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学术园地

Inheritance and Molecular Mapping of New Genes Conferring Late Blight and CPB Resistance in Mexican Wild Potato Species *Solanum pinnatisectum*

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Abstract： Late blight (*Phytophthora infestans*) and Colorado potato beetle (CPB) are the most disastrous disease and insect problem of potatoes. Development of high levels of late blight and CPB resistant potatoes has become a high priority for the Canadian potato breeding program. New genes for late blight and CPB resistance have been identified in a wild 1EBN diploid Mexican species *Solanum pinnatisectum* which has been successfully transferred via protoplast fusion to the cultivated potato background. However, development of new potato cultivars with late blight and CPB resistance has been a difficult and cumbersome task. To accelerate the isolation of the resistant genes, molecular DNA markers tightly linked to the resistance are needed. In this study, a susceptible diploid potato *S. cardiophyllum* was selected as the male parent to cross with *S. pinnatisectum*. The F_1 and BC_1 populations were assessed for resistance to late blight and CPB by the detached leaf method. Disease test using US-8/A2 mating type isolate revealed that all of the F_1 individuals were resistant to the late blight. The ratio of late blight resistant plants to susceptible plants was 1 : 1 in BC_1 populations. The results confirmed that a single dominant gene *Rpi1* for late blight resistance was present in *S. pinnatisectum*. For CPB resistance, a 1:3 resistant to susceptible ratio in BC_1 populations confirmed polygenic inheritance. Molecular marker analysis combined with bulked segregating analysis (BSA) was carried out in the F_1 and BC_1 populations for fine mapping late blight resistant gene *Rpi1* in *S. pinnatisectum*. Twenty seven specific PCR primers were designed from sequence information of EST and STS markers located between RFLP markers TG20A and CP56 on potato chromosome VII. Several new DNA markers showed the linkage relation with the late blight resistant gene *Rpi1*. The analysis of a large number of individual plants from BC_1 populations indicated that two markers S1c9 and GP127~300 on chromosome VII are flanking the late blight resistant gene *Rpi1* with a genetic distance of 1.17 cM and 3.89 cM, respectively. These markers were used to screen two bacterial artificial chromosome (BAC) libraries. Several BAC clones 90~125 kb in size linked with late blight resistance were isolated which will be used to isolate late blight resistance genes through the map-based cloning strategy. Development of molecular matkers closely linked with CPB resistance is on going.

Key Words：molecular mapping; late blight resistance gene; Mexican wild species; *Solanum pinnatisectum*

Potato (*Solanum tuberosum* L.) is the fourth most important food crop in the world after wheat, maize and rice[1]. Late blight and Colorado potato beetle (CPB) are the most devastating disease and insect pest in potato production. They can cause severe losses to the potato producer[2]. Colorado potato beetle can reduce yields by 30%~50%[3]. For late blight, under suitably wet or humid conditions, the disease can spread rapidly within the crop, resulting in defoliation and plant death, and yield losses can reach 100%. Tubers can also be infected. Storage of diseased tubers can result in infection of other tubers and cause extensive crop loss. Control of the disease and the insect pest mainly depend on the extensive

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use of chemical pesticides. However, pest resistance to insecticides, food safety, and environmental concerns have increased interest in alternative methods of disease and insect control. The development of new cultivars with high levels of resistance to both late blight and CPB, or even moderate resistance to the disease and the insect, would significantly benefit potato production. Recently, several new resistance genes were identified in Mexican wild diploid species, such as *S. bulbocastanum* and *S. pinnatisectum*[4-6]. *S. pinnatisectum* Dunal (2n = 2x = 24, 1EBN) is a wild Mexican diploid 1EBN potato species, which carries high levels of resistance to late blight and CPB[6,7]. The transfer of useful resistance genes from *S. pinnatisectum* to another potato gene pool has significant value in proving genetic and molecular marker information on late blight and CPB resistance traits. Incorporation of these genes into commercial potato cultivars would greatly enhance the breeding program and provide significant advantages to both the producers and processors, such as a reduced requirement for chemical treatment of tubers, reduced losses associated with disease and insect control and less tuber desiccation. However, conventional selection of disease and insect resistance clones is very slow and time consuming since it involves screening thousands of clones in the greenhouse and in the field for up to 6 months in order to identify the few resistance clones.

