MUHAMMAD UMAIR AHSAN

CANDIDATE GENE IDENTIFICATION IN *Lotus japonicus* USING INSERTIONAL MUTAGENSIS AND GENETIC MAPPING

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2013
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APPROVAL PAGE

This is to certify that I have read this thesis written by MUHAMMAD UMAIR AHSAN and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science in Genetics & Bioengineering.

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July 2013

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M.S. Thesis – Genetics & Bioengineering
2013

Thesis Supervisor: Assoc. Prof. Mustafa Fatih ABASIYANIK

ABSTRACT

Lotus japonicus is a model legume plant, which has the ability to fix atmospheric nitrogen by symbiosis with rhizobia. The forward genetics approach uses a particular phenotype to identify which gene is responsible for certain changes. Up to now, a number of symbiotic and non symbiotic genes were discovered in lotus. Insertional mutagenesis is one of the most powerful tools to investigate gene function. For generating the insertional mutations on large scale, application of transposable elements can be an alternative approach. To facilitate progress in this field, we perform forward genetic approach for LORE1-tagged mutant population and image-based quantitative phenotyping of plant responses to microbial inoculation followed by association analysis based on available genotypic information from *L. japonicus* natural accessions. Lotus retrotransposon 1 (LORE1) belongs to Gypsy-type retrotransposon and is 5kbp long. LORE1 can be derepressed during tissue culture, which leads to a few new insertions in the next generations. It was shown that LORE1 inserts randomly, without sequence preferences. By adopting Sequence Specific Amplified Polymorphism (SSAP) procedure we are able to find some good candidate genes in different LORE1 mutated lines. *L. japonicus* natural accessions are infected with Sclerotinia and were afterward subjected to association mapping analysis. With generated Manhattan plot it is quite obvious that no significant association is found but these results can unveils some clues for further studies.

Keywords: Sequence Specific Amplified Polymorphism, LORE1, Insertional Mutagensis, symbiosis, Genetic mapping, association analysis

Lotus japonicus'DA INSERSIYONEL MUTAGENESIS VE GEN HARITALAMA TEKNIKLERINI KULLANARAK ADAY GEN IDENTIFIKASYONU

MUHAMMAD UMAIR AHSAN

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ÖZ

Lotus japonicas, rhizobia ile simbiyoz sonucu atmosferik azot fiksasyonu yeteneğine sahip model bir baklagil bitkisidir. Ileri genetik çalışmalarda, belirli fenotip bazlı değişikliklerden sorumlu genleri belirlemek için kullanır. Şimdiye kadar, Lotus bitkisinde simbiyotik ve simbiyotik olmayan birçok gen keşfedilmiştir. Insersiyonel mutagenez, gen fonksiyonu araştırmalarında kullanılan güçlü araçlardan biridir. Büyük ölçekte insersiyonel mutasyonlar üretmek için, alternatif bir yaklaşım olarak, transpoze edilebilir elemanlar uygulanabilir. Bu alanda ilerleme kaydedebilmek için, LORE1 etiketli mutant populasyon için ileri genetik uygulamalar yapıldı ve mikrobiyal aşılamalara bitki tepkisi görüntü tabanlı nicel fenotipleme analizi ile yapıldı. Bunu takiben *L. japonicus*'un doğal accessionlarindaki uygun genotipik bilgiler baz alınarak asosisasyon analizi yapıldı. Lotus retrotransposon 1 (LORE1) Gypsy-tipi retrotranspozonlardandır ve 5kbp uzunluğundadır. LORE1 doku kültürü sırasında stress olabilir ve bu sonraki jenerasyonlarda birkaç yeni insertsiyona yol açar. LORE1'in sekans ayrımı yapmadan, rastgele insersiye olduğu belirlenmiştir. Sekans spesifik amplifiye edilmis polimorfizm (SSAP) prosedürünu kullanarak, mutasyona uğramış farklı LORE1 hatlarında birkaç uygun aday gen bulabiliriz. *L. Japonicus* doğal aksesyonlari Sclerotinia ile enfekte edilmiş ve daha sonra asosisasyon haritalama analizine tabi tutulmuştur. Oluşturulan Manhattan plot sonucu belirli bir anlamlı ilişki bulunamamakla birlikte bu sonuçlar daha ileri çalışmalar için önemli ipuçları sağlayabilir.

Keywords: Sekans spesifik amplifiye edilmiş polimorfizm, Insersiyonel mutagenez, simbiyoz, genetik haritalama, asosisasyon analizi.

Dedicated
To my beloved Country Pakistan

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SYMBOL/ABBREVIATION	
SSAP	Sequence specific amplifying polymorphism
LORE1	LOTUS RETROTRANSPOSON 1
T-DNA	transfer DNA
CARB	Centre for Carbohydrate Recognition and Signalling, Denmark
EMS	Ethyl methanesulfonate
MNU	N-Nitroso-N-methylurea
TE	Transposable Elements
TIRs	Terminal Inverted Repeats
LTR	Long Terminal Repeat
ENV	non-Envelop Protein
LINEs	Long interspersed elements
SINEs	Short interspersed elements
TSDs	Target Site Duplication

CHAPTER 1

INTRODUCTION

Legumes contribute substantially to the general food supply worldwide. They are cultivated for either food or forage and are a main source of protein-rich plant material. Due to the fixation of dinitrogen in symbiosis with rhizobia, growth of legumes is independent of external addition of reduced nitrogen. In a survey conducted for approximately 3000 legume species, 90% have the ability to form nitrogen- fixing nodules (Allen and Allen, 1981).The estimates shows that it saves US$10 billion for commercial chemicals. Legumes are the enrich source of 33% of the dietary protein nitrogen needs of human being. Also, they contribute to generation of 35% of all vegetable oil (Graham and Vance 2003). To study and anlyzing better the genetics of nitrogen fixing process and symbiosis the model plant was emerge.

In 1992, Handberg and Stougaard suggested *Lotus japonicus* as a model legume. *Lotus japonicus*, a model legume plant, can be able to fix atmospheric nitrogen by symbiosis with rhizobia. *Lotus japonicus* has important features, which make it easy to study. Lotus is a diploid plant having a small genome (~480 Mbp). Large-scale sequencing of *Lotus japonicus* was initiated in 2000. Uptil now, a large part of the Lotus genome is available with the annotation of genes (Sato et al. 2006). It is an autogamous plant, producing numerous seeds within 3-4 months. It is easy to transform with *Agrobacterium tumenfaciens* as well as crossing and vegetative propagation (Handberg and Stougaard 1992). Numbers of genes involved in symbiosis were discovered by forward genetics approach - map based cloning, (Sandal et al. 2006). But still lots of genes are yet to be unveiled.

Insertional mutagenesis is the most powerful tool to investigate gene function. In plants, *Agrobacterium* T-DNA and maize transposable elements have been extensively used as insertional mutagens (An et al., 2005). In *Arabidopsis thaliana* Over 225,000 independent Agrobacterium transferred DNA (T-DNA) insertion were examined and through this T-DNA tagging, near saturation mutagenesis of the Arabidopsis genome has been achieved. Retrotransposons, genomic elements which are quite similar to retroviruses, are the potential future of insertional mutagenesis. Lotus retrotransposon 1 (LORE1) is an endogenous retrotransposon which belongs to Gypsy-type retrotransposon and is 5kbp long. Well suited for insertion mutagenesis, LORE1 can be derepressed during tissue culture, leads to some new insertions in the next generations, randomly and without sequence preference.

This study is concerned with identification of genes using different forward genetic approaches in *Lotus japonicus.* The first one is phenotypic screening of LORE1-tagged mutant populations for aberrant nodulation phenotypes followed by identification of causal mutations through sequence specific ampliphied polymorphism (SSAP). The second is image-based quantitative phenotyping of plant responses to microbial inoculation followed by the association analysis based on available genotypic information from *L. japonicus* natural accessions.

