Studies on RAPD Molecular Markers Associated with Resistance to Powdery Mildew in Pea (*Pisum sativum* L.)

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Summary

Random amplified polymorphic DNA (RAPD) analysis is the alternative tool for identifying specific molecular markers associated with powdery mildew resistance genes. Molecular marker among 8 Taiwan's pea varieties with various levels of resistance to powdery mildew were investigated in this study. A total of 241 primers were used to detect the DNA polymorphism among eight varieties, out of which 35 primers produced discriminating, and reproducible DNA patterns were selected. Nine specific RAPD molecular markers associated with resistance to the powdery mildew were identified which were presented only in the resistant and/or intermediate resistant varieties. The results of morphological traits of 8 pea varieties were clustered into two groups with genetic similarity among varieties ranged from 0.54 to 0.84, whereas genetic traits were clustered into two groups with genetic similarity ranged from 0.61 to 0.77 by RAPD analysis. The results of PCR analysis revealed that *er1* and *ER3* DNA fragments could be amplified from the genomic DNAs of 8 pea varieties, regardless of the different sensitivity to powdery mildew disease of pea varieties.

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Introduction

Pea (*Pisum sativum* L.) is an important commercial edible legume as vegetable and dry seeds grown worldwide for human food and animal feed. Various parts of pea plants can be consumed, including leaves, seeds, shoots, sprouts and pods. This plant is known for high protein content suitable for vegetarian food and be able to fix nitrogen for improve soil nitrogen status.

Powdery mildew, caused by the obligate parasitic fungus *Erysiphe pisi*, is the main important disease affecting both quality and quantity of pea plants (Tiwari *et al*., 1998). The spores of powdery mildew exist as dust white color coating on leaves, stem and pod surfaces. Appressoria of fungi penetrate into epidermal cells and uptake nutrients from plants. (Falloon *et al*., 1989; Clark and Hall, 1998) This disease affects biomass yield, number of pod per plant, number of seed per pod, plant height and number of node (Gritton and Ebert, 1975), therefore 25-50 % yield was reduced (Munjal *et al*., 1963).

Utilizing disease resistant varieties is the most efficient strategy for disease controlling. Genetic resistance to powdery mildew in pea by a single recessive gene '*Er-er*' was first reported by Harland in 1948. In addition, two independent powdery mildew resistant genes, *er1* and *er2*, were reported (Heringa *et al.* 1969; Tiwari *et al*. 1997). A new powdery mildew resistant gene '*Er3*' was identified from *Pisum fulum* and applied in the breeding program of *Pisum sativum* (Fondevilla *et al*., 2007).

Screening of breeding populations for disease resistance by phenotype appearances is difficult especially when weather conditions do not favour fungal growth (Janila and Sharma. 2004). Linked molecular markers are alternative way helpfully finding the resistance gene. Powdery mildew resistance gene *er-1* in pea was confirmed by RAPD technique (Tiwari *et al.*, 1998). The RAPD and SCAR markers linked to the powdery mildew resistance genes identified by Janila and sharma. (2004) were suggested to be used in marker-assisted selection in pea breeding programs. The resistant *er-3* gene was identified and validated by RAPD and SCAR markers (Fondevilla *et al.*, 2008). RFLP, RAPD/SCAR and SSR markers had been linked to *er1* and *er2* on linkage group VI (Tonguc, and Weeden, 2010).and III (Katoch *et al.*, 2010), respectively.

Identification of RAPD molecular markers associated with resistance to the powdery mildew in pea would greatly facilitate in breeding program for better pea cultivar. The objectives of this study are to investigate the presences of *er*1 and *Er*3 genes in Taiwan's pea varieties, and to identify the RAPD molecular markers associated with resistance to the powdery mildew in pea.

Materials and Methods

1. Plant materials

Eight varieties of pea, namely Taichung No. 11, Taichung No. 12, Taichung No. 13, Taichung No. 14, Taichung No. 15, Native white flower, Good Farmer's No. 2, and Nung yu ta chia No.2, were collected from Taichung District Agricultural Research and Extension Station (TDAIS), Council of Agriculture, Executive Yuan, Taiwan, ROC. The characteristics of 8 varieties including genetic background, phenotype characteristics and sensitivity to powdery mildew disease were shown on Table 1. Plants were grown in the greenhouse at Department of Horticulture, National Chung Hsing University, Taichung, Taiwan, ROC. For each variety, three plants were grown in one pot and a total of five pots were used in one experiment. The experiment was repeated twice in fall and winter season, 2010 and 2011. The horticultural characteristics including blooming days at first node, total flowering days, color of flowers, length of leaves, width of leaves, length of internodes, number of nodes, length of branching, eye blue at leaf axil, plants height, length of pods, width of pods, shape of pods, texture of pods, pods per plant, seeds per pod, pod bearing percentage, and sensitivity to powdery mildew disease were recorded.

