EVALUATION ON IN VITRO SOMATIC EMBRYOGENESIS AND OPTIMIZATION OF GENETIC TRANSFORMATION VIA TRANSIENT EXPRESSION FOR PEPPER (Piper nigrum L.)

Chen Teck You

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Chen Teck You

A thesis submitted in fulfilment of the requirement for the degree of Master of Science (Plant Genetic Transformation)

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ABSTRACT

Black pepper (Piper nigrum L.) is one of the important spices in the world. Pepper has numerous uses and functions to consumers however the occurrence of pests and diseases have become main problem to pepper cultivation. Conventional approach of pepper breeding is hampered by absence of pest and disease resistance genes in the species. Molecular biological approach of plant breeding could assist and complement the conventional approach of breeding for pest and disease resistance. In order to adopt molecular biological approach of pepper breeding, the present study was conducted to develop an efficient regeneration and genetic transformation system for pepper. A successful regeneration of somatic embryos was obtained using micropylar of seeds cultured on MS medium supplemented with 0.3% activated charcoal. When maintenance extended to four months, cyclic secondary somatic embryo formation on root pole explants enabled mass production of pepper plantlets. The in vitro generated somatic embryos were used as source of explants for developing transformation system whilst a number of somatic embryos were left to grow into plantlets to determine the genetic relatedness from field grown mother plants. For developing a transformation system for pepper, Agrobacterium-mediated and particle bombardment approaches were applied by initially monitoring the transient gene expression. However, due to explants browning problem, the GUS reporter gene was found unsuitable for use in Agrobacterium-mediated transformation technique. Consequently, GFP reporter gene was employed in particle bombardment method and transient green fluorescent spots were discovered on transformed explants. The p35SCaMV-sgfpS65T construct co-bombarded with pCambia 1300 showed the highest transient expression spots among all the GFP
constructs examined. After optimization of bombardment parameters, 1.0 µm microcarriers, 900 psi pressure and 6 cm distance were found optimal for producing green transient fluorescent on explants. However, no single transgene was detected from *in vitro* regenerated putative transformed plantlets via PCR analysis. Based on the result from RAPD analysis, no genetic variation was detected among mother plants and somatic embryo plantlets themselves respectively. However, small polymorphism was found when compared mother plant and somatic embryo plantlets using OPD 05, OPM 04 and OPP 16 primers.

Key words: *Piper nigrum* L., direct somatic embryogenesis, green fluorescent protein (GFP), β-glucuronidase (GUS), transient gene expression
PENILAIAN EMBRIOGENESIS SOMATIK SECARA IN VITRO DAN PENGOPTIMUMAN SISTEM TRANSFORMASI GENETIK MELALUI EKSPRESI SEMENTARA UNTUK LADA (Piper nigrum L.)

ABSTRAK

sementara gen. Oleh sebab masalah keperangan eksplan, gen penanda GUS didapati tidak sesuai untuk kegunaan teknik transformasi 'berperantaraan Agrobacterium'. Akibatnya, gen penanda GFP digunakan dalam kaedah 'penembakan zarah' dan tompok pendarfluor hijau sementara ditemui pada eksplan diubahsuai. Vektor p35SCaMV-sgfpS65T ditembak bersama dengan pCambia 1300 menunjukkan pengekspressan sementara tertinggi di antara semua vektor-vektor GFP yang telah diperiksa. Setelah pengoptimuman parameter penembakan, pembawa mikro bersaiz 1.0 µm, tekanan 900 psi helium dan jarak 6 sm didapati optimum bagi menghasilkan pendarfluor hijau sementara pada eksplan. Namun, tiada satu transgen dikesan dari anak pokok ubahsuai putatif yang diregenerasi secara in vitro melalui analisis PCR. Berdasarkan keputusan dari analisis RAPD, tiada variasi genetik dikesan antara tanaman induk dewasa, dan juga masing-masing di antara anak pokok embrio somatik. Namun, polimorfisme kecil didapati apabila tanaman induk dewasa dibandingkan dengan anak pokok embrio somatik dengan menggunakan pencetus OPD 05, OPM 04 dan OPP 16.