CHAPTER 2

LITERATURE SURVEY

2.1 LEGUMES CHARACTRISTICS

Leguminosae (Fabaceae) is the second most important plant family for humankind after Gramineae. Around 12% to 15% of the Earth's arable surface is under cultivation of Legumes for both grain and forage purposes (Graham and Vance 2003). Legumes have the ability to develop root nodules and to fix atmospheric nitrogen by symbiosis with the help of compatible rhizobium bacteria. With this function of fixing Nitrogen, Legumes plays key role in sustainable agriculture and the nitrogen cycle. It has been calculated that legumes saves US$10 billion for commercial chemicals. Grain legumes are the source of 33% of the dietary protein nitrogen needs of humans. Also they contribute to production of 35% of all vegetable oil (Graham and Vance 2003). To develop better understanding regarding the genetics of nitrogen fixing process and symbiosis the need of model legume plant emerges. In 1992, Handberg and Stougaard suggested *Lotus japonicus* as a model legume plant (Handberg and Stougaard 1992).

Figure 2.1 Distribution of Lotus species around globe (Joseph H. K. 1999).

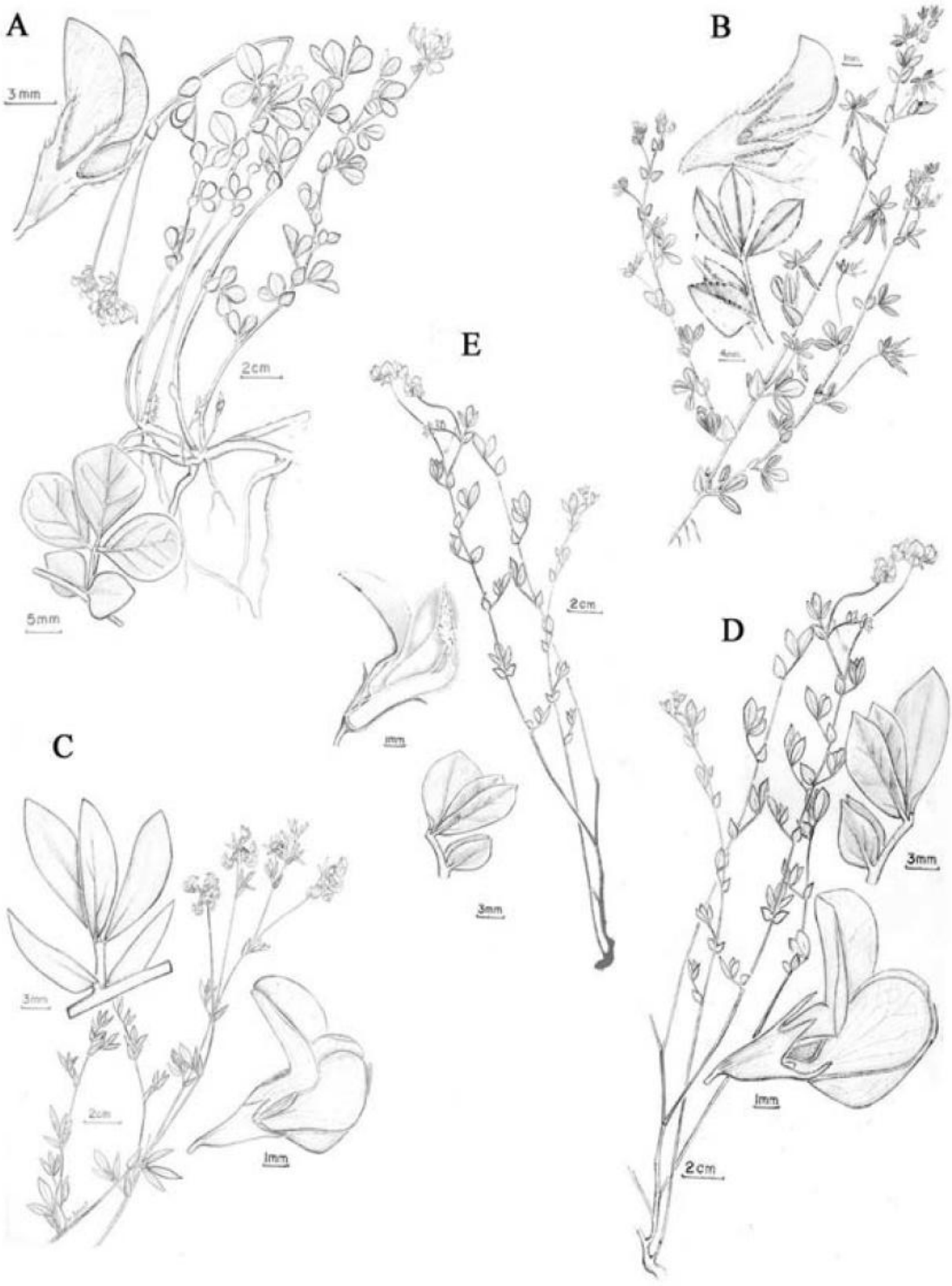

Figure 2.2 Botanical drawing of agriculturally important Lotus species (A) *L. uliginosus*; B) *L. subbiflorus*; C) *L. glaber*; D) *L. Corniculatus*, and (E) *L. Japonicas* (Márquez et al. 2005).

2.1.1 *Lotus japonicus*

Lotus japonicus belongs to *Leguminosae* family and it has the ability to fix atmospheric nitrogen by symbiosis with rhizobia. It is easy to study *Lotus japonicus* due to important features that it bears (Table 1). It is a diploid plant with a small genome (~480 Mbp). Large-scale sequencing in *Lotus japonicus* was initiated in 2000. A large part of the Lotus genome is available with the annotation of genes (Sato et al. 2006). On KAZUSA Institute webpage all the information is published online. It is an autogamous plant, able to produce numerous seeds within 3-4 months. Interestingly, it is easy to transform with *Agrobacterium tumenfaciens,* to cross and to propagate vegetatively (Handberg and Stougaard 1992). Through forward genetics approach - map based cloning, a number of genes involved in symbiosis were discovered (Sandal et al. 2006).

Table 2.1 *L. japonicus* model plant features (Handberg and Stougaard, 1992).

Growth characteristics	Primary plant small Bushy plant after secondary shoot formation, and branching Perennial Period from seed to flowering, 7 weeks Re-growth from stem base/top root Plant multiplication from nodal sections Generation time, seed to seed, 3-4 months Small seeds; 1,2 g per 1000 seeds
Propagation	Large flowers Self-fertile No seed scattering Hand pollination possible Approximately 20 seeds per pod Approximately 6000 seeds per plant
Genome characteristics	Diploid, $2n = 12$ 0.5 pg DNA per haploid genome
Tissue culture	Regeneration from callus Hygromycin and kanamycin selection Regeneration of transgenic plants *Agrobacterium tumefaciens* transformation Transfer of hygromycin resistance to $\mathbf{F_1}$
Nodulation	Fast growing *Rhizobium loti* Determinate nodules

2.2 INVESTIGATION OF GENE FUNCION

To examine gene function two approaches can be used i.e., forward and reverse genetics (Figure 2.3). Forward genetics approach refers to the selection a particular phenotypic trait of interest and then exploring the genetic locus or loci that control that specific trait. While, the reverse genetics allows to investigate the function of the particular gene by introducing the mutation and analyzing the subsequent phenotype (Nancy et al. 2002, Peters et al. 2003). Nowadays a variety of tools facilitating the introduction of the mutation are available. Physical and chemical mutagens were widely used for the purpose of creating induce mutation in any organism. Ethyl methanesulfonate (EMS), an alkyIating agent is commonly used in chemical mutagenesis (Koh et al. 2001). By alkylating the guanine bases EMS can cause point mutations leading to the base mispairing (Talebi et al, 2012). The alkylated guanine pairs with thymine, ultimately causing the G/C to A/T transitions. Other chemical mutagens, widely used are the sodium azide (Az) and N-methyl-N-nitrosourea (MNU). Sodium Azide induces A/T to G/C transition, while MNU is causing the G/C to A/T transition. The pH is an important factor upon which the effect of sodium Azide depends on. Also it was shown that Az can be mutagenic only to certain organisms. X-rays, fast neutrons and gamma rays are the most commonly used physical mutagens. They can cause DNA breaks and also the formation of abasic sites, which leads to substitutions and deletions (Sparrow et al. 1974, Kurowska et al. 2011).