Molecular techniques provide the opportunity to develop DNA markers closely linked to the genes that control a particular trait which can then be used to rapidly and precisely identify clones that possess the trait. Development of DNA markers for disease and insect resistance would reduce the time required to produce resistant cultivars by several years and considerably increase breeding efficiency. Markers may be used to develop marker assisted selection assays that reduce the time required for screening advanced breeding lines from months to days. Markers also represent a starting point for positional cloning of the disease and insect resistance genes to facilitate insertion of the gene into existing varieties of various crops. Isolated genes may be modified to improve the level of resistance to the diseases that the resistance is effective against. This study contributes to the genetic analysis and molecular mapping of the resistance gene to late blight and CPB in *S. pinnatisectum* in order to clone these resistant genes by a map-based cloning approach.

1 Materials and Methods

1.1 Plant materials

A susceptible diploid potato *S. cardiophyllum* (*S. cph*) was selected as the male parent to cross with *S. pinnatisectum* (*S. pnt*). The resistant F_1 plants were back crossed with susceptible *S. cph* in order to generate diploid progenies segregating for resistant genes to late blight and CPB. F_1 and BC_1 populations were maintained vegetatively as in vitro tissue culture plantlets and tubers since their first propagation as seedlings. About 900 lines from backcross populations were selected to analyse the genetics of resistance to late blight and CPB by using detached leaf methods.

1.2 Detached leaf assay for evaluating late blight resistance

Inoculum was prepared from P1801C.16 of *Phytophthora infestans* (US-8/A2 mating type). The isolate was cultured on Rye-A medium[8] to produce sporangia in the dark for 12 days at 10°C. Then sterile distilled water was added into the cultured isolate, and mycelium was stirred using a sterilized glass rod and filtered through two layers of cheesecloth. The sporangial suspension was placed at 8~12°C for 2~3 h to induce the release of zoospores. The inoculum was diluted with distilled water to a final concentration of 30 000 sporangia per ml using a haemocytometer at 100 × magnification.

A detached leaf assay was used for the late blight test. Young plantlets in tissue culture tubes were transplanted into Root-trainer (RT), and cultured in a Growth Cabinet (GC) for 30 d. Seedlings were transplanted from RT into pots, and then grown in a greenhouse for 1~2 months. Two compound leaves were cut for the late blight test, which were selected from the top 3rd~ 5th leaf on each plant's main branch. Each compound leaf with 5 leaflets was inserted into the prepared moist vermiculite

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in a plastic tray that was prepared by adding 800 g of vermiculite and 2 L dd H_2O. Inoculum was sprayed on the surface of all leaflets. Finally, the plastic tray was covered with white plastic, and the sides were sealed with sticky tape. Trays with inoculated compound leaves were incubated in the growth cabinet at18 /6h and 20/18℃ day/night regime for about 15 days. Plant resistance evaluations were performed after 8 and 15 days.

1.3 Bioassay for CPB resistance.

Adult beetles used for the CPB test were obtained from a single, summer generation, from the experiment fields at the Lethbridge Research Centre, Alberta, Canada. A choice test bioassay was used for screening F_1 and BC_1 progenies for resistance to CPB consumption[9]. In this study, resistance percentage of the test lines over the control was measured using the formula $R = 100 \times [C/(C+L)]$, where C and L indicated the amount of damage to the control and test line respectively in each petridish.

1.4 Development of molecular markers linked with late blight resistance

DNA for marker analysis was extracted from 2 g of leaves for each potato plant by a SDS method and was purified by phenol and chloroform. DNA was quantified by Smart Spec™ Plus Spectrophotometer (Bio-Rad), and qualified by electrophoresis on 0.8% agrose gel. 100 ng·μL^{-1} DNA stock solution and 10 ng·μl^{-1} DNA working solution were prepared for each parent and BC_1 plant. Fifteen resistant bulks (Rb) were made by bulking 10 resistant plants; and 18 susceptible bulks were made by bulking 10 susceptible plants in each bulk.

Forty five pairs of SSR and specific PCR primers on potato Chromosome VII and VIII were searched and synthesized from 25 SSR markers and from sequence information of EST and STS markers located between TG20A and CP56 on Chromosome VII (PoMaMo Database). All of these primers were screened against resistant and susceptible parents, resistant F_1 hybrid and resistant and susceptible bulks. If polymorphisms existed between two bulks, further certification was then done by screening individual resistant and susceptible plants.

