Allelic Diversity of the Population of *Phytophthora infestans* in China

Ying Li and Sanwen Huang
IVF-CAAS, Beijing
China

Ruofang Zhang
Inner Mongolia University
China

Chengzhong Lan
Fujian Academy of Agricultural Sciences
China

Yanli Yang
Yunnan Agricultural University
China

T. van der Lee, G.J.T Kessel and E. Jacobsen
WUR
The Netherlands

Guanghui Jin
Heilongjiang August First Land Reclamation University
China

Zhijian Zhao
Yunnan Agricultural Academy
China

S. Kamoun
Sainsbury Laboratory
UK

Keywords: late blight, avirulence, allele mining, population genetics

Abstract

Introduction of resistance genes from wild *Solanum* species into potato cultivars is considered the most promising and environmentally safe approach to achieve late blight resistance. An R-gene stacking breeding program using cisgenesis is planning to trial its products in China. To adapt this approach to local conditions, we propose to assess the allelic diversity of known avirulent genes of *P. infestans* from the intended introduction regions of the GM-potatoes in China. So far, we have a large (~100 isolates) and geographically diverse collection of *P. infestans*. We measured diversity in the isolates based on sequences and functions of several known *Avr* genes. The work relied on molecular and modern computer tools to examine the dynamics of the pathogen population structure and evolutionary relationships among different genotypes. We aim to give an ecological assessment of the Chinese population of *P. infestans*, which would be a guideline for the release of cisgenetic plants with stacked R genes.

INTRODUCTION

The introduction of resistance genes from wild *Solanum* species into potato cultivars is considered the most promising and environmentally safe approach to achieve late blight resistance. Cisgenesis is the genetic modification of a recipient plant with a natural gene from a crossable (sexually compatible) plant (Jacobsen and Schouten, 2007; Schouten et al., 2006). Although transgenesis and cisgenesis both use the same genetic modification techniques, cisgenesis involves only genes from the same species or from a close relative, and these genes could also be transferred by traditional breeding techniques (Schouten et al., 2006). Therefore, cisgenesis is similar to traditional breeding to some extent; however, it overcomes the incompatibility bottlenecks of traditional breeding. For instance, cisgenesis is a particularly efficient method for cross-fertilizing heterozygous plants that propagate vegetatively, such as potato. The *R3a* resistance gene was cloned from *Solanum demissum* (Huang et al., 2005a). In the meantime, more resistance (R) genes from other wild potato species have been cloned (Armstrong et al., 2005). These natural resistant genes would enable us to use cisgenesis to render existing susceptible elite potato varieties resistant by stacking cloned R genes.

A number of wild potato species, such as *S. demissum*, coevolved with *Phytophthora infestans*, and have provided the primary germplasm for breeding late blight resistance in cultivated potatoes. At least 11 R genes that originated from *S. demissum*

have been identified for use in potato breeding. All of these 11 R genes confer race-specific hypersensitive resistance. Potato cultivars possessing such R genes are not resistant to all races of the pathogen. These race-specific R genes provide only short-lived resistance in the field as new virulent races of the pathogen rapidly overcome the resistance encoded by single race-specific resistance genes. Gene stacking is a term that is used in the context of genetically modified crops, but is not a new idea in plant breeding. Gene stacking is the combination of desired traits into one line. Theoretically, by stacking resistant genes, durable resistance could be achieved. Biotechnology is the quickest and easiest way to stack genes. However, the major limitation is the number of genes and the combinations of genes that can be stacked into a crop plant. Late blight has been the most important disease of potato on a worldwide basis and it is very destructive in China. We propose to stack R genes of late blight into potato varieties and to test them in the field in China. The overarching purpose is to help solve the food crisis problem in an environmentally friendly way.

The resistance response of *Solanum* to *P. infestans* is determined by the interaction of resistance proteins with effector proteins secreted by *P. infestans*. A genetic model for the interaction of resistant gene and avirulent gene (R-AVR) is based on a gene-for-gene hypothesis. The recognition event involving the products of the R and AVR genes triggers host defense responses, including a localized host cell death or hypersensitive response (HR) that limits the spread of the pathogen from the infection site. AVR products are diverse, and quickly selected under the selection pressure for a functional effector. The high degree of diversity of AVR products has made it difficult to manage disease through resistant breeding. To inspect and foresee the process of AVR evolution, we need to understand that the antagonistic relationship between R and AVR genes results in a coevolutionary process (Dodds et al., 2006). *P. infestans* is notorious for its ability to change in response to R genes, and such adaptive changes may be accelerated by the genome plasticity of *P. infestans* (Jiang et al., 2006; Liu et al., 2005). We identified the plasticity on sequences and functions of several known AVR genes among Chinese isolates. We will use molecular tools to examine the dynamics of the pathogen population structure and the evolutionary relationships among the genotypes. To adjust approaches to local conditions, it is necessary to understand the allelic diversity of known avirulent genes of *P. infestans* in the intended introduction regions of GM-potatoes. We aim to give an ecological assessment of the *P. infestans* population in China, which would serve as guidelines for the release R-gene stacked cisgenetic plants.