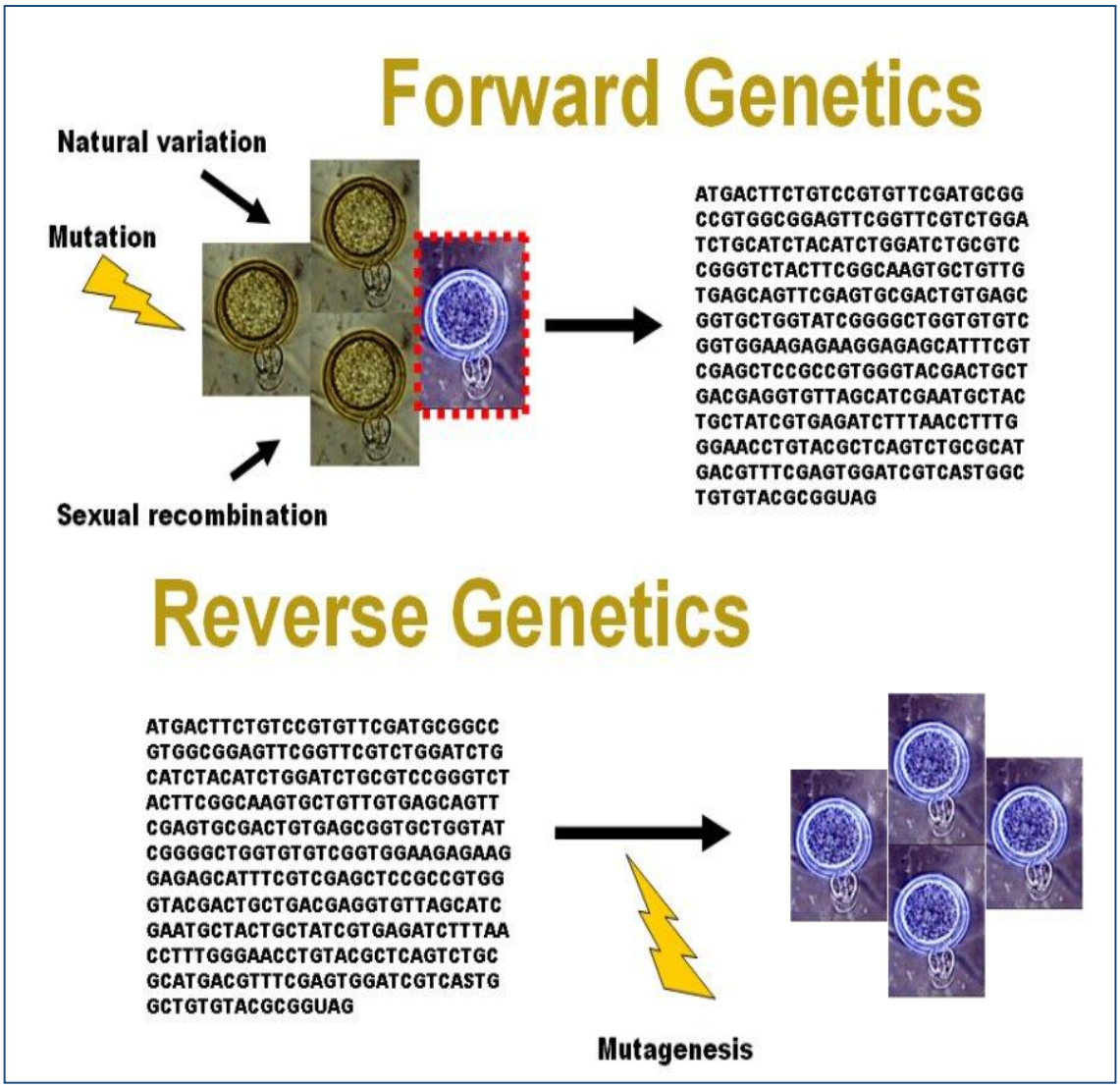

Figure 2.3 Major differences between forward and reverse genetics. The blue oospore symbolizes an observable phenotype that may have been produced via any one of a number of pathways (mutation, recombination etc.). This is the starting point for A) forward genetics where the investigator proceeds from the phenotype to characterize the underlying genetic difference. B) Illustrates reverse genetics where the sequence for a gene is known but the gene's function is unknown and the investigator disrupts the gene and investigates the resulting phenotype (Melinda et al. 2005).

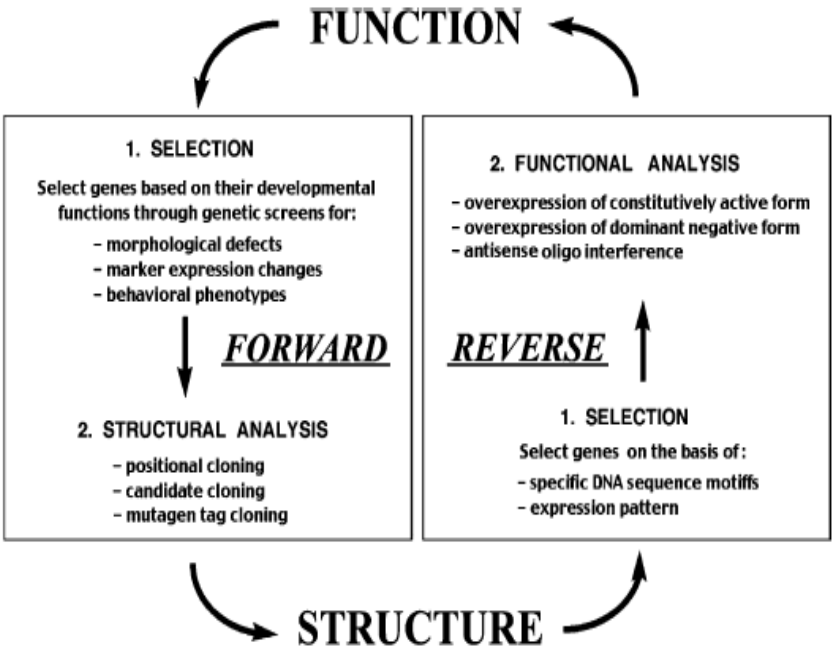

Figure 2.4 Flow chart of Forward and reverse genetics approaches in zebra fish (Malicki et al. 2002).

2.2.1 Insertional Mutagenesis

Insertional mutagenesis is one of the most powerful tools to investigate gene function. The main advantage of using this mutagenic approach is the possibility to generate loss of function mutations. *A. tumefaciens* (a plant-pathogenic bacterium) has the ability to transfer a segment of DNA (T-DNA) into a host genome of interest; this phenomenon has been widely used to create insertion mutants in a wide range of plant species (Tzfira & Citovsky 2006). T-DNA tagging mediated by *Agrobacterium tumefaciens* is a successful method utilized in Arabidopsis. A small genome and easy transformation permits tagging of a large number of *Arabidopsis thaliana* genes (Alonso et al. 2003). The plants generated by T-DNA mutagenesis usually carry 1 to 3 insertions per line. So by using this phenomenon more than 100,000 plants would be needed to saturate Arabidospis genome with mutation in at least 95% of all genes. More than 200,000 mutations were created for Arabidopsis by using insertional mutagenesis and were placed in Arabidopsis stock centers (Jander et al. 2002; Alonso et al. 2003).