For CAPS marker analysis, fifteen restriction endonucleases (*Mse* I, *Eco*R I, *Eco*R V, *Hin*d III, *Bam*H I, *Dra* I, *Alu* I, *Hin*f I, *Rsa* I, *Tas* I, *Taq* I, *Hha* I, *Hae* III, *Vsp* I, *Bst*U I) were used to digest PCR products amplified from S. pnt1, S. cph.1 with loci specific primers GP127, TG216R, TG216F, CT54, Ppc, RGA, C2_At3g14910 and C2_At3g15290 respectively.

PCR amplification reactions were carried out in 25 μl reaction mixtures containing 10 mM TRIS-Cl, pH8.3, 50 mM KCl, 2 mM $MgCl_2$, 100 μM of each dNTP, 200 nM primers, approximately 20 ng template DNA and 1 Unit Taq DNA polymerase (Fermentas, Canada). An Eppendorf Mastercycler gradient was used for the PCR. For SSR, the cycling program consisted of an initial 4 min denaturation step at 94℃, followed by 36 cycles of 94℃ (30 s), 53℃ (30 s), 72℃(50 s), and a final 5 min extension step at 72℃. For specific PCR, the cycling program consisted of an initial 2 min denaturation step at 94℃, followed by 3 cycles of 94℃ (1 min), 50℃ (1 min), 72℃(2 min), then by 36 cycles of 94℃ (30 s), 50℃(30 s), 72℃(1 min), and a final 5 min extension step at 72℃. PCR products were size-separated on a 3% agarose gel (SSR) or 1.7% agarose gel (specific primers), and stained with ethidium bromide and visualized on a Gel Imaging system (Bio-Rad).

2 Results

2.1 Genetic analysis of resistant gene for late blight in *S. pinnatisectum*

Nineteen out of twenty F_1 lines produced from *S. pnt* × *S. cph* were highly resistant to late blight (Table 1). Nine hundred and thirty one plants from BC_1 populations derived from a resistant F_1 plant backcrossed with the susceptible parent *S. cph* were tested for resistance to late blight using the detached leaf method. The results showed that 440 plants were resistant and 491 plants were susceptible to late blight (Table 1). The ratio between resistant and susceptible plants was 1 : 1 in the BC_1 populations. It confirmed that a single dominant gene *Rpi1* for late blight resistance was present in *S. pinnatisectum*.

2.2 Genetic analysis of resistant gene for CPB in *S. pinnatisectum*

Colorado potato beetle resistance was also assessed in F_1 hybrids and BC_1 populations. Nine out of twenty F_1

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hybrids were resistant to CPB. Segregation ratios of resistant to susceptible plants was 1∶3 in the BC_1 populations (Table 1). Using the mean resistance percentage data at 8 h after putting beetles in the dish, out of the 345 backcross lines, 81 lines showed high or moderate resistance over the control, and 264 lines showed similar or moderate susceptibility over the control. The Chi-square test supported a significant 1∶3 ratio in this back crossing population, indicating the presence of resistant and susceptible genes in the heterozygous condition within the parents. This observation indicates that resistance in *S. pinnatisectum* to CPB may be controlled by two independently segregating genes having two respective loci, acting in a complementary epistemic manner.

Table 1 The results of late blight and CPB test in F_1 and BC_1 populations

Item		Total plants	Resistance	Susceptible	R/S ratio
Late blight	F_1	20	19	1	19:1
	BC_1	931	440	491	1:1
CPB	F_1	20	9	11	1:1
	BC_1	345	81	264	1:3

2.3 Molecular marker linked with late blight resis-tance

After screening twenty five pairs of SSR primers on potato Chromosome VII and VIII, a chromosome VII primer S041 was found flanking gene *Rpi1* at a genetic distance of 20 cM. This result confirmed that gene *Rpi1* was located on Chromosome VII.

