comments on previous versions of the manuscript, and S. Ahmed for English corrections.

REFERENCES

1. **Benbouza, H., J. M. Jacquemin, J. P. Baudoin, and G. Mergeai.** 2006. Optimization of a reliable, fast, cheap and sensitive silver staining method to detect SSR markers in polyacrylamide gels. Biotechnol. Agron. Soc. Environ. **10:**77–81.
2. **Chen, W. J., et al.** 2007. At least two origins of fungicide resistance in grapevine downy mildew populations. Appl. Environ. Microbiol. **73:**5162–5172.
3. **Delmotte, F., et al.** 2006. Microsatellite DNA markers for *Plasmopara viticola*, the causal agent of downy mildew of grapes. Mol. Ecol. Notes **6:**379–381.
4. **Delmotte, F., et al.** 2008. Single nucleotide polymorphisms reveal multiple introductions into France of *Plasmopara halstedii*, the plant pathogen responsible of sunflower downy mildew. Infect. Genet. Evol. **8:**534–540.
5. **Dutech, C., et al.** 2007. Challenges of microsatellite isolation in fungi. Fungal Genet. Biol. **44:**933–949.
6. **Feau, N., M. J. Bergeron, D. L. Joly, F. Roussel, and R. C. Hamelin.** 2007. Detection and validation of EST-derived SNPs for poplar leaf rust *Melampsora medusae* f. sp. *deltoidae*. Mol. Ecol. Notes **7:**1222–1228.
7. **Giraud, T., J. Enjalbert, E. Fournier, F. Delmotte, and C. Dutech.** 2008. Population genetics of fungal diseases of plants. Parasite **15:**449–454.
8. **Giresse, G., D. Tourvielle de Labrouhé, S. Richard-Cervera, and F. Delmotte.** 2007. Twelve polymorphic expressed sequence tags-derived markers for *Plasmopara halstedii*, the causal agent of sunflower downy mildew. Mol. Ecol. Notes **7:**1363–1365.
9. **Gobbin, D., I. Pertot, and C. Gessler.** 2003. Identification of microsatellite markers for *Plasmopara viticola* and establishment of high throughput method for SSR analysis. Eur. J. Plant Pathol. **109:**153–164.
10. **Jaillon, O., et al.** 2007. The grapevine genome sequence suggests ancestral hexaploidization in major angiosperm phyla. Nature **449:**463–467.
11. **Rousset, F., and M. Raymond.** 1997. Statistical analyses of population genetic data: new tools, old concepts. Trends Ecol. Evol. **12:**313–317.
12. **Tyler, B. M., et al.** 2006. *Phytophthora* genome sequences uncover evolutionary origins and mechanisms of pathogenesis. Science **313:**1261–1266.