Application of transposable elements can be an alternative approach for generating the insertional mutations on large scale. Transposons are mobile genetic

elements and in the genome they can multiply using a variety of mechanisms. There are two classes of these transposable elements: DNA transposons and retrotransposons. DNA transposons (TE) belongs to the class II of transposable elements and they were observed to move into the novel places in the genome, but the disadvantage is that they generate unstable insertions, because of the usage of a cut and paste mechanism by them (Figure 2.6). TE typically consists of terminal inverted repeats (TIRs), which may differ in length. The transposase, which is able to recognize the TIR and cut both strands on the ends, initiates the transposition of this element. One of the well-known transposons is Ac/Ds being discovered in maize. On the other hand as compared to DNA transposons, retrotransposons, belongs to class I are moving in a copy and paste mechanism by mRNA intermediate and reverse transcription thus generating stable copies in the genome (Wessler 2006; Tadege et al. 2008). Retrotransposons are divided into two groups: One is that are flanked by Long Terminal Repeats (LTR) retrotransposons and the others are non-LTR retrotransposons. LTR retrotransposon can be very short fragments, from a few hundred base pair (bp) up to 25 kbp. They consist of two direct long terminal repeats (LTR), which vary in length from 100 bp to several kbp long. They have wide presence in plants, but not present in the animals. Most of the time they behave like an autonomous elements which means that they encode the proteins necessary for their transposition. They consist of pol genes and gag genes. Pol gene encodes the polyprotein which fulfills the function of reverse transcriptase, aspartic proteinase, integrase and RNase H, while gag gene encodes the capsid-like protein. Moreover, LTR retrotransposons posses: packaging, reverse transcription, dimerization and integration signals. Among this subclass of retrotransposons, two big superfamilies are characterized: Ty1 or *Copia* and Ty3 *Gypsy*. They are moving in the genome using similar mechanisms, but the major structural difference between copia and gypsy groups is in the order of integrase and reverse transcriptase in the polyprotein.

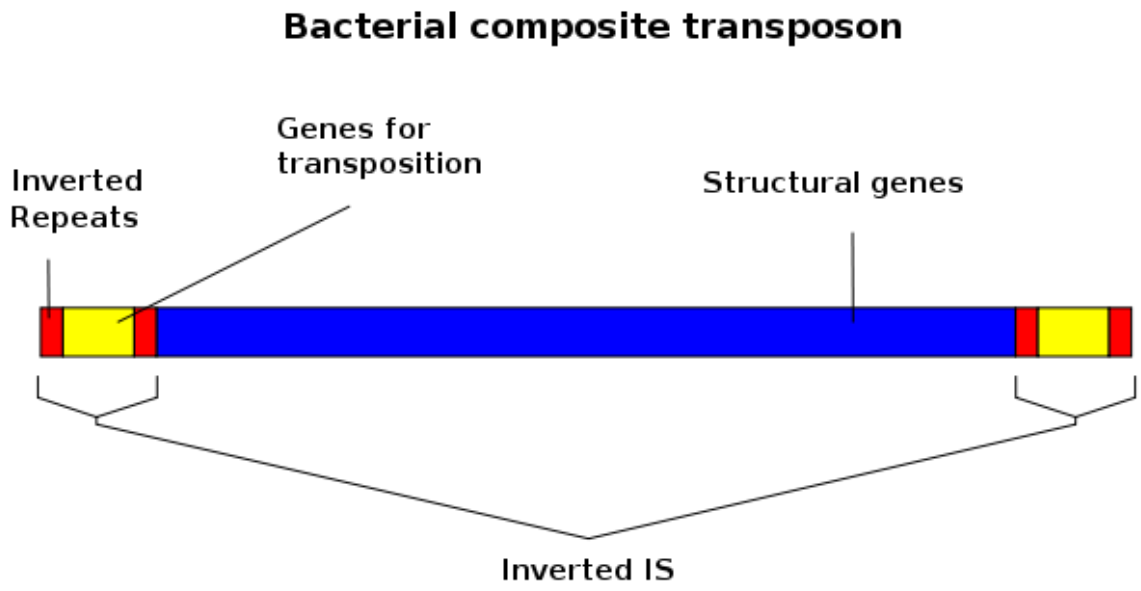

Figure 2.5 Graphical illustration of a bacterial DNA transposon (Jacek F.H. 2007).

Previous studies suggest that the retroviruses had their origin in the *Gypsy* group elements. It is more likely that retroviruses evolved from this group ultimately acquiring the envelope protein (ENV) and other regulatory sequences. The group of non-LTR retrotransposon consists of autonomous long interspersed elements (LINEs) and non-autonomous short interspersed elements (SINEs) (Wessler 2006; Wicker et al. 2007). LINEs are the extremely diverse subclass of retrotransposons and they can be a few kbp long. They were found in all Eukaryots. Typically, they encode reverse transcriptase and nuclease. On the other hand, SINEs are short, typically 80 to 500 bp long elements. They are able to generate the 5 to 15 bp target site duplication (TSDs). Despite the fact that they belong to class I transposable elements they have different origin. It was suggested that they evolved from random insertions of polymerase III transcripts. One of the most known SINE is Alu element present in humans (Wicker et al. 2007).

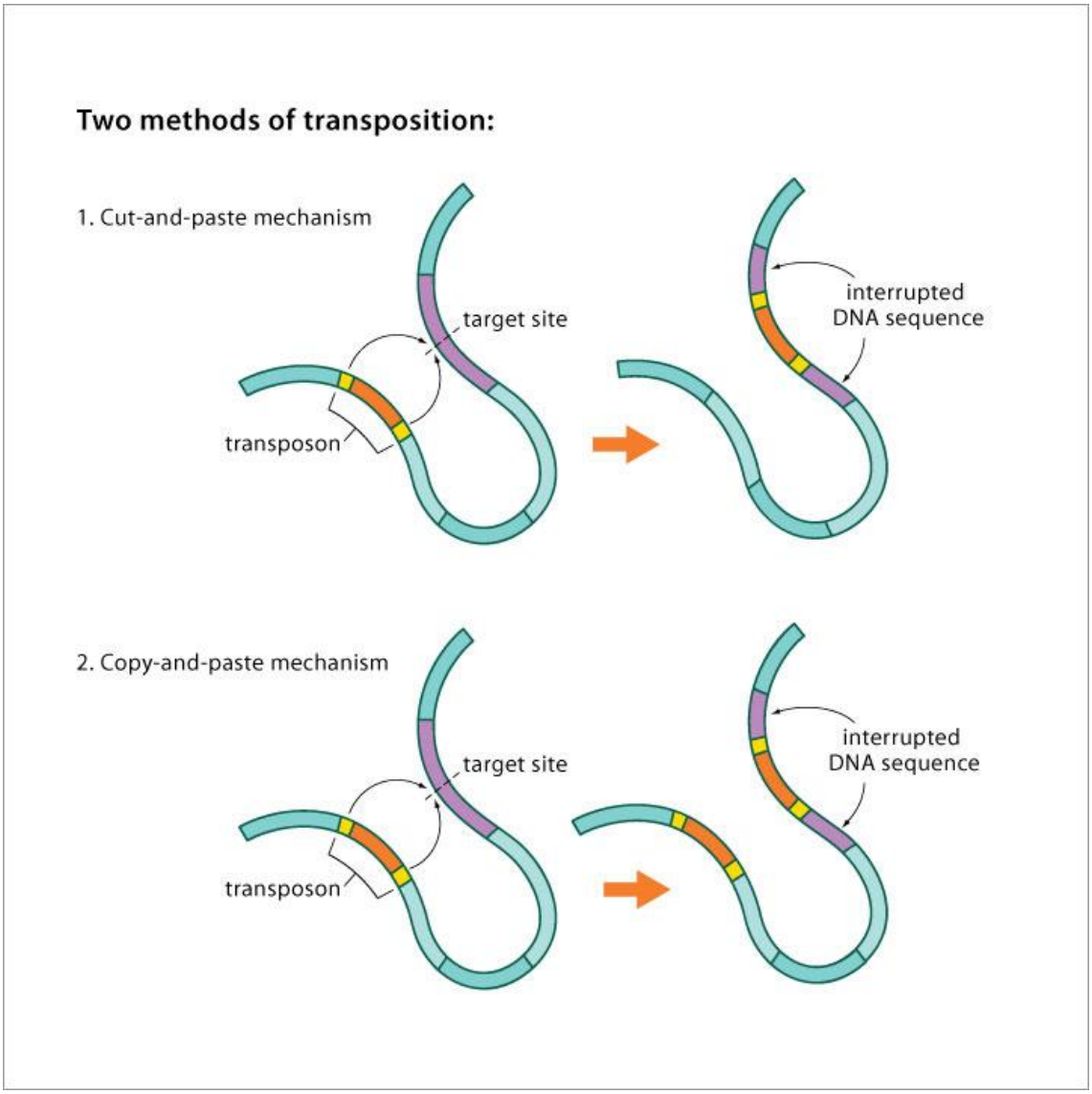

Figure 2.6 Methods of transposition, 1. Cut and paste Mechanism, 2. Copy and paste Mechanism.