Among twenty locus specific PCR primers, marker S1c9 showed polymorphisms not only between two parents (resistant and susceptible), but also between resistant and susceptible bulks (Figure1). Through further screening by PCR using DNA samples from individual resistant and susceptible plants, thirteen recombinants were found in the segregating populations. From combination of a marker GP127 and different restriction endonucleases, a 300 bp fragment was observed to be linked with late blight resistant gene at a genetic distance of 1.17 cM . Based on these results, a genetic map of late blight *Rpi1*-encompassing region can be constructed on chromosome VII. The gene *Rpi1* located between specific marker S1c9, CAPS marker GP127-300 and At3g at the distance of 3.89, 1.17 and 2.99 cM, respectively.

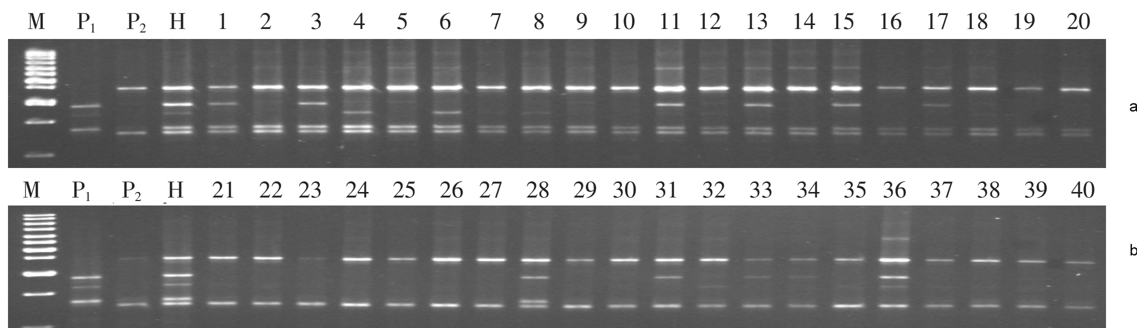

Figure1 PCR profile of S1c9 in two parents, resistant individuals (a) and susceptible individuals (b) of BC_1 population.

M: 1kb DNA Ladder; P_1: resistant parent; P_2: susceptible parent;
H: hybrid of $P_1 \times P_2$; 1-20: resistant individuals; 21-40: susceptible individuals.

3 Discussion

The production of sexual intra- and inter-specific hybrids and various diploid potato genetic stocks is the first step in the transfer of useful resistance genes from *S. pinnatisectum* to another potato gene pool, and has significant value for marker development. Previous research indicated that the Mexican species *S. pinnatisectum* is very difficult to hybridize through sexual inter-species crossing. Hybridization between *S. pinnatisec-*

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tum and *S. cardiophyllum* was only successful when *S. pinnatisectum* was used as the female parent. However, hybrid F_1 is easily backcrossed with both parents: *S. pinnatisectum* and *S. cardiophyllum*.

The availability and production of a large number of backcross (BC) generations provides a unique genetic stock at the diploid level for genetic and molecular study for late blight and CPB resistance[10]. Natural plant populations and breeding populations of crop plants show qualitative and quantitative phenotypic variation for resistance to pests and pathogens. Qualitative resistance is characterized by two distinct phenotype classes, resistant and susceptible, and follows Mendelian inheritance. In contrast, quantitative resistance is characterized by continuous phenotypic variation ranging from high susceptibility to high resistance among the recombinant individuals within a progeny. Such resistance is controlled by more than one gene. In case one gene controls resistance to late blight, the segregation of a back cross population would be 1∶1. If it is two genes that control resistance, the segregation of a backcross population would be 1∶3 [11]. The disease analysis of the back cross populations confirmed that the late blight resistance is controlled by one gene while CPB resistance was controlled by two genes. Quantitative trait loci (QTL) associated with late blight resistance has been mapped in the potato. However, late blight resistance in *S. pinnatisectum* does not appear to be due to a large number of QTLs on different chromosomes. The quantitative resistance for CPB has been observed in the BC_1 populations. These observations indicated only one major chromosomal region that associates with late blight resistance on chromosome VII, while CPB resistance may be controlled by two genes.

Incorporating multiple resistance genes into a single genotype may be accomplished through the use of a combination of different components of late blight and CPB resistance from wild species with other sources found in cultivated potato. Because the introgression of genes from wild species to cultivated potato requires great effort, the use of genotypes that have multiple resistance genes as parents for the crossing would be advantageous. The availability of *S. pinnatisectum* late blight and CPB resistance genes, along with other recently identified resistance genes in some North American potato cultivars and breeding lines may enable breeders to develop cultivars with resistance to late blight and CPB through gene pyramiding[12-14]. The basic genetic architecture of late blight and CPB resistance gene in *S. pinnatisectum* may be used as a reference framework for achieving enhanced control over late blight and CPB.

