CHAPTER III
MATERIALS AND METHODS

3.1 Plant materials

Forty seven *H. tuberosus* genotypes (Table 3.1) obtained from Kaentawan Research Project, Department of Plant Science and Agricultural Resources, Faculty of Agriculture, Khon Kaen University. These *H. tuberosus* genotypes were collected from the Plant Gene Resources of Canada (PGRC), Canada and cultivated in Khon Kaen university farm.

Table 3.1 Plant materials used in this study.

Accession	Name	Origin	Germplasm collection	Year
JA-27	DHM-7	Canada	PGRC	1970
JA-28	DHM-13	Canada	PGRC	1970
JA-29	DHM-14	Canada	PGRC	1970
JA-30	DHM-16	Canada	PGRC	1970
JA-31	DHM-18	Canada	PGRC	1970
JA-32	DHM-19	Canada	PGRC	1970
JA-34	DHM-22	Canada	PGRC	1970
JA-35	W-97	Canada	PGRC	1970
JA-36	W-106	Canada	PGRC	1970
JA-42	75005	Canada	PGRC	1976
JA-43	75004-52	Canada	PGRC	1976
JA-44	A-3-6	Canada	PGRC	1976
JA-45	HM hybrid-A-4	Canada	PGRC	1976
JA-46	DHM-14-3	Canada	PGRC	1976
JA-47	DHM-14-6	Canada	PGRC	1976
JA-48	DHM-15	Canada	PGRC	1976
JA-49	7513A	Canada	PGRC	1976
JA-50	W-97	Canada	PGRC	1976
JA-54	Unknown	USA	PGRC	1978
JA-55	Unknown	USA	PGRC	1978
JA-58	Intress	USSR	PGRC	1979
JA-59	VOLZSKIJ-2	USSR	PGRC	1979
JA-60	JAMCOVSKIJ KRASHYJ	USSR	PGRC	1979

Table 3.1 Plant materials used in this study (cont.)

Accession	Name	Origin	Germplasm collection	Year
JA-61	VADIM	USSR	PGRC	1979
JA-66	FR. MAMMOTH WHITE	USA	PGRC	1981
JA-69	TUB-364 USDA-ARS-SR	USA	PGRC	1983
JA-70	TUB-365 USDA-ARS-SR	USA	PGRC	1983
JA-71	TUB-675 USDA-ARS-SR	USA	PGRC	1983
JA-72	TUB-676 USDA-ARS-SR	USA	PGRC	1983
JA-73	TUB-709 USDA-ARS-SR	USA	PGRC	1983
JA-74	TUB-847 USDA-ARS-SR	USA	PGRC	1983
JA-78	FUSEA U 60	France	PGRC	1983
JA-87	242-63	France	PGRC	1984
JA-88	TOPINSOL 63	USSR	PGRC	1984
JA-91	KIEVSKII	USSR	PGRC	1984
JA-92	INDUSTRIE	USSR	PGRC	1984
JA-95	NACHODKA	USSR	PGRC	1984
JA-97	D19-63340	France	PGRC	1984
JA-98	242-62	France	PGRC	1984
JA-100	105-62G2	France	PGRC	1984
JA-105	357303 VOLGA 2	USSR	PGRC	1989
JA-106	83-001-1 (37X6)	Canada	PGRC	1983
JA-107	83-001-2 (37X6)	Canada	PGRC	1983
JA-108	83-001-3 (37X6)	Canada	PGRC	1983
JA-109	83-001-4 (37X6)	Canada	PGRC	1983
JA-110	83-001-5 (37X6)	Canada	PGRC	1983
JA-111	83-001-6 (37X6)	Canada	PGRC	1983

3.2 DNA extraction and purification

A small quantity of young fresh leaf material from *H. tuberosus* (Table 3.1) was collected and isolated by a modified Tai and Tanksley (1990) method. The tissue was ground with a homogenizer and 0.7 ml of extraction buffer (100mM Tris.HCL pH8, 50mMEDTA pH8, 0.5M NaCl, 1.25% SDS, 8.3mM NaOH, 0.38% Na bisulfite) was added and mixed by vortexing. The sample was incubated at 65 °C for 30 min and 0.22 ml of 5M potassium acetate added and mixed well. The tube was placed on ice for 40 min, then centrifuged for 3 min. The supernatant was transferred to the new tube. The DNA was precipitated by adding 0.7 volume of isopopanol. Mixing well and centrifuged