2. DNA isolation and quantification

Genomic DNA was extracted from fully expended mature leaves by the CTAB (cetyltrimethyl-ammonium bromide) method. The DNA amount was measured by spectrophotometer at 260 and 280 nm.

3. Primers and PCR reaction

A total of 241 arbitrary 10-mer oligonucleotide primers (UBC Set and Operon Kit) were used to detect the DNA polymorphism among eight varieties by RAPD. The PCR was performed in a 25µl reaction mixture containing 10X dream Taq buffer, 0.2 mM dNTPs, 0.2 µM primers, 1 units of dream Taq DNA polymerase, 2 ng genomic DNA. The conditions for thermal cycling were denaturation at 95 ℃ for 5 min followed by 40 cycles of denaturation at 95℃ for 45 s, annealing at 40 ℃ for 45 s, and extension at 72 ℃ for 1 min 30 s, finally at 72 ℃ for 10 min. Following amplification, 5 µg of DNA was loaded in a 1% agarose gel, electrophoresed, and stained with ethidium bromide. The size of the amplification products were visualized under UV light, and verified by comparison to DNA size markers.

4. Design primers

Following primer sets were designed according to NCBI Gene Bank database and synthesized, including er1: 5’ AGAGGTTTAGGTTTGCAAGGGA 3’ (forward), 5’ TGGCCTGCTTGAATAC GGTG 3’ (reverse) amplifying DNA fragments of 818- and 758-bp; Er3-1: 5’ CAGAAGCGGATG AGGCGGAG 3’ (forward), 5’ AAGAAGGTGGAAGGCGGTCG 3’ (reverse) amplifying DNA fragment of 508-bp; Er 3-2: 5’ CCGTCGGTAGTAAAAAAAAC 3’ (forward), 5’ GCTGGGGAA AGAAGGATGCA 3’ (reverse) amplifying DNA fragment of 777-bp.

5. Data analysis

Amplified markers and horticultural characteristic were scored as either present (1) or absent (0) for each sample. A genetic relationship and phenotype relationship dendrograms among varieties were constructed by a UPGMA cluster analysis of the genetic similarity matrix using software NTSYS-PC (version 2.1).

Results

The phenotype characteristics, genetic background, and sensitivity to powdery mildew disease of eight pea varieties collected from Taichung District Agricultural Research and Extension Station (TDAIS) were shown on Table 1. There were two types of flower colors among 8 varieties, white: Taichung No. 11, 13, 14, 15, Native white flower, Good Farmer’s No. 2; purple: Taichung No. 12, Nung yu ta chia No.2. Eye blue at leaf axil was present in Taichung No. 11, 12, Nung yu ta chia No.2, Good Farmer’s No. 2, but not in Taichung No. 13, 14, 15, Native white flower. There were three types of pods shape, flat sticky: Taichung No. 14, 15; flat crisp: Taichung No. 11, 12, Native white flower, Good Farmer’s No. 2, Nung yu ta chia No.2; swell: Taichung No. 13.

Sensitivity to powdery mildew disease could be classified into 3 groups, resistance (score 1): Taichung No. 15; intermediate resistance (score 2): Taichung No. 12, 14, Good Farmer’s No. 2; susceptible: (score 3~5): Taichung No. 11, 13, Native white flower, Nung yu ta chia No.2 (Table 1). We also found that powdery mildew disease was more severe in spring (February- April) than winter season (October- January). Plants were infected on organ from lower to upper part of plants, heavily infected leaves become dry out and turned brown, prior to harvest.

Phenotypic characteristics were used to analyze phenotypic relationship among 8 varieties of pea (Fig. 1). The results of phenotypic characteristics analysis show that the coefficients of similarity were between 0.54 and 0.84, and 8 varieties could be separated into two groups, Taichung No. 14 (D), Taichung No. 15 (E), Native white flower (F), in one group characterized by early

Table 1. The phenotype characteristics, genetic background, and sensitivity to powdery mildew disease of eight pea (*Pisum sativum* L.) varieties.