MATERIALS AND METHODS

Isolates and Specific Effector Primers

The isolates used were: 24 from Inner Mongolia, 15 from Northeast China and 10 from Fujian. Reference isolates from the Netherlands were generously provided by Dr. Francine Govers and Dr. Vivianne G.A.A. Vleeshouwers. The avirulent genes used are *INF1*, *Avr3a*, *Avrblb1* and *Avrblb2*. The specific primer sequences and vector controls (pGR106) were provided by Dr. Sophien Kamoun and Dr. Vivianne G.A.A. Vleeshouwers (Table 1).

Isolates and DNA Extraction

Isolates used in this study were chosen to represent the known range of geographic locations and hosts of this pathogen. Isolates were grown in rye medium on a rotary shaker at room temperature (RT) for approximately 7 days. Genomic DNA was isolated from 20 mg of lyophilized mycelium using the PUREGENE DNA isolation kit and protocol (Gentra, Minneapolis, MN) and eluted in 50 µl ultra-pure water. DNA extracts were stored at -20°C.

Allelic Diversity of Avirulent Genes

PCR reactions were carried out in a 15-µl reaction system, containing ~100 ng

DNA, 2.25 pmol of each primer, 3 mM of each dNTP, 0.6 units Taq-poplymerase, 10 mM Tris-HCl (pH 9), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100 and 0.01% (w/v) gelatine. The PCR protocol started with 5 min at 95°C. The 35-cycle amplification profiles were as follows: 30-sec DNA denaturation at 94°C, 40-sec annealing and variable elongation (depending on the primers, Table 1) at 72°C. The PCR was finalized by an extra 5-min elongation step at 72°C.

RESULTS

Allelic Diversity of Avirulent Genes

In our work, four known effectors were studied, *INF1*, *Avr3a*, *Avrblb1*, and *Avrblb2*. *INF1* is an elicitin of *P. infestans* inducing host cell death. It has been reported that the INF1 gene is highly conserved and functions in a cell death-induced pathway. Within all isolates tested, no differences were found in *INF1* nucleotide sequences. Our results indicate that the *INF1* gene is conserved in *P. infestans* and that it plays a key role in the cell death pathway. The *Avr3a* gene has two alleles, $Avr3a^{KI}$ and $avr3a^{EM}$ (Table 2, positions 239 and 310, respectively). So far, all isolates tested were heterozygous at the *AVR3a* locus. This means that potato varieties with *R3a* gene will not be resistant to *P. infestans* in China. There were other differences in sequences: both isolates 99018 and 428-2 had a special "X" amino acid sequence (Table 2). EM/KI positions of 99018 were consistent with published information; the isolate had two alleles and was avirulent on R3 (Huang et al., 2004, 2005b). The isolate 428-2 was virulent on *R3* (Huang et al., 2004, 2005b) and only had $avr3a^{EM}$ in our sequence information. It can be concluded that the special SNP in isolates 99018 and 428-2 was not functional, and could be ignored in our further research. The isolate 82001 was a questionable strain. It had two alleles at the locus, but its race test showed compatible interaction with the *R3* differential.

Little has been published on *Avrblb1* and *Avrblb2* genes. *P. infestans* has two *ipiO* genes, *ipiO1* and *ipiO2*, which are closely linked and located in an inverted orientation. From the sequence information (provided by Dr. Sophien Kamoun), *ipiO1* is an avirulence gene, and can be referred to as *Avrblb1*. After analyzing all sequences of the Chinese collection, both *ipiO1* and *ipiO2* loci were found within all isolates, but none were found similar to the virulent allele (*avrblb1*, Table 3).

The two known genes, *Avrblb2.1* and *Avrblb2.2,* are closely located in *P. infestans* (Sophien Kamoun, pers. commun.). Unfortunately, the recessive allele of *AVRblb2* is not known. After comparison of sequencing data (Table 4), there were several unique SNPs between *Avrblb2.1* and *Avrblb2.2*. Chinese isolates shared both SNPs and represented many novel polymorphic sites (Table 4), suggesting that Chinese isolates possessed the structures of *Avrblb2.1* and *Avrblb2.2* and also had several unique amino acids all of which were only found in Fujian isolates.

DISCUSSION

According to sequence differences of virulent and avirulent genes, it is not surprising why some R genes have durable resistance and others not. The sequence differences probably affect evolution of pathogen fitness. The virulent allele of *AVRblb1* is rare (personal contact with Dr. Sophien Kamoun) in nature, and it was absent in our *P. infestans* collection. The *Rpi-blb1* gene apparently confers broad-spectrum resistance to *P. infestans* as virulent alleles have not yet been found. Another explanation for the durable resistance of *Rpi-blb1* is that potato varieties in China do not have parental background from *S. bulbocastanum* from which the *Rpi-blb1* gene was cloned. The isolates from Chinese fields have not undergone any selection pressure for virulence on the *Rpi-blb1* gene. The durable resistance of the *Rpi-blb1* gene and the fitness penalty of virulence are important aspects in developing a strategy for resistance breeding in potato .