In plants, transposons were widely and successfully applied as insertional mutagens in recent years. In 2003, the population of 43,776 plants containing *Mutador* insertions was generated for *Zea mays* (May et al. 2003; McCarty et al. 2005). In *Medicago truncatula* the exogenous *Tnt1* retrotransposon, originating from *Nicotiana tabacum,* was used. *Tnt1* belongs to the LTR retrotransposon subclass. Figure 2.7 shows the Schematic representation of Tnt1 tagging in *Medicago truncatula*. The tobacco long-terminal repeat retrotransposon Tnt1 was first introduced into *M. truncatula* by Agrobacterium-mediated transformation. A homozygous line with low-copy-number Tnt1 inserts (three copies) was grown in soil to bulk up the seeds; these seeds are being

used to make all the transposed insertion lines. Seeds are germinated and grown in soil (for R108) or on agar medium (for A17); leaf explants are cultured on auxin-containing medium for five weeks. Calli are formed from individual leaf explants and plants are regenerated from individual calli via somatic embryogenesis. Tnt1 activation and transposition occur during the process of callus formation and also probably during embryogenesis. Thus, each plant derived from individual callus represents an independently transposed Tnt1 line. Seeds from each regenerated plant will be stored for forward genetic screens. DNA extracted from the leaves of each line can be used either to identify flanking plant sequences using Tnt1-specific primers by inverse PCR or can be stored for future use to identify the mutated gene in a desired mutant identified through a forward-genetic screen. DNA from different lines can also be pooled together in a systematic fashion so that it can be conveniently screened for any gene of interest using Tnt1 and gene-specific primers by a reverse genetics approach. It was shown that it can be successfully used to generate approximately 7,600 lines, conferring around 190,000 insertions (Tadege et al. 2008). In *Oryza sativa,* the *Tos17* retrotransposon was applied and it was shown that it can generate 5-30 copies per transformed plant. The drawback of this element is a presence of hot spots (Hirochika et al. 1996). In *Petunia* it was shown that the endogenous *dTph1* element is able to generate approximately 10,000 insertions in 1,000 individuals (Vandenbussche et al. 2008). In Lotus, Ac/Ds element originated from maize was used, in order to identify the new mutants. Unfortunately the procedure was very laborious and it did not generate many insertions (Fukai et al. 2008).

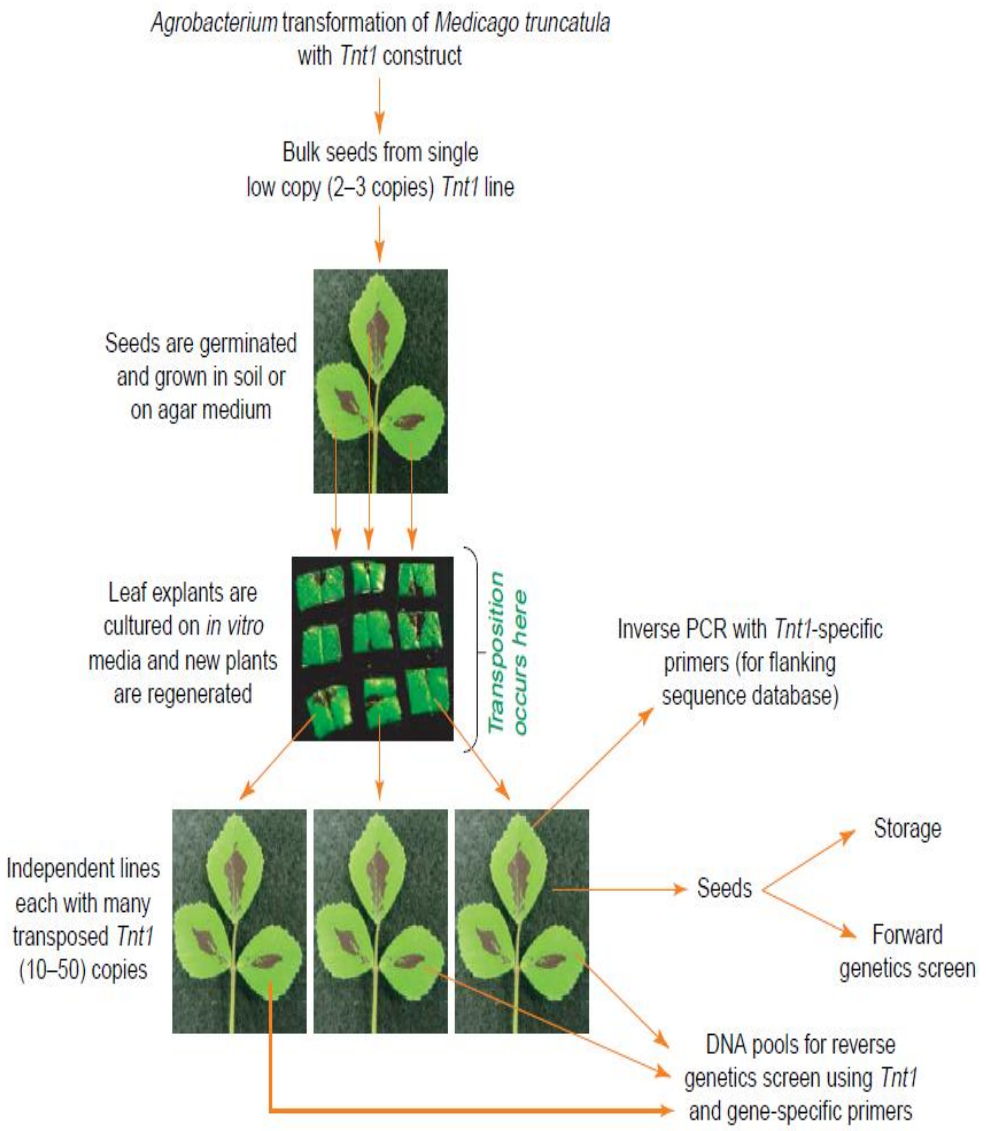

Figure 2.7 The schematic representation of Tnt1 tagging in Medicago truncatula (Million et al., 2005).

2.2.1.1 *LORE1 as an Insertional Mutagenesis Tool*

Lotus japonicus retrotransposon 1 (LORE1) is an endogenous retrotransposon, which belongs to the *Gypsy* family. LORE1 is 5,041 bp long and it consists of two direct LTRs (Figure 2.8). LORE1 is present in 9-10 copies in the Lotus genome (Madsen et al. 2005).

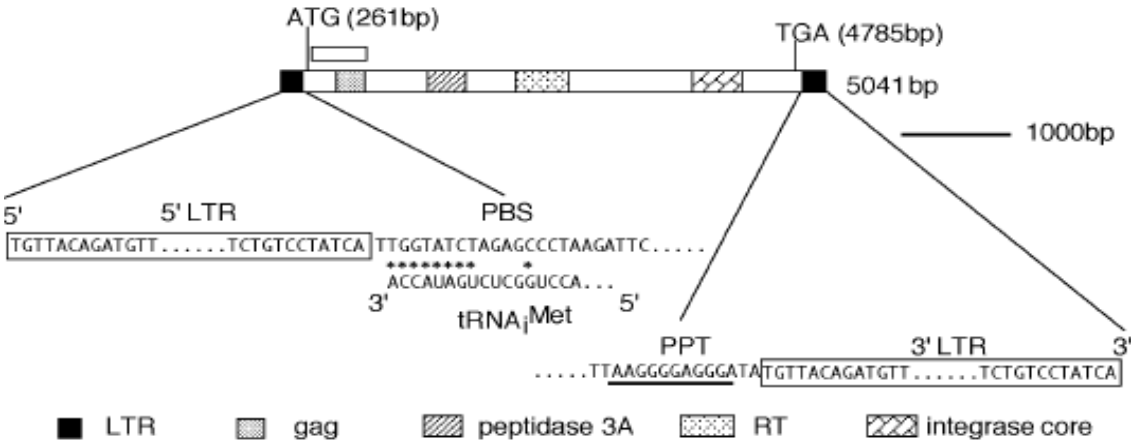

Figure 2.8 The Schematic representation of LORE1a. Black boxes, long terminal repeats (LTRs). While the Other signatures mark regions showing homology with characteristic retrotransposon proteins.