Code	Variety (Genetic Background)	Phenotype Characteristics (Usage)	Sensitivity to Powdery Mildew (1~5) (Resistance → Susceptible)
A	Taichung No. 11 (Odeme × Melting Sugar，1980)	(pod)	Susceptible (4)
B	Taichung No. 12 (Taichung No. 11 × Manoa Sugar，1988)	(pod)	Intermediate (2)
C	Taichung No. 13 (Sugar Snap × Knight，1988)	(sweet seeds)	Susceptible (5)
D	Taichung No. 14 (Satsuma × Taichung 78-203，1998)	(seeds)	Intermediate (2)
E	Taichung No. 15 (80-73 × Wusui，2003) (80-73: F6 (Wusui × Mexique))	(leaves, shoots, sprouts)	Resistance (1)
F	Native white flower (在來白花)	(pod)	Susceptible (3)
F	Good Farmer's No. 2 (好農家2號)	(pod)	Intermediate (2)
G	Nung yu ta chia No.2 (農友大英1號)	(pod)	Susceptible (4)

blooming, white flower, large leaf, and no eye-blue at leaves axil, while Taichung No. 11 (A), Taichung No. 12 (B), Taichung No. 13 (C), Good Farmer's No. 2 (F), and Nung yu ta chia No.2 (G) in another group.

A total of 241 arbitrary 10-mer oligonucleotide primers (UBC Set and Operon Kit) were used to detect the DNA polymorphism among eight varieties, out of which only 35 primers produced clearly polymorphism among them and gave reproducible banding patterns (Table 2). An average of 8 bands with a range of 4-12 bands was amplified from each primer. A total of 265 clearly bands, 218 (83.2%) were shown polymorphic bands. The sizes of most amplified DNA fragments ranged from 100-4,000 bp. An example of a RAPD pattern obtained from primer UBC 63 was show in Figure 2. The results of RAPD analysis show that the coefficients of similarity were between 0.61 and 0.77 (Fig. 3). Eight varieties could be divided into two groups according to their genetic relationships, Taichung No. 11 (A), Taichung No. 12 (B), Taichung No. 13 (C), and Taichung No. 14 (D) in one group, and Taichung No. 15 (E), Native white flower (F), Good Farmer's No. 2 (G), and Nung yu ta chia No. (H) in the second group. Furthermore, neither the phenotypic characteristics nor the RAPD markers assessed the cluster analysis of UPGMA, showed the relationships among groups following the powdery mildew resistance.

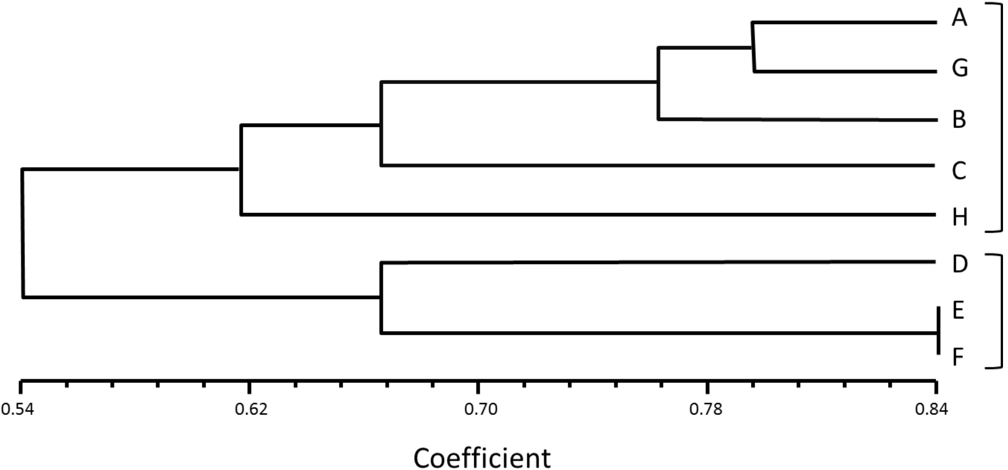

Fig 1. Dendrogram illustrating genetic similarity among 8 varieties of pea calculated by phenotypic characteristics. A~H: Varieties codes are the same as shown in Table 1.

Table 2. List of primers used to produce polymorphic DNA bands among 8 pea varieties.