Kata kunci: Piper nigrum L., embriogenesis somatik secara langsung, protein pendarfluor hijau (GFP), β-glucuronidase (GUS), pengekspressan sementara gen
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iv</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td>vi</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xiv</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xvi</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xx</td>
</tr>
<tr>
<td>CHAPTER 1 INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>CHAPTER 2 LITERATURE REVIEW</td>
<td>6</td>
</tr>
<tr>
<td>2.1 Black pepper</td>
<td>6</td>
</tr>
<tr>
<td>2.1.1 The origin, ecology and distribution of pepper</td>
<td>7</td>
</tr>
<tr>
<td>2.1.2 General morphology</td>
<td>9</td>
</tr>
<tr>
<td>2.1.3 Pest and diseases</td>
<td>12</td>
</tr>
<tr>
<td>2.1.4 Contamination in <em>in vitro</em> culture</td>
<td>15</td>
</tr>
<tr>
<td>2.1.5 Browning of explants</td>
<td>18</td>
</tr>
<tr>
<td>2.2 Somatic embryogenesis</td>
<td>19</td>
</tr>
<tr>
<td>2.2.1 Somatic embryogenesis in pepper</td>
<td>21</td>
</tr>
<tr>
<td>2.2.2 <em>In vitro</em> pepper regeneration</td>
<td>22</td>
</tr>
<tr>
<td>2.2.3 Somatic embryos in plant genetic transformation</td>
<td>22</td>
</tr>
<tr>
<td>2.3 <em>Agrobacterium tumefaciens</em> in plant transformation</td>
<td>24</td>
</tr>
</tbody>
</table>

viii
2.3.1 Vector and marker genes
2.3.2 Overgrowth-control antibiotics for Agrobacterium
2.3.3 Genetic transformation study in pepper
2.3.4 GUS reporter system

2.4 Particle bombardment
2.4.1 Transformation using particle bombardment
2.4.2 Co-bombardment transformation
2.4.3 Green fluorescent protein (GFP)
2.4.4 Application of GFP as selectable marker

2.5 Polymerase chain reaction
2.5.1 Random amplified polymorphic DNA (RAPD)
2.5.2 Application of RAPD in Piper species
2.5.3 Detection of somaclonal variation using RAPD

CHAPTER 3
MATERIALS AND METHODS

3.1 Plant materials
3.2 Surface sterilization protocol
3.3 Preparation of culture media
3.4 Direct somatic embryogenesis
3.4.1 Induction of somatic embryos from zygotic embryo
3.4.2 Induction of somatic embryos from seed
3.4.3 Induction of somatic embryos from seed coat
3.4.4 Effect of different genotypes of seed on somatic embryogenesis
3.4.5 Formation of somatic embryos from root pole
3.4.6 Effects of culture conditions

3.5 Indirect somatic embryogenesis
   3.5.1 Induction of somatic embryos from zygotic embryo
   3.5.2 Induction of somatic embryos from seed

3.6 Assessment of hygromycin resistance as antibiotic selectable marker

3.7 Agrobacterium-mediated transformation
   3.7.1 Agrobacterium tumefaciens strain and plasmid vector
   3.7.2 Preparation of bacterial growth media
   3.7.3 Co-cultivation medium

3.8 Agrobacterium-mediated transformation protocol
   3.8.1 Streaked Agrobacterium tumefaciens on APM medium
   3.8.2 Inoculation and co-cultivation
   3.8.3 Effects of Agrobacterium culture condition (optical density) on transient expression of GUS in Agrobacterium transformation
   3.8.4 GUS histochemical assay

3.9 Microprojectile bombardment
   3.9.1 GFP gene constructs
   3.9.2 Preparation of Luria Bertani (LB) medium
   3.9.3 Isolation of plasmid DNA

3.10 Preparation of materials and parameters
   3.10.1 Preparation of microcarrier
   3.10.2 Coating of microcarrier with plasmid DNA
   3.10.3 Macrocarrier preparation
   3.10.4 Bombardment procedure
   3.10.5 Detection of transient GFP expression in P. nigrum
3.11 Effects of different constructs affecting transient expression

3.11.1 Effects of different pressures and distances on transient expression of GFP using pCambia 1302

3.11.2 Effects of different pressures and distances on transient expression of GFP using pCambia 1304

3.11.3 Effects of different pressures and distances on transient expression of GFP using pHBT-sgfpS65T and pCambia 1300

3.11.4 Effects of different pressures and distances on transient expression of GFP using p35SCaMV-sgfpS65T and pCambia 1300

3.11.5 Effects of pressures and size of microcarriers affecting transient expression

3.11.6 Effects of pressures on transient expression

3.11.7 Analysis of putative transformed plantlet

3.12 Genetic fidelity assessment of in vitro P. nigrum through RAPD

3.12.1 Plant materials obtained for DNA extraction

3.12.2 DNA extraction

3.12.3 DNA quantification

3.12.4 Agarose gel electrophoresis

3.12.5 Random amplified polymorphic DNA (RAPD) assay

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Direct somatic embryogenesis