In the present study, one SSR marker S041, one EST marker S1c9 (AGPaseB-a), and two CAPS markers GP127-300 and At3g-100 were identified with linkage with the late blight resistance gene. The *Rpi1* gene was mapped on chromosome VII between the CAPS marker GP127-300 and EST marker S1c9 at genetic distances of 1.17 and 3.89 cM, respectively. The identification of a closely linked marker: CAPS marker GP127-300 in this study provides the possibility for cloning *Rpi1* using a map-based cloning approach. There are two strategies for using the marker for cloning the late blight resistance gene. The first strategy is to collect and purify the PCR products produced from resistant and susceptible parents using the markers, and then sequencing them separately. It is possible to find some SNP markers closely linked with *Rpi1* gene. The second strategy is that the PCR products produced from *S. pinnatisectum* (resistant parent) are used as probes to screen BAC library constructed with *S. pinnatisectum* [15]. The selected BAC clones should then be sequenced. Finally, the *Rpi1* gene can be cloned by a long-range (LR)-PCR approach. The successful example is that the other late blight resistant gene *RB* was cloned from *S. bulbocastanum* by using a map-based approach in combination with a long-range-PCR strategy[16].

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墨西哥野生马铃薯 *Solanum pinnatisectum* 抗晚疫病及抗马铃薯甲虫新基因的遗传分析与分子标记

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摘　要：马铃薯晚疫病（*Phytophthora infestans*）和科罗拉多马铃薯甲虫（CPB）是马铃薯生产中最为严重的病虫害。培育高抗晚疫病和甲虫的马铃薯品种是加拿大马铃薯育种工作的重要组成部分。目前，我们实验室在二倍体 1EBN 墨西哥野生种中已鉴定出抗马铃薯晚疫病和甲虫的新基因，并利用原生质体融合技术成功的将其转移到栽培品种中。但是，培育出抗晚疫病和抗甲虫的马铃薯新品种仍然是一项艰难而繁杂的工作。为了加快分离抗性基因，建立与抗性基因紧密关联的 DNA 分子标记至关重要。本研究以感病的二倍体马铃薯品种 *S. cardiophyllum* 作为父本，与带有抗性基因的墨西哥野生种 *S. pinnatisectum* 杂交。用叶片离体鉴定的方法测试 F_1 和 BC_1 代群体的抗病性，从而筛选抗晚疫病和抗甲虫的植株。US-8/A2 交配型病菌测试显示所有的 F_1 代植株都表现出抗晚疫病，而在 BC_1 群体中抗病与感病植株的比例为 1∶1。这个结果证明，在墨西哥野生种 *S. pinnatisectum* 中存在一个抗晚疫病的单显性基因 *Rpi1*。马铃薯甲虫抗性检测中，BC_1 群体的抗虫性分离比例为 1∶3. 这表明其对甲虫的抗性是由多基因遗传控制的。在 F_1 和 BC_1 群体中利用分子标记结合集团分离分析法（BSA）对 *S. pinnatisectum* 中的晚疫病抗性基因 *Rpi1* 进行精细作图。根据马铃薯第 7 条染色体上 RFLP 标记 TG20A 和 CP56 之间的 EST 和 STS 标记的序列信息，合成了 27 对特异性 PCR 引物。获得一些与抗晚疫病基因 *Rpi1* 相关联的新的 DNA 标记。对 BC_1 群体中大量的个体植株进行的分析表明，在马铃薯第 7 条染色体上位于抗晚疫病基因 *Rpi1* 两侧的两个标记 S1c9 和 GP127-300，它们与 *Rpi1* 基因的遗传距离分别为 1.17 cM 和 3.89 cM。这些标记被用来筛选两个细菌人工染色体（BAC）文库，并分离出与晚疫病抗性相关的 90–125 kb 的 BAC 克隆，这些克隆将在后续的工作中通过图位克隆的方法而用于分离晚疫病抗性基因。同时分离与甲虫抗性紧密相关的分子标记的工作正在进行中。

关键词：分子标记；抗晚疫病基因；墨西哥野生种；*Solanum pinnatisectum*

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