for 3 min. The supernatant was poured off and the pellet rinsed with 70% ethanol, then the pellet was resuspended in 300 µl of T_5E (50mM Tris-HCl pH 8.0, 10mM EDTA) by briefly vortexing and incubated at 65 °C for 5 min, then vortexing again. 150 µl of 7.4M ammonium acetate was added and mixed well before centrifuged for 3 min and removed the supernatant to the new tube. The DNA was precipitated by mixing with 330 µl of isopopanol and centrifuged for 3 min. The pellet was rinsed with 70% ethanol and resuspended in 100 µl of T5E by vortexing and incubated at 65 °C for 5 min then vortexing again. DNA was resuspended in 150 µL of Tris-EDTA (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The purity and quality of genomic DNA were detected after digested with RNaseA (Sigma), and quantitated on 1% agarose gel against known concentration of ladder DNA, (100bp DNA ladder plus, Vivantis). Genomic DNA was resuspended in water and stored at –20°C.

3.3 Genetic diversity of *H. tuberosus* based on SRAP markers

3.3.1 DNA amplification by SRAP primers and detection

The selected primers were based on previous reports (Li and Quiros, 2001). SRAP primer combinations (Table 3.2); 1) ME2-EM5, 2) ME2-EM6, 3) ME2-EM8, 4) ME5-EM5, 5) ME5-EM6, 6) ME5-EM8, 7) ME7-EM5, 8) ME7-EM6 and 9) ME7-EM8 were used to amplify genomic DNA of 47 *H. tuberosus* genotpyes.

Table 3.2 Sequences of the SRAP primers used in this study

Primer	Type	Sequence	%GC	Tm (°C)
ME2	Forward	TGAGTCCAAACCGGAGC	58.8	59.6
ME5	Forward	TGAGTCCAAACCGGAAG	52.9	57.2
ME7	Forward	TGAGTCCTTTCCGGTCC	58.8	59.6
EM5	Reverse	GACTGCGTACGAATTCAA	44.4	55.3
EM6	Reverse	GACTGCGTACGAATTCCA	50.0	57.6
EM8	Reverse	GACTGCGTACGAATTCAC	50.0	57.6

A total of 10 µl PCR reaction mixture was composed of 1x *Taq* buffer (75 mM Tris-HCl (pH 8.4), 20 mM $(NH_4)_2SO_4$, and 0.01% Tween 20: Fermentas), 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, 0.5 µM of each primer, 0.4 unit/10 µl of *Taq* DNA polymerase (Fermentas, U.S.A), and 30 ng of template DNA. PCR amplification was carried out in a the Gradient Palm Cycler PCR machine (Corbett Research, Australia) programmed for pre-denaturalization of 3 min at 95 ^{O}C and 5 cycles (or otherwise stated) of 1 min at 95 ^{O}C, 1 min at 35 ^{O}C, and 2 min at 72 ^{O}C, followed by 35 cycles of 1 min at 95 ^{O}C, 1 min at 50 ^{O}C, and 2 min at 72 ^{O}C, finally by one cycle of 5 min at 72 ^{O}C. The SRAP products were analyzed by a 1.5% agarose gel electrophoresis, ethydium bromide stained and visualized by Electrophoresis Gel Photodocumentation System (Vilber Lourmat, Japan). In addition, the PCR products also were analyzed by electrophoresis on 10% (w/v) polyacrylamide gel and revealed DNA bands by a gel silver staining. 100 bp DNA ladder plus, Vivantis was used as a molecular size standard.

3.4 Genetic diversity of *H. tuberosus* based on EST-SSR markers

3.4.1 Searching for Jerusalem artichoke EST databases for resistance gene homologs

Jerusalem artichoke transcript assemblies (TAs) that were BLAST annotated in the Compositae Genome Database (CGPdb; http://www.cgpdb.ucdavis.edu/) were screened to identify unigenes involved in resistance genes (homologous to disease resistance genes) using "disease" or "resistance" keyword searches. The candidate EST sequences were assembled using CAP3 (Huang and Madan 1999) and aligned using ClustalW system (Higgins et al., 1994) to identify redundant sequences and were performed BLASTX analysis against the NCBI Protein Database to substantiate the homology of the selected *H. tuberosus* ESTs to genes involved in resistance identified in *Helianthus* or *Arabidopsis* species (Altschul et al., 1997; http://www.ncbi.nlm.nih.gov). These detailed data on the homology of R gene were used to classify the EST sequences into subgroups.