4.1.1 Induction of somatic embryos from zygotic embryo

4.1.2 Induction of somatic embryos from seed

4.1.3 Induction of somatic embryos from seed coat

4.1.4 Effect of seeds of different genotypes on somatic embryogenesis
4.1.5 Formation of somatic embryos from root pole 76
4.1.6 Effects of culture conditions 78

4.2 Indirect somatic embryogenesis 80
4.2.1 Induction of somatic embryos from zygotic embryo 80
4.2.2 Induction of somatic embryos from seed 82

4.3 Assessment of hygromycin resistance as antibiotic selectable marker 85

4.4 Effects of Agrobacterium culture concentration (optical density) on transient expression of GUS in Agrobacterium transformation 87

4.5 Effects of different constructs affecting transient expression 90
4.5.1 Effects of different pressures and distances on transient expression of GFP using pCambia 1302 90
4.5.2 Effects of different pressures and distances on transient expression of GFP using pCambia 1304 94
4.5.3 Effects of different pressures and distances on transient expression of GFP using pHBT-sgfpS65T and pCambia 1300 96
4.5.4 Effects of different pressures and distances on transient expression of GFP using p35SCaMV-sgfpS65T and pCambia 1300 98
4.5.5 Effects of pressures and size of microcarriers affecting transient expression 102
4.5.6 Effects of pressures on transient expression 106
4.5.7 Analysis of putative transformed plantlets 109

4.6 Genetic fidelity assessment of pepper and its in vitro regenerated clones through RAPD 111
4.6.1 Genomic DNA 111
4.6.2 PCR-RAPD for mother and in vitro plantlets 113
4.6.3 Polymorphisms verification 125
LIST OF TABLES

Table 2.1  Induction of somatic embryogenesis through different pathways and plant growth regulators. 20
Table 2.2  Transgenic plant species transformed by different Agrobacterium-mediated and particle bombardment techniques using GUS as reporter gene. 30
Table 2.3  Transgenic plant species using particle bombardment with different reporter gene, selectable marker gene and target tissue. 32
Table 3.1  Antibiotics selection for different GFP gene constructs. 53
Table 3.2  Sequences of the selected Operon primers. 66
Table 4.1  Percentage of somatic embryos formation from zygotic embryo explants cultured on MS medium supplemented with 0.38 µM ABA and 0.3% activated charcoal respectively. 68
Table 4.2  Percentage of somatic embryos developed from seed coat cultured on MS or SH medium supplemented with 0.3% activated charcoal respectively at day 140. 70
Table 4.3  Percentage of somatic embryos generated from seed coat cultured on MS, MS supplemented with 0.3% activated charcoal and SH media at day 90 and day 150 respectively. 72
Table 4.4  Percentage of seed coats of two varieties developed somatic embryos on MS and SH medium supplemented with 0.3% activated charcoal at day 90. 74
Table 4.5  Mean number of somatic embryos cultured on MS medium supplemented with 0.3% activated charcoal against duration. 77
Table 4.6  Mean numbers of somatic embryos formed in different culture conditions at day 90. 80
Table 4.7  Percentage of somatic embryos developed from zygotic embryos cultured on MS medium incorporated with ABA at different concentrations at day 30. 82
Table 4.8  Summary of GFP gene constructs expressed transient expression. 91
Table 4.9 Mean number of explants expressed green fluorescent spots with different combinations of pressure and distance two weeks after bombardment.

Table 4.10 Mean number of explants expressed green fluorescent spots with different combinations of pressure and distance at two weeks of post-bombardment.

Table 4.11 Mean number of explants expressed green fluorescent spots with different combinations of pressure and distance at two weeks of post-bombardment.

Table 4.12 Mean number of explants expressed green fluorescent spots with different combinations of pressure and distance at two weeks of post-bombardment.

Table 4.13 Expression of transient expression spots on *P. nigrum* explants bombarded with different sizes of microcarriers.

Table 4.14 Mean number of explants expressed green fluorescent spots at different pressures bombarded with microcarriers of size 1.0 and 1.6 µm respectively.

Table 4.15 Mean number of explants expressed green fluorescent spots with different combinations of pressure and distance at two weeks of post-bombardment.