Primers	Sequence	No. of amplified bands	No. of polymorphic bands (%)
OPA-02	TGCCGAGCTG	6	5 (83.3)
OPA-04	AATCGGGCTG	11	9 (81.8)
OPC-16	CACACTCCAG	11	7 (63.6)
OPC-2	GTGAGGCGTC	9	4 (44.4)
OPG-11	TGCCCGTCGT	5	4 (80.0)
OPJ-20	TGCCCGTCGT	9	9 (100)
OPM-07	CCGTGACTCA	6	5 (83.3)
OPP-08	CCGTGACTCA	8	8 (100)
OPQ-20	TCGCCCAGTC	6	5 (83.3)
OPT-05	GGGTTTGGCA	7	4 (57.1)
OPT-07	GGCAGGCTGT	12	9 (75.0)
OPU-07	CCTGCTCATC	6	6 (100)
OPV-05	TCCGAGAGGG	6	3 (50.0)
OPV-10	GGACCTGCTG	7	5 (71.4)
OPV-12	ACCCCCCACT	8	4 (50.0)
OPV-19	GGGTGTGCAG	4	3 (75.0)
OPAB-4	GGCACGCGTT	8	7 (87.5)
OPAB-7	ACGGCGATGA	5	4 (80.0)
OPAI-11	ACGGCGATGA	7	7 (100)
OPAK-10	CAAGCGTCAC	6	5 (83.3)
OPAN-10	ACAACTGGGG	8	8 (100)
OPAU-14	CACCTCGACC	12	9 (75.0)
OPAX-01	GTGTGCCGTT	8	5 (62.5)
UBC-05	CCTGGGTTCC	6	6 (100)
UBC-18	GGGCCGTTTA	6	6 (100)
UBC-24	ACAGGGGTGA	4	4 (100)
UBC-34	CCGGCCCCAA	9	8 (88.9)
UBC-51	CTACCCGTGC	7	7 (100)
UBC-63	TTCCCCGCCC	12	10 (83.3)
UBC-67	GAGGGCGAGC	5	5 (100)
UBC-84	GGGCGCGAGT	11	10 (90.9)
UBC-85	GTGCTCGTGC	8	5 (62.5)
UBC-88	CGGGGGATGG	7	7 (100)
UBC-106	CGTCTGCCCG	8	8 (100)
Total		265	218 (83.2)

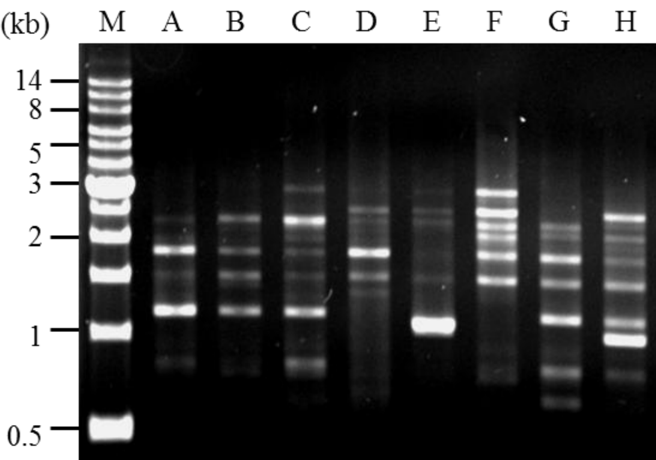

Fig. 2. RAPD banding pattern generated with primer UBC- 63 using pea DNAs as template. M: DNA molecular weight marker. A~H: Varieties codes are the same as shown in Table 1.

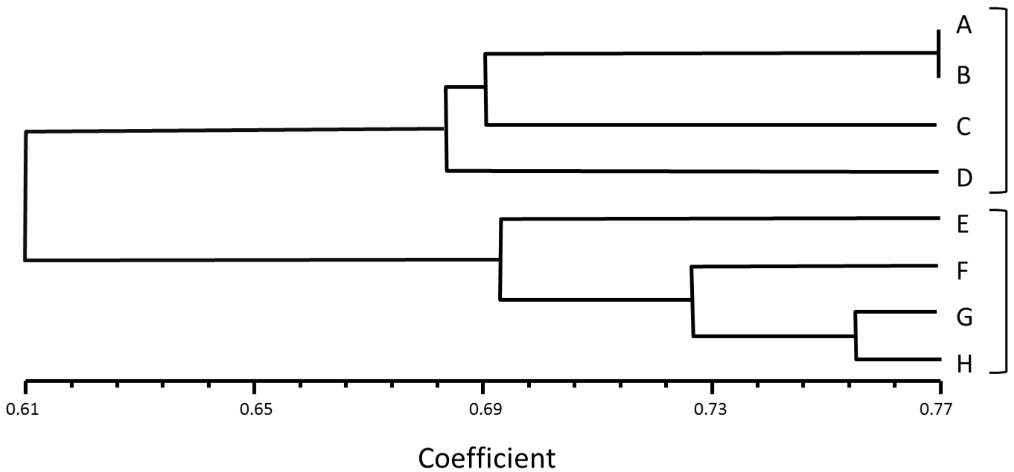

Fig 3. Dendrogram illustrating genetic similarity among 8 varieties of pea calculated by RAPD analysis. A~H: Varieties codes are the same as shown in Table 1.