3.4.2 Sequence and genetic diversity analysis

To determine whether EST sequences encoded which conserved motifs, The candidate EST on sequences were translated in six frames using the expert protein analysis system (ExPASy) on proteomics server of the Swiss Institute of Bioinformatics (SIB) (http://cn.expasy.org/). Each peptide sequences were scanned against the nonredundant (nr) protein database by BLASTP searches (Altschul et al., 1990) for the presence of known domains. A multiple alignment analysis was performed with ClustalW for nucleotide sequences and amino acid translations of the EST sequences. Phylogenetic analyses were performed on nucleotide sequences and amino acid translations of the EST sequence data using the neighbor-joining method as implemented in ClustalW with default analysis parameters.

3.4.3 Searching for the microsatellites

To obtain an idea about putative functions of SSR containing genes, these *H. tuberosus* ESTs were used to identify and characterize SSRs using the WebSat software (http://wsmartins.net/websat). This computer program was run under windows, using a FASTA-formatted sequence file containing multiple sequences. The parameters were set for detection of di-, tri-, tetra-, penta- and hexanucleotide motifs. Single nucleotide repeats were not selected, because they were generally not considered as useful as polymorphic markers.

3.4.4 Designing primer for EST-SSRs

Primers were designed to flank SSR region of each EST of *H. tuberosus* using the Primer 3 (Whitehead Institute of Biomedical Research - http://www.genome. wi.mit.edu /cgi-bin/ primer/primer3_www.cgi) with modification. The major parameters for primer design were set as follows: primer length from 19 to 21 nucleotides, PCR product size ranges from 100 to 300 bp, optimum annealing temperature at 52-55 °C and 50% GC contents. These primers were then tested against genomic DNA of *H. tuberosus*. Polymorphisms were detected by PCR amplification with these specific flanking primers.

3.4.5 PCR amplification by EST-SSR primers and detection

The PCR amplification was done in a final volume of 10 μL containing 50 ng of DNA, 0.6 units of *Taq* DNA polymerase, 1x reaction buffer (75 mM Tris-HCl (pH 8.4), 20 mM $(NH_4)_2SO_4$, and 0.01% Tween 20: Fermentas), 1 mM of MgCl2, 150 mM of each dNTPs, and 0.1 mM of each primer. Amplification was done in a thermal cycler (Corbett Research, German). using a PCR procedure, which consisted of denaturing at 95°C for 3 min, followed by 35 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min 30 s followed by a final extension at 72°C for 8 min. After the PCR reaction, the amplification products were resolved by electrophoresis on 10% polyacrylamide gels. Bands were revealed using a gel silver-staining and bands were analyzed.

3.4.6 DNA cloning, sequencing and analysis

3.4.6.1 Recombinant plasmid construction

In order to clone putative R-gene PCR fragments (from 3.4.5) into a bacterial cloning vector, pGEM-T easy vector (Promega, USA) were used. The PCR product was excised and eluted using GF-1 gel recovery kit (Vivantis, Malaysia) according to manufacturer protocol and ligated to linearized T-overhang pGEM-T easy vector. The recombinant plasmids were transformed into *E.coli* JM109 for plasmid amplification as described in 3.4.6.2.

3.4.6.2 Bacterial transformation and selection

Competent cells were prepared prior to bacterial transformation. *E. coli* strain JM109 was used as host cells. *E. coli* starter was made from a single colony of the bacteria grown at 160 rpm (Takigen incubator, Japan) overnight at 37 ^{O}C in 2 ml LB medium (1% Bacto Tryptone, 0.5% Bacto Yeast extract and 0.5% NaCl). Starter (1 ml) was transferred into 250-ml LB medium and subsequently incubated at 37 ^{O}C for 3-4 hrs or until the cell density reached OD_{600} 0.3-0.5. The culture was then incubated on ice for 15 min before centrifuged at 3,500 rpm (Sorvall Super T21 Centrifuge, Dupont U.S.A.) for 15 min at 4^{O}C. The cell pellet was washed with 0.4 volume of cold RFI

buffer (50mM Mn.4H_2O, 30mM CH_3COOK, 10mM KCl, 10 mM $CaCl_2$, 15% glycerol, pH 5.8) to 1 volume of culture medium and centrifuged at 3,500 rpm for 15 min at 4°C. The resulting pellet of cells was then resuspended in small amount of RFII buffer (10mM MOPS, 30mM KCl, 75mM $CaCl_2$, 15% glycerol, pH 6.8), kept on ice for 30-60 min then aliquots of 100 µl were transferred into 1.5 ml tubes and stored at -80°C until used.