Up to now, the whole or partial sequence of 9 is available. Nucleotide sequence polymorphisms were observed between the copies, which allowed distinguishing and naming them alphabetically: LORE1a - LORE1i. LORE1 is an autonomous element, which means it consists of gag and pol genes (Wessler 2006). It was shown that LORE1 can be epigenetically de-repressed during tissue culture. The de-repression is stochastic and independent of antibiotic selection and presence or absence of transgenes. What is more, once activated, it can transpose for at least two generations. LORE1d, e, f, h and i was mainly found in the heterochromatin region, while LORE1a, b, c and g were observed to insert in the regions which does not resemble heterochromatin. LORE1a was shown to be the most active out of all the LORE1 elements. The insertions are inherited and they do not seem to have region preferences. LORE1 is more often transposing to the gene-rich places. Analysis of insertion-sites shows no nucleotide composition preferences. It was suggested that it is inserting in the gene-rich region, because the DNA is more available for transcriptional regulation. Fusion of LORE1a 5'LTR with GUS indicates that LORE1 is active in pollen (Fukai et al. 2010; Eigo et al., 2012; Urbanski et al. 2012). Native insertion of LORE1a is in kinase gene, which shows homology to a mitogen-activated protein kinase gene (Madsen et al. 2005).

Application of transposable elements is an approach use for the generation of insertional mutagenesis in large scale. Transposable elements can be described in two

classes; DNA transposons and retrotransposons. Retrotransposons belongs to class I are working by a copy and paste mechanism by mRNA intermediate and reverse transcription, generates stable copies in the genome (Wessler 2006; Tadege et al. 2008).

Lotus japonicus retrotransposon 1 (LORE1) is an endogenous retrotransposon, can be easily used as a tool for mutagenesis. The founder line G-329-3 which carried the active retroelement was used to perform large-scale mutagenesis. In R2 generation approximately 2, 4 new insertions were observed (Urbanski et al. 2012).

2.2.1.2 Snowy Cotyledon Gene

During early development seedlings undergo a transition from heterotrophic to autotrophic growth. This transition to autotrophic growth requires a light-induced rapid transformation of etioplasts to chloroplasts that seems to be restricted to cotyledons and normally does not occur in true leaves. Several of mutants in Arabidopsis which have pale yellow cotyledon but green leaves were identified and dubbed snowy cotyledon. Allelism tests of these mutants revealed two different loci i.e., sco1 and sco2 (Albrecht et al. 2005). Backcrossing of homozygous sco1 mutants with wild-type plants confirmed that the phenotype of the mutant was due to one recessive mutation. The mutated SCO2 gene (Snowy cotyledon) was identified using a map-based cloning strategy (Zhao et al., 2007). The Figure 2.9 shows the phenotypes of Arabidopsis wild-type and a cyo1 mutant. All were grown under continuous light at 238C on 1/2 MS agar plate medium with 1.5% sucrose. It is clear from the figure that the mutant has albino (white) cotyledon phenotype, while wild type has normal cotyledon. Also in the cyo1 mutant plant complemented with the CYO1 genomic DNA clone the phenotype is quite similar with that of wild-type. So it was concluded that CYO1 is responsible for the white cotyledon in the Arabidopsis plant. CYO1 has a C4-type zinc finger domain similar to that of Escherichia coli DnaJ. CYO1 is expressed mainly in young plants under light conditions. Recombinant CYO1 speed up the disulfide bond reduction in the model substrate insulin and renatures RNase A is an indication that CYO1 may have protein disulfide isomerase activity (Shimada et al. 2007)

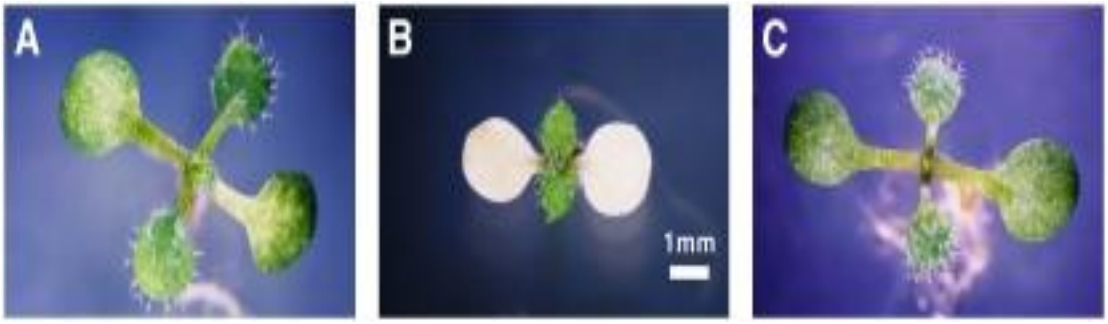

Figure 2.9 In Arabidopsis A) a 10-d-old wild-type plant, (B) a cyo1 (SCO2) mutant plant with albino phenotype, (C) and a cyo1 mutant plant complemented with the CYO1 genomic DNA clone.

2.2.1.3 Subtilase Family

Serine peptidases also called as proteolytic enzymes that mainly depend on a serine residue for catalytic activity, fall into more than 20 families that can be grouped into six families. Three of them are the chymotrypsin, subtilisin and carboxypeptidase C families which share a common reaction mechanism based on the 'catalytic triad' comprising a serine, a histidine residue, and an aspartic acid. However, the protein folds of these enzymes, however, are quite different. Subtilases family belongs to subtilisin-like serine proteases. The subtilisin family is the second largest serine protease family characterised to date. Over 200 subtilases are known, more than 170 of which with their complete amino acid sequence (Siezen et al. 1997). The subtilisin-like serine protease, SbtM1 is strongly and specifically induced during arbuscular mycorrhiza (AM) symbiosis in *Lotus japonicas* (Takeda et al. 2011).

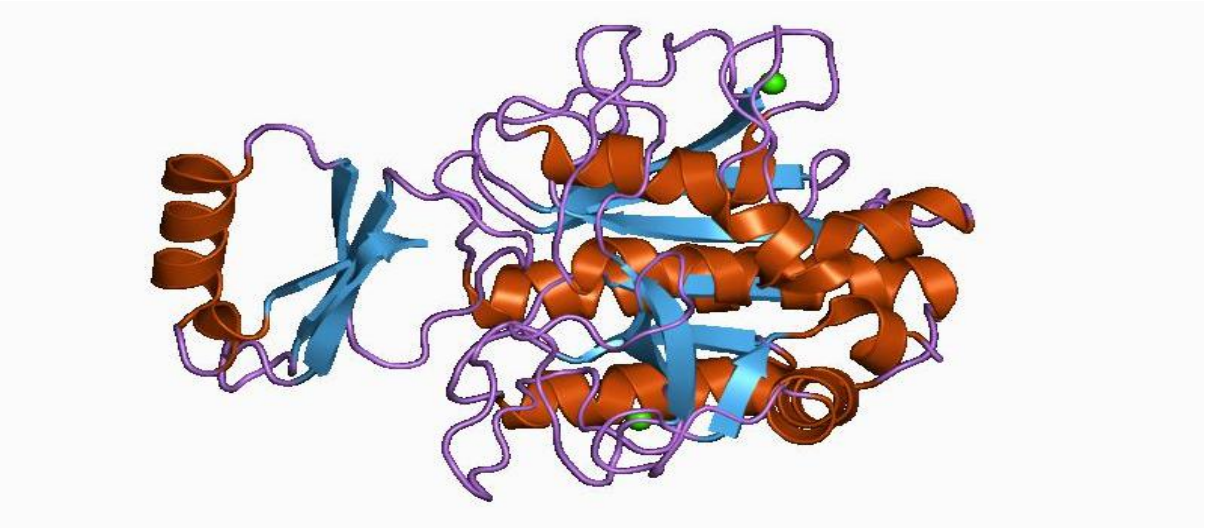

Figure 2.10 Structure of the complex formed between subtilisin Carlsberg and eglin c, an elastase inhibitor from the leech *Hirudo medicinalis* (Bode et al. 1987).