Table 4.16 Comparison of polymorphisms through amplified RAPD fragments using different primers between mother plant cv. Semongok Aman and the somatic embryo plantlets regenerated.
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 2.1</td>
<td><em>Piper nigrum</em> plants growing in the field.</td>
<td>7</td>
</tr>
<tr>
<td>Figure 2.2</td>
<td>Pepper berries.</td>
<td>12</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>T-DNA region of the GFP constructs used in particle bombardment of black pepper.</td>
<td>52</td>
</tr>
<tr>
<td>Figure 4.1 (a-c)</td>
<td>Induction of somatic embryos from zygotic embryo.</td>
<td>68</td>
</tr>
<tr>
<td>Figure 4.2 (a-c)</td>
<td>Induction of somatic embryos from seed.</td>
<td>70</td>
</tr>
<tr>
<td>Figure 4.3 (a-c)</td>
<td>Induction of somatic embryos from seed coat.</td>
<td>72</td>
</tr>
<tr>
<td>Figure 4.4 (a-c)</td>
<td>Effect of seeds of different genotypes on somatic embryogenesis.</td>
<td>75</td>
</tr>
<tr>
<td>Figure 4.5 (a-c)</td>
<td>Formation of somatic embryos from root pole.</td>
<td>78</td>
</tr>
<tr>
<td>Figure 4.6 (a-b)</td>
<td>Effects of culture conditions on colour of somatic embryos.</td>
<td>80</td>
</tr>
<tr>
<td>Figure 4.7 (a-b)</td>
<td>Induction of somatic embryos from zygotic embryos derived calli.</td>
<td>82</td>
</tr>
<tr>
<td>Figure 4.8 (a-i)</td>
<td>Induction of somatic embryos from seed coat.</td>
<td>84</td>
</tr>
<tr>
<td>Figure 4.9</td>
<td>Effect of hygromycin concentration on non-transformed <em>P. nigrum</em> explants.</td>
<td>86</td>
</tr>
<tr>
<td>Figure 4.10 (a-c)</td>
<td>Assessment of hygromycin resistance as antibiotic selectable marker.</td>
<td>86</td>
</tr>
<tr>
<td>Figure 4.11 (a-f)</td>
<td>Effects of different optical densities on Agrobacterium-mediated transformation.</td>
<td>88</td>
</tr>
<tr>
<td>Figure 4.12</td>
<td>A green spot detected on explant bombarded with pCambia 1302 at 900 psi and 9 cm one day after bombardment.</td>
<td>92</td>
</tr>
<tr>
<td>Figure 4.13</td>
<td>Numerous green spots observed on explant bombarded with pCambia 1302 at 650 psi and 3 cm one day after bombardment.</td>
<td>92</td>
</tr>
<tr>
<td>Figure 4.14</td>
<td>Non-transformed explant showed red autofluorescence</td>
<td>92</td>
</tr>
</tbody>
</table>
of the chlorophyll.

Figure 4.15 Detection of transient expression on somatic-embryo explant bombarded with pCambia 1304 at 650 psi and 3 cm 10 days after bombardment.

Figure 4.16 Transient expression as indicated by green spots in explant bombarded with pHBT-sgfpS65T construct with 900 psi and 6 cm were observed at one day after bombardment.

Figure 4.17 Detection of green fluorescent spots on explant bombarded at 650 psi one day after bombardment.

Figure 4.18 Detection of green fluorescent spots on explant bombarded at 900 psi one day after bombardment.

Figure 4.19 Detection of green fluorescent spots on explant bombarded at 1100 psi one day after bombardment.

Figure 4.20 Green fluorescent spots were clearly observed on bombarded explant one day after bombardment with 1.0 µm microcarriers.

Figure 4.21 Fluorescent spots become faint on growing explant at day 6 of post bombardment with 1.0 µm microcarriers.

Figure 4.22 A green transient spot was detected on explant one day after bombardment with 1.6 µm microcarriers.

Figure 4.23 The green spot fluoresced brighter at day 3 of post bombardment with 1.6 µm microcarriers.

Figure 4.24 Somatic-embryo explant bombarded at 650 psi showed multiple green fluorescent spots at one day of post bombardment.

Figure 4.25 Somatic-embryo explant bombarded at 900 psi showed multiple green fluorescent spots at one day of post bombardment.

Figure 4.26 Somatic-embryo explant bombarded at 1100 psi showed multiple green fluorescent spots at one day of post bombardment.