Nevertheless, nine specific RAPD molecular markers amplified by OPAI 11, OPT 7, OPC 16, UBC 106, and OPA 4 primers might associate with resistance to the powdery mildew which were presented only in the resistant and/or intermediate resistant varieties, including OPAI 11-a, OPAI 11-b, OPAI 11-c, OPT 7-a, OPC 16-a, UBC 106-a, UBC 106-b, OPA 4-a, and OPA 4-b (Fig. 4).

In order to verify whether the presence of powdery mildew resistance genes in these 8 pea varieties, PCR analysis was performed using *er1* and *ER3* specific primers and pea DNAs as template. The results of PCR analysis revealed that *er1* and *ER3* DNA fragments could be amplified from the template DNAs of 8 pea varieties, regardless of the different sensitivity to powdery mildew of pea varieties (Fig. 5).

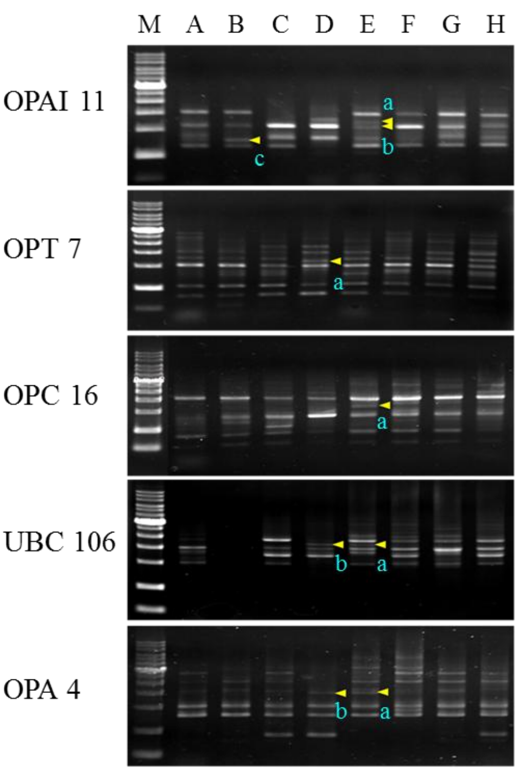

Fig. 4. RAPD banding pattern generated with primer OPAI 11, OPT 7, OPC 16, UBC 106, and OPA 4 using pea DNAs as template. The yellow arrows designated the specific RAPD molecular markers presented only in the resistant and/or intermediate resistant varieties. M: DNA molecular weight marker. A~H: Varieties codes are the same as shown in Table 1.

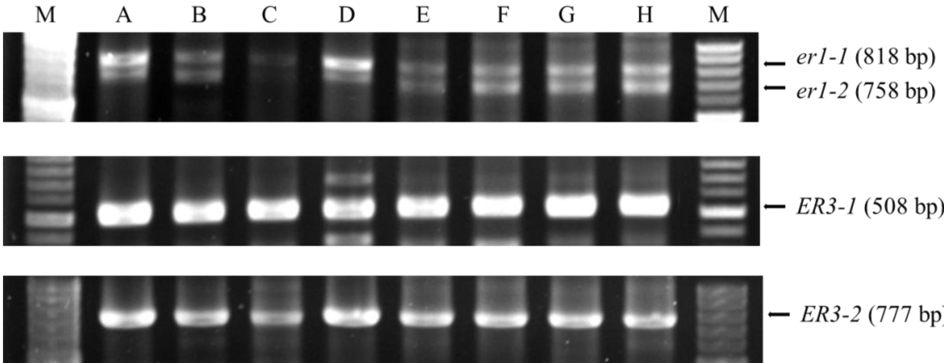

Fig. 5. Detection of *er1* and *Er3* genes in 8 varieties of pea. M: DNA molecular weight marker. A~H: Varieties codes are the same as shown in Table 1.