2.4 ASSOCIATION MAPPING

Association mapping (linkage disequilibrium mapping) is a method of mapping quantitative trait loci (QTLs) that takes advantage of historic linkage disequilibrium to link observable characteristics (phenotypes) to the genetic constitution of organisms (genotypes). Association mapping is rapidly becoming an important method to explore the genetic architecture of complex traits in plants and offers unique opportunities for studying resistance to insect herbivores. Genomewide association (GWA) studies are a promising route to associate phenotypes with genotypes, at a genome-wide level, using unrelated (from same species) individuals. Association mapping allows screening of many different wild and cultivated populations for genes involved in complex plant traits (Karen et al. 2012). Understanding the genetic basis of phenotypic variation is one of the key goals in evolutionary biology. Family-based quantitative trait locus (QTL) mapping which uses well characterized pedigrees and association mapping which uses linkage disequilibrium among numerous individuals of different populations are the most commonly used tools for dissecting the genetic basis of phenotypic trait variation (Brotman et al. 2011, Balasubramanian et al. 2009, Ingvarsson et al. 2011).

Table. 2.2 Comparison of family-based (QTL) and population-based association mapping methods that aims to unravel the genetic basis of complex traits in plants (Karen et al. 2012).

	QTL mapping	**Candidate gene association mapping**	**Genome-wide association mapping**
Main advantages	No population structure effects Identification of rare alleles Few genetic markers required	Allows fine mapping Relatively low costs	Allows untargeted fine mapping (blind approach) Detection of common alleles
Main disadvantages	Limited genetic diversity Not always possible to create crosses Cannot distinguish between pleiotropic and physically close genes	Detailed functional knowledge of trait is required No novel traits will be found	Confounding effects due to population structure Will miss rare and weak effect alleles
General requirements	Small ‘original population size’, low number of genetic markers, many replicates needed Generated mapping material, e.g. F2 population, (AI-)RILs, MAGIC lines, NILs, HIFs, etc.	Large population size, small number of genetic markers, the bigger the population size, the less replicates needed Prior genetic and biochemical knowledge on trait of interest Prior knowledge on LD, nucleotide polymorphism, breeding system and population structure	Large population size, many genetic markers, the bigger the population size, the fewer replicates needed Prior knowledge on LD, nucleotide polymorphism, breeding system and population structure
Recent case study in Arabidopsis	QTL mapping with AI-RILs on flowering time two AI-RIL populations (approximately 280 individuals each) 181 and 224 markers 12 to 70 replicates	Candidate gene approach on flowering time 251 accessions 51 SNPs 10 replicates per accession	Whole-genome approach on multiple phenotypic traits 199 accessions in total 216 150 SNPs Four replicates in general

CHAPTER 3

MATERIALS AND METHODS

3.1 FORWARD SCREENING OF LORE1 LINE

Forward screening of LORE1 line has been performed in the green house of Department of Molecular Biology & Genetics, Aarhus University Denmark. Thanks to Niels we were able to get the mutated lines having probable candidate gene of interest. So the leaves of plants from the selected lines were taken and were subjected to following procedure:

3.1.1 DNA extraction

DNA extraction was performed by CTAB method (Murray and Thompson 1980). Leaf material was collected in blue base/8 strip plates. The samples were grind using vortex and 1 porcelain beads (9, 3 mm) in each tube. 400 µl of preheated CATB buffer was added. Samples were incubated at 65°C for 30 min and mixed regularly. Subsequently, 400 µl of chloroform:isoamylalcohol (24:1) was added, subsequently vortexed until the formation of uniform emulsion. Samples were centrifuged for 30 min at 2717 rcf at 4°C. Subsequently, about 4 ml of water-phase was moved to the fresh 13 ml tube. 0.6 µl of RNAse H (10 mg/ml) was added, vortexed and they were incubated for 30 min at 37°C. The samples were place on the ice for 5 min and then 0.6 volumes (about 300 µl) of ice-cold isopropanol were added and content was mixed. Samples were incubated at -20°C overnight. After that samples were centrifuged for 30 min at 2717 rcf at 4°C. The isopropanol was discarded and 400 µl of 70% ethanol was added in the samples. The content was mixed gently and centrifuged again for 30 min at 271 rcf at 4°C. 70% EtOH was discarded and pellet was dried for 30 min. Subsequently, the

obtained pellet was resuspended in 20 µl of TE buffer and the content was mixed. Samples were stored overnight at 4°C. Supernatant was moved to the 96-well plate and 0. 8% gel was run to check the quality.

3.1.2 Sequence Specific Amplified Polymorphism (SSAP) for LORE1

Using CTAB method DNA was extracted and by performing PCR, subsequently run on the agarose gel, the quality of DNA was tested. For PCR reaction the following PCR mix is used (Table 3.1) and the PCR conditions (Table 3.2).

After that the DNA from each sample was treated with restriction enzymes: MboI and Csp6I in corresponding buffer for 3h at 37°C. The reaction was performed in 20 µl: 4 µl of plant DNA, 2 µl buffer 10x (buffer B for Csp6I, while buffer R for MboI), 13 µl of H_2O and 1 µl of Restriction enzyme. For inactivation of restriction enzymes, the samples were incubated for 20 min at 80°C. Adaptor is made by mixing SPLlong oligo with appropriate SPLshort oligo (Table 3.3)

Table 3.1 PCR mixture used for DNA quality test.

	1 x
Forward primer (100 µM)	0.05 µl
Reverse primer "P2" (100 µM)	0.05 µl
dNTP	0.1 µl
10x Taq polymerase buffer	2 µl
Taq polymerase (5u/µl)	0.1 µl
DNA	1 µl
ddH2O	To 20 µl

Table 3.2 PCR conditions adopted in DNA quality test.

Temperature	Duration	Cycles
95°C	3 min	
95°C	30 sec	5 Cycles
72°C	1 min 15 sec	
95°C	30 sec	Touchdown 10 cycles
72-68°C	30 sec	
72°C	45 sec	
95°C	30 sec	15 cycles
68°C	30 sec	
72°C	45 sec	
72°C	10 min	

Table 3.3 SPLlong oligo and SPLshort oligo for MboI and Csp6I.

LORE1_SSAP_MboI	GATCACCACTAGTGTCGACACCAGTCTCTAATTTTTTTTTTCAAAAAAA
LORE1_SSAP_Csp6I	TAACCACTAGTGTCGACACCAGTCTCTAATTTTTTTTTTCAAAAAAA
SPL long strand	CGAAGAGTAACCGTTGCTAGGAGAGACCGTGGCTGAATGAGACTGGTGTCGACACTAGTGGT

Oligos were mixed and adding annealing buffer to 1x final concentration. For 1µg digested sample 15pmol of ready adaptor is needed. The tube mixed oligos and buffer was inserted to heating block (90°C) and incubated for 3 minutes. The heating block with tube was removed and placed in hot block until the temperature goes down to 30°C. Afterward the tube containing adaptor was placed in ice.