Figure 4.27 Genomic DNA extracted from Semongok Aman and Kuching cultivars mother plants.
Figure 4.28 (a-c) Twenty-seven genomic DNA extracted from in vitro derived somatic embryo plantlets.

Figure 4.29 Amplified RAPD markers produced by mother plant and cv. Kuching using OPD 05 primer.

Figure 4.30 (a-c) Amplified RAPD pattern produced by somatic embryos derived plantlets regenerated from root pole using OPD 05 primer.

Figure 4.31 Amplified RAPD markers produced by mother plant and cv. Kuching using OPD 18 primer.

Figure 4.32 (a-c) Amplified RAPD pattern produced by somatic embryos derived plantlets regenerated from root pole using OPD 18 primer.

Figure 4.33 Amplified RAPD markers produced by mother plant and cv. Kuching using OPL 14 primer.

Figure 4.34 (a-c) Amplified RAPD pattern produced by somatic embryos derived plantlets regenerated from root pole using OPL 14 primer.

Figure 4.35 Amplified RAPD markers produced by mother plant and cv. Kuching using OPM 04 primer.

Figure 4.36 (a-c) Amplified RAPD pattern produced by somatic embryos derived plantlets regenerated from root pole using OPM 04 primer.

Figure 4.37 Amplified RAPD markers produced by mother plant and cv. Kuching using OPP 16 primer.

Figure 4.38 (a-c) Amplified RAPD pattern produced by somatic embryos derived plantlets regenerated from root pole using OPP 16 primer.

Figure 4.39 Amplified RAPD markers produced by mother plant and cv. Kuching using OPR 01 primer.

Figure 4.40 (a-c) Amplified RAPD pattern produced by somatic embryos derived plantlets regenerated from root pole using OPR 01 primer.

Figure 4.41 Comparison of amplified PCR-RAPD fragments between mother plant and in vitro regenerated plantlets using OPR 01 primer.
Figure 4.42  Comparison of amplified PCR-RAPD fragments between mother plant and *in vitro* regenerated plantlets using OPL 14 primer.

Figure 4.43  Comparison of amplified PCR-RAPD fragments between mother plant and *in vitro* regenerated plantlets using OPD 18 primer.

Figure 4.44  Comparison of amplified PCR-RAPD fragments between mother plant and *in vitro* regenerated plantlets using OPD 05 primer.

Figure 4.45  Comparison of amplified PCR-RAPD fragments between mother plant and *in vitro* regenerated plantlets using OPM 04 primer.

Figure 4.46  Comparison of amplified PCR-RAPD fragments between mother plant and *in vitro* regenerated plantlets using OPP 16 primer.
LIST OF ABBREVIATIONS

& and
°C Degree centigrade
% Percentage
µg Microgram
µl Microliter
µM Micromolar
µm Micron
2,4-D 2, 4-dichlorophenoxyacetic acid
ABA Abscisic acid
AC Activated charcoal
ANOVA Analysis of variance
BA 6-benzyladenine
BAP 6-benzylamino purine
bp Base pair
CaCl₂ Calcium chloride
CAT Chloramphenicol acetyltransferase
CaMV Cauliflower mosaic virus
cm Centimetre
CRD Complete randomized design
CMV Cucumber mosaic virus
cv. Cultivar
dNTPs Deoxynucleotide triphosphates
EDTA Ethylenediaminetetra-acetic acid
e.g. For example
et al. and others
GUS β-glucuronidase
HCl Hydrochloric acid
Hg Mercury
HgCl₂ Mercury chloride
HPT Hygromycin phosphotransferase
i.e. that is to say
Inc. Incorporated
ISSRs Inter simple sequence repeats
kb Kilobase pairs
K₃Fe(CN)₆ Potassium ferricyanide
K₄Fe(CN)₉.3H₂O Potassium ferrocyanide
l Liter
LS Linsmaier and Skoog medium
M Molar
m Meter
mg Milligram
MgCl₂ Magnesium chloride
min Minute
ml Millilitre
mm Millimetre
mM Millimole
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS</td>
<td>Murashige and Skoog medium</td>
</tr>
<tr>
<td>N</td>
<td>Normality</td>
</tr>
<tr>
<td>NAA</td>
<td>( \alpha )-naphthalene acetic acid</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaOCl</td>
<td>Sodium hypochlorite</td>
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<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>((\text{NH}_4\text{)}_2\text{SO}_4)</td>
<td>Ammonium sulfate</td>
</tr>
<tr>
<td>NPA</td>
<td>Neutralized phosphorous acid</td>
</tr>
<tr>
<td>NPT II</td>
<td>Neomycin phosphotransferase II</td>
</tr>
<tr>
<td>PGR</td>
<td>Plant growth regulator</td>
</tr>
<tr>
<td>ppm</td>
<td>Part per million</td>
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<tr>
<td>PPT</td>
<td>Phosphinothricin acetyl transferase</td>
</tr>
<tr>
<td>PVP</td>
<td>Polyanlyl pyrrolidone</td>
</tr>
<tr>
<td>rbcS</td>
<td>Ribulose bisphosphate carboxylase</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
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<tr>
<td>SH</td>
<td>Schenk and Hildebrandt medium</td>
</tr>
<tr>
<td>spp.</td>
<td>species</td>
</tr>
<tr>
<td>ssp.</td>
<td>subspecies</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate-EDTA</td>
</tr>
<tr>
<td>Taq</td>
<td><em>Thermophile aquaticus</em></td>
</tr>
<tr>
<td>T-DNA</td>
<td>Transfer-DNA</td>
</tr>
<tr>
<td>Ti</td>
<td>Tumor inducing</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume/volume</td>
</tr>
<tr>
<td>VIGS</td>
<td>Virus-induced gene silencing</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight/volume</td>
</tr>
<tr>
<td>X-gluc</td>
<td>5-bromo-4-chloro-3-indolyl-( \beta )-D-glucuronide</td>
</tr>
<tr>
<td>YAC</td>
<td>Yeast artificial chromosome</td>
</tr>
</tbody>
</table>
CHAPTER 1
INTRODUCTION