Literature Cited

Armstrong, M.R., Whisson, S.C., Pritchard. L. et al. 2005. An ancestral oomycete locus

contains late blight avirulence gene Avr3a, encoding a protein that is recognized in the host cytoplasm. Proc. Natl. Acad. Sci. USA 102:7766-7771.
Dodds. P.N., Lawrence, G.J., Catanzariti, A.M. et al. 2006. Direct protein interaction underlies gene-for-gene specificity and coevolution of the flax resistance genes and flax rust avirulence genes. Proc. Natl. Acad. Sci. USA 103:8888-8893.
Huang, S., van der Vossen, E.A., Kuang, H. et al. 2005a. Comparative genomics enabled the isolation of the R3a late blight resistance gene in potato. Plant J. 42:251-261.
Huang, S., Vleeshouwers, V.G.A.A., Werij, J.S. et al. 2004. The R3 Resistance to *Phytophthora infestans* in Potato is Conferred by Two Closely Linked R Genes with Distinct Specificities. Molecular Plant-Microbe Interactions 17:428-435.
Huang, S., van der Vossen, E.A.G., Kuang, H. et al. 2005b. Comparative genomics enabled the isolation of the *R3a* late blight resistance gene in potato. The Plant Journal 42:251-261.
Jacobsen, E. and Schouten, H.J. 2007. Cisgenesis strongly improves introgression breeding and induced translocation breeding of plants. Trends Biotechnol. 25:219-223.
Jiang, R.H., Weide, R., van de Vondervoort, P.J. and Govers, F. 2006. Amplification generates modular diversity at an avirulence locus in the pathogen Phytophthora. Genome Res. 16:827-840.
Liu, Z., Bos, J.I., Armstrong, M. et al. 2005. Patterns of diversifying selection in the phytotoxin-like scr74 gene family of *Phytophthora infestans*. Mol. Biol. Evol. 22:659-672.
Schouten, H.J., Krens, F.A. and Jacobsen, E. 2006. Cisgenic plants are similar to traditionally bred plants: international regulations for genetically modified organisms should be altered to exempt cisgenesis. EMBO Rep. 7:750-753.

Tables

Table 1. Overview of specific effector primers used to assess diversity in a sample of isolates of *Phytophthora infestans* from China.

Gene	Forward/ Reverse	Sequence (5-3)
INF1	INF1-F	CGCATCGATGAACTTNCGNGCTCTGTTCGCTGC
	INF1-R	GATGCGGCCGCTNANAGNGACGCNCACGTAGACGAGAA
AVR3a	AVR3a-F	GAAAATCGATGGACCAAACCAAGGTCTTGGTG
	AVR3a-R	'GGACTAGTCAGTGAGCCCCAGGTGCATCAGGTA
AVRblb1	AVRblb1-F	CCATCGATGGTTTCATCCAATCTCAACACCGCCG
	AVRblb1-R	GATGCGGCCGCTATACGATGTCATAGCATGACA
AVRblb2	AVRblb2-F	CGCATCGATGTTCCCAATCCCCGACGWGTCTCGC
	AVRblb2-R	GATGCGGCCGCGTCTACCCCTTTCTCGAAGTCGTA

Table 2. Alignment of *AVR3a* DNA and protein sequences.

		83	239	310	363	370	416
DNA	AVR3a	C	A	T	T	C	A
	Avr3a	C	G	G	T	C	C
	Chinese[1]	T	G	G	T	C	C
	99018	T	A/G	T/G	T/C	C/G	C
	428-2	T	G	G	T/C	C/G	C
	82001	T	A/G	T/G	T	C	C
Protein	AVR3a	L	K	I	L	R[2]	L
	avr3a	L	E	M	L	R[2]	M
	Chinese[1]	L	E	M	L	R[2]	M
	99018	L	K/E	I/M	L	R[2]/G	M
	428-2	L	E	M	L	R[2]/G	M
	82001	L	K/E	I/M	L	R[2]	M

1. One summed-up sequence from Chinese collection.
2. Nucleotide ambiguity code R is A & G; K is T & G; Y is C & T; S is G & C; X is unknown.

Table 3. Alignment of *AVRblb1* protein sequences.

	3x	46	7X	87	9X	117	122	12X	128	129	134	135	143
ipiO1	V	N	S	L	G	S	R	L	H	L	A	S	K
ipiO2	V	N	S	L	G	L	I	L	H	L	G	S	N
Chinese isolates	V	N	S	L	G	X	X	L	H	L	X	S	X
H30P04	X	X	X	X	X	X	X	L	X	L	X	S	X
RD6-39-6-avrblb1	G	N	A	L	A	S	A	Y	H	P	A	G	N

Table 4. Alignment of *AVRblb2* protein sequences.

	28	40	43	47	62	68	69	70	76	78	84	88	95
Avrblb2.1	V	V	P	V	G	V	V	Q	V	K	G	E	A
Avrblb2.2	V	V	P	I	G	V	A	Q	V	R	S	A	A
Chinese isolate	X	X	X	X	X	X	X	X	X	X	X	X	X