Bacterial transformation was made by heat shock transformation. Competent cells were thawed on ice before adding 2 µl (10-50 ng) extracted recombinant plasmid or ligation mixture, mixed gently by flicking and incubated on ice for 30 min. After that, the mixture was heat-shocked at 42°C for 30-45 sec, which never exceeded 45 sec, before kept on ice for 2 min. LB media (900 µl) was added in and incubated at 37°C for 2 hrs with agitation at 160 rpm. The transformed cells were spreaded onto either IPTG-X-Gal LB-ampicillin agar or LB-amplicilin agar plates for selection.

Selection of recombinant pGEM-T easy transformed cells was made using blue-white colony screening. White colony of positively transformed cells grown onto LB-ampicillin agar plate, in the presence of 100 mM IPTG (40 µl) and 20mg/ml X-Gal in DMFO (80 µl), were selected. Positive clones (white colonies) were then analyzed in order to select the expected sized clones using T7 and SP6 primers.

3.4.6.3 DNA sequencing and analysis

Nucleotide sequencing of PCR amplified fragments were performed using the dideoxy-mediated chain termination method (Sanger et al., 1977). Sequencing of the target DNA fragment was conducted at the Department of Biochemistry, Faculty of Medicine, Khon Kaen University by using the ALF express automatic DNA sequencer (Pharmacia Biotech, Sweden). The nucleotide sequences were analyzed and aligned by using the standard Blast system in GenBank database, ClustalW version 1.8.3 and BioEdit version 7.0.4.1.

3.5 Data analysis

Amplified fragments were defined as a single character. The allelic data for all genotypes were scored in the form of binary data matrix (BDM) where 1 represented

presence and 0 (zero) absence of bands. The binary data matrix were used to calculate the polymorphic information content (PIC) values as follows (Anderson et al., 1993).

$$PIC = 1 - \sum_{i=1}^{k} P_i^2$$

where, k is the total number of alleles detected for a microsatellite marker and *Pi* is the frequency of the i^{th} allele in the set of analysed genotypes.

The binary data were also used to derive similarity coefficients, and three most commonly used; the Simple matching, Jaccard and Dice coefficients (Table 3.3) were compared among 47 *H. tuberosus* genotypes. Dendrograms were developed through Unweighted Pair Group Method with Arithmetic (UPGMA), Weighted Pair Group Method using Arithmetic averages (WPGMA), Complete Linkage and Single Linkage (Jackson et al., 1989; Duarte et al., 1999) clustering methods over the original matrices calculated from Simple Matching, Dice and Jaccard similarity coefficients in NTSYS Pc 2.1 program. Clustering methods and similarity coefficients were tested by applying SIMQUAL, SAHN, TREE procedures in NTSYS Pc 2.1. As the clustering method in SAHN module, WARN was selected in the option of UPGMA, WPGMA, Single Linkage and Complete Linkage and Tie Method (Rohlf, 2000). Cophenetic correlation coefficients were calculated by using COPH and MXCOMP procedures for each combination. The correlation matrices calculated show the goodness of fit of cluster analysis in accordance with the similarity matrix.

Table 3.3 Similarity cœfficient studies

Coefficients	Similarity expression	Source
Simple matching (SM)	m/n	Sokal and Sneath, 1963
Jaccard (J)	a/(n-d)	Jaccard, 1908
Dice (D)	2a/(2a + b + c)	Dice, 1945

In the above table a, b, c, d, m, n and u are defined as follows for a two-way frequency table for all pairs of two objects i and j (Table 3.4):

Table 3.4 Two-way frequency table for all pairs of two objects i and j

		j	
		+	–
i	+	a	b
	–	c	d

Where (+) = presence band, (–) = absence band, m = a + d (number of matches), u = b + c (number of "unmatches"), and n = u+m (total sample size).