Black pepper (*Piper nigrum* L.) is one of the important spices in the world. It is also known as the “King of Spices” having numerous uses such as spice, food seasoning, preservative and medicine which provide valuable benefits to consumers (Ravindran, 2000). Besides, the spice aroma and taste of pepper increase consumers’ appetite for food and enhance their digestion system. Botanically, the fruit of pepper is a drupe but it is more commonly known as berry. Pepper of commerce is categorized into black and white pepper based on the colour of the products, the pepper corns. However, both of them came from the same plant. White pepper corns are the dried seeds prepared by removal of pericarp of ripe berries after retting in water while black pepper corns are the dried fruits with pericarp intact (Ravindran, 2000). The high commercial value of pepper has placed it as economically important crop for countries like Vietnam, India, Indonesia and Malaysia.

In Malaysia, pepper is an important agricultural crop especially in the state of Sarawak contributing up to 95% of pepper production in the country. Malaysian pepper is mainly exported to Singapore, Japan, Taiwan, South Korea, Germany, USA and the Netherlands. In year 2010, Vietnam was the largest pepper producer producing 90,000 tonnes of pepper while Malaysia produced 23,500 tonnes of pepper is the sixth major pepper producer (International Pepper Community, 2010). In India, over 195,050 hectares pepper cultivation areas produced 5340 tonnes of pepper annually in average whereby Kerala state contributed 96% of pepper production (Farooqi et al., 2005).
The cultivation of pepper globally as well as in Malaysia has many constraints affecting production. In India, according to Sarma and Kallo (2004), the main factors that contributed to low pepper production were low yield, high cost of production, insufficient supply of healthy planting materials for replanting or new planting as well as biotic (diseases and pests) and abiotic stresses (drought) which led to the loss of crop. In addition, inadequate planting material of high yielding varieties also result low pepper production (Nair and Gupta, 2003). However, in Sarawak, Malaysia, the main constraint is high cost of production which includes input for plant maintenance and input for pests and diseases control (Kueh, 1986).

The major pests and diseases of *Piper nigrum* are pepper weevil (*Lophobaris piperis*), Pepper tingid bug (*Diconocoris hewetti*), root-knot nematodes (*Meloidogyne incognita, M. javanica and M. arenaria*), phytophthora foot-rot (*Phytophthora capsici*), fusarium wilt (*Fusarium* spp.), black berry disease (*Colletotrichum capsici, C. piperis and C. gloeosporioides*), velvet blight (*Septobasidium* sp.) and viruses (Mammootty and Neema, 2006). These pests and diseases are difficult to control or eliminate by chemicals and cultural practices (Kueh, 1986). The resistance genes to these major pests and diseases are not available from the varieties within the *P. nigrum* species. Therefore, in order to reduce or completely eradicate these obstacles, resistance to major pests and diseases need to be developed.

The development of resistance to pests and diseases can be carried out either through conventional breeding approach or development of transgenic plants through genetic