Discussion

In this study, we investigated resistant and susceptible phenotype of Taiwan's pea 8 varieties using 241 universal primers (UBC Set and Operon Kit) to verify specific molecular markers associated with resistance to powdery mildew disease. In addition, 3 primers for detection of *er1* and *Er3* genes were also designed according to NCBI GeneBank. The phenotypic appearances confirmed the previous studied on assessment of resistance to powdery mildew in these pea varieties which were asserted under greenhouse using natural infected in plants. Pea plants were more severely infected with powdery mildew in spring season than those in winter season. Disease would be appeared on plant when the environmental condition favored to disease pathogen. The powdery mildew spreads rapidly in dry weather when humidity is high and nights are cool. (Fondevilla *et al.*, 2006).

Janila and Sharma (2004) reported that after inoculation of powdery mildew fungi to resistant plants, infection was absent or localized in small patched only on the foliage but it never spread to the stem. Our observations on the occurrence, development, and symptom of powdery mildew disease in the greenhouse were consistent with the previous reports. Therefore, plants could be clearly distinguished the sensitivity to powdery mildew disease into three groups, (1) susceptible varieties, including Taichung No. 11 (A), Taichung No. 13 (C), Native white flower (F), and Nung yu ta chia No. 2 (H), (2) intermediate resistant varieties, including Taichung No. 12 (B), Taichung

No. 14 (D), and Good Farmer' s No. 2 (G), and (3) resistant varieties, Taichung No.15 (E) (Table 1). The results of phenotypic characteristics analysis of 8 pea varieties were clustered into 2 groups with genetic similarity ranged from 0.54 to 0.84. Taichung No. 14, Taichung No. 15, and Native white flower were clustered into the same group, and they were early blooming, white flower, large leaf, and no eye-blue at leaves axil.

Out of 241 arbitrary 10-mer oligonucleotide primers (UBC Set and Operon Kit) used, only 14.5% of primers (35) produced clearly polymorphism among eight pea varieties and gave reproducible banding patterns (Table 2). The percentage of polymorphic bands generated by 35 primers was 83.2%. The results of genetic traits of 8 pea varieties were clustered into 2 groups with genetic similarity ranged from 0.61to 0.77 by RAPD analysis. A great genetic diversity existed in 8 pea varieties which reflected that the distinct varieties had been used in the breeding program. However, neither the phenotypic characteristics nor the RAPD analysis assessed the cluster analysis of UPGMA, showed the relationships between clustered groups and sensitivity to powdery mildew. We had identified nine specific RAPD molecular markers, OPAI 11-a, OPAI 11-b, OPAI 11-c, OPT 7-a, OPC 16-a, UBC 106-a, UBC 106-b, OPA 4-a, and OPA 4-b, might associate with resistance to the powdery mildew which were presented only in the resistance and/or intermediate resistant varieties. Further studies were needed to investigate the possible application of these RAPD molecular markers in the marker-assisted selection in pea breeding programs. The results of PCR analysis revealed that powdery mildew resistance genes, *er1* and *ER3*, could be amplified from the template DNAs of 8 pea varieties, regardless of the different sensitivity to powdery mildew of pea varieties. It indicated that the *er-1* and *ER-3* genes may not be the universal molecular markers for the traits of powdery mildew resistance in pea.

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豌豆(*Pisum sativum* L.) 抗白粉病相關之 RAPD 分子標誌的研究

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關鍵字：RAPD、白粉病、RAPD 分子標記、豌豆

摘要：逢機增幅多型性 DNA (RAPD) 分析是尋找與抗白粉病相關基因的特定分子標誌的一個替代方法。本研究以對白粉病具有不同抗性的 8 種台灣豌豆品種為材料，探討與抗白粉病相關之 RAPD 分子標記。在總計 241 條引子中選出 35 條引子能增幅出具有清晰及重複性的 DNA 條帶。我們確認出 9 個獨特的 RAPD 分子標誌，只存在具抗性及/或中間型抗性的豌豆品種。外表性狀調查結果顯示，8 種豌豆品種可分成遺傳相似度為 0.54~0.84 之二個群組。RAPD 親源分析結果顯示，可分成遺傳相似度為 0.61~0.77 之二個群組。PCR 分析抗白粉病基因(*er1* 及 *ER3*)之結果顯示，在 8 種具有不同抗性的台灣豌豆品種之基因組 DNA 均能偵測到 *er1* 及 *ER* 之 DNA 片段。

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