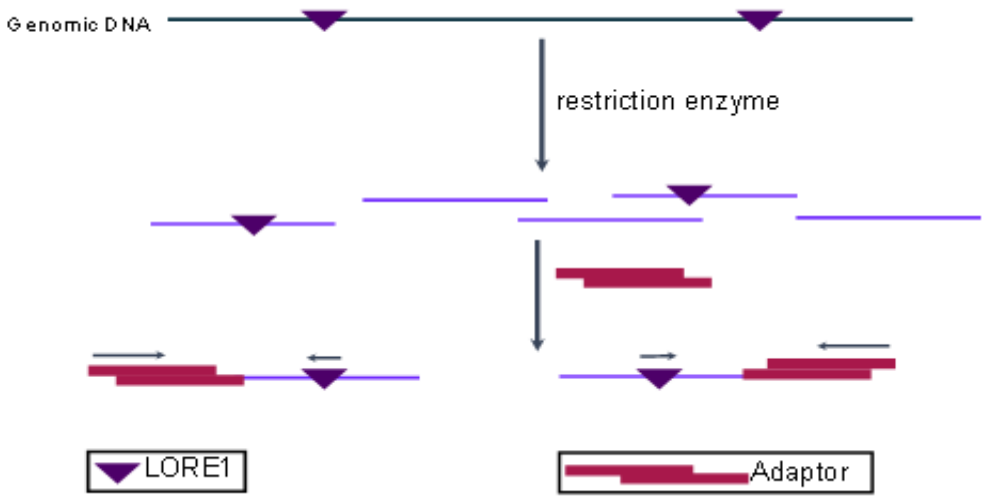

Figure 3.1 Working flow of Adopter ligation after the activity of restriction enzyme (Credit: Anna Malopsizy).

For Ligation: 1 µl T4 DNA ligase, adapter (50pmol/ µl) 0.3µl, 1.3 µl of 30 mM ATP and 22.4µl of H_20 was added to each sample. After that for 1h they were incubated at Room Temperature followed by the inactivation process of incubating at 65°C for 20 minutes. PEG precipitation was performed to remove the traces of adapter. The ligation reaction was transferred to 150µl PEG selection solution: 2 µl of 1M $MgCl_2$, 51 µl of 35% PEG 8000 and 97 µl of H_2O was added. Samples were vortexed followed by incubation at room temperature for 10 min. The samples were put on spinning at maximum speed for 30 min. The supernatant was carefully removed. 195 µl of 70% of ethanol was added into the sample followed by vortex process on slow speed. Then the Samples were centrifuged at maximum speed for 15 min, EtOH was removed and leaved until dry. The Samples were dissolved in 50 µl of H_20 and vortexed at low speed. Approximately 6 µl of DNA was used in PCR reaction.

For 1st PCR reaction the following PCR mix (Table 3.4) is used and PCR conditions (Table 3.5) were adopted.

Table 3.4 The 1st PCR reaction mixture.

LORE1 SSAP 1st PCR	1 x
PM4399 "Splink1" primer (100 µM)	0.05 µl
PM4152 primer "P2" (100 µM)	0.05 µl
dNTP	0.1 µl
10x Taq polymerase buffer	2 µl
Taq polymerase (5u/µl)	0.1 µl
DNA	6 µl
ddH2O	11.7 µl

Table 3.5 PCR conditions of 1st PCR reaction.

Temperature	Duration	Cycles
95°C	3 min	
95°C	30 sec	Touchdown 10 cycles
72-68°C	30 sec	
72°C	1 min 30 sec	
95°C	30 sec	15 cycles
68°C	30 sec	
72°C	1 min 30 sec	
72°C	7 min	

For nested PCR reaction the following PCR mix (Table 3.6) is used and PCR conditions (Table 3.7) were adopted.

Table 3.6 Nested PCR reaction's mixture.

LORE1 SSAP 2nd PCR	1 x
PM4400 "Splink2" primer (100 µM)	0.05 µl
PM4153 primer "P3" (100 µM)	0.05 µl
dNTP	0.1 µl
10x Taq polymerase buffer	2 µl
Taq polymerase (5u/µl)	0.1 µl
DNA	1 µl
ddH2O	16.7 µl

Table 3.7 Nested PCR conditions.

Temperature	Duration	Cycles
95°C	3 min	
95°C	30 sec	Touchdown 10 cycles
72-68°C	30 sec	
72°C	1 min 30 sec	
95°C	30 sec	20 cycles
68°C	30 sec	
72°C	1 min 30 sec	
72°C	7 min	

Then 5 µl of samples were run on 5% polyacrylamide gel.

Table 3.8 Explanation of commonly use terms in SSAP.

Splink 1	Primer used in first PCR for LORE1
Splink 2	Primer used in nested PCR or LORE1
P2	Primer used in first PCR for 5‘LTR LORE1
P3	Primer used in nested PCR for 5‘LTR LORE1
LORE2_SSAP1	Primer used in first PCR for 5‘LTR LORE2

3.1.3 Polyacrylamide Gel Preparation

Volumes of reagents used to cast 5% polyacrylamide gel are: 16.6 µl of 30% Acrylamide, 72.7 µl of Water, 10 µl of 5x TBE, 0.85 µl of 10% ammonium persulphate. 35 µl of TEMED (N,N,N`,N`-tetramethylethylenediamine) to the 100 acylamide solution and mixed by swirling. The glass plates and spacers were prepared for pouring the gel. 50ml syringe was used to pour the solution into the space between the two glass plates, after filling the space the combs were inserted between the head of the glass plates. The acrylamide solution was allowed to polymerize for 60 minutes at room temperature.The Gel were attached to the electrophoresis tank, using large bulldog paper clips on the sides. The reservoirs of electrophoresis tank was filled with 0.5x TBE, combs were removed and Pasteur pipette was used to flush out the wells with 0.5x TBE. 5 µl of DNA samples were mixed with 6 x gel loading dye and the mixture were loaded into the wells using a drawn-out glass micropipette. The electrodes were then connected to the power pack (40 mAmp current for 2 Gels). The Gel was allowed to run until the marker dyes have been migrated about half of the Gel. After that the glass plates were detached and laid on the bench. The upper glass plate was lifted with the help of thin spatula while the Gel remained on the lower plate. Then the Gel and its attached plate were submerge in staining solution (200 5 µl ethidium bromide in 0.5 x TBE) for 15 minutes. Photographs of Gel were taken by putting it on ultraviolet transilluminator. The photographs were analysed and the candidate band were identified.

3.1.4 Band Identification

The suspected band for mutations were cut from the gel with clean scalpel and were putted into 1.5ml tube and labelled. The polyacrylamide gel was crushed with the help of pipette tip, further on 100 µl of TE buffer is added in the tube containing gel, vortexed. After that the mixture was incubated for 10 minutes in a 95°C heating block, and then the sample was vortexed, further cooled on ice and spun for 2 minutes at maximum speed to precipitate gel fragments.

3.1.5 EXO/SAP Method

PCR product must be cleaned up prior to cycle-sequencing in order to remove remaining primers and unincorporated dNTPs. So EXO/SAP method was adopted. For 5 tubes the following concentrations were used: water 34.2 µl, 10x buffer 12.5 µl, Exonuclease (EXO) 1.65 µl, shrimp alkaline phosphate (SAP) 1.65 µl.

3.1.6 Band Sequencing

To perform sequencing the suspected band gel was further treated by adding: 1 µl of Big Dye sequencing mixture, 1.5 µl of 5x sequencing buffer, 1 µl of primer (PM4153, PM4400), 6.5 µl of DNA and water (1 µl of DNA). And the samples were run for sequencing.

3.2 ASSOCIATION MAPPING

3.2.1 Seed Treatment

The proper number of seeds were taken and put in the tube. Seeds were prepared for germination by scarifying seed coats in concentrated sulfuric acid (S6). Then by washing step 3 times in water and 1:30 dilution of sodium hypochloride treatment for 20 min. After that, seed were washed again in sterile deionized water 3 times. The seeds were left in small amount of ddH_20 on shaking platform overnight at 4°C. Subsequently they were sow out on the wet paper on the round plate and left for germination for 2 day. The plates were put vertically at 21°C room to generate straight roots. In the meantime the plates with ¼ B&D medium and 0.8% agar noble were prepared by

pouring the solution on the slope. 10 Germinating seeds were put on the plates and were closed by parafilm. All the above work was done in the flow band. The plant plates were placed in control room. The photoperiod was 16 h light and 8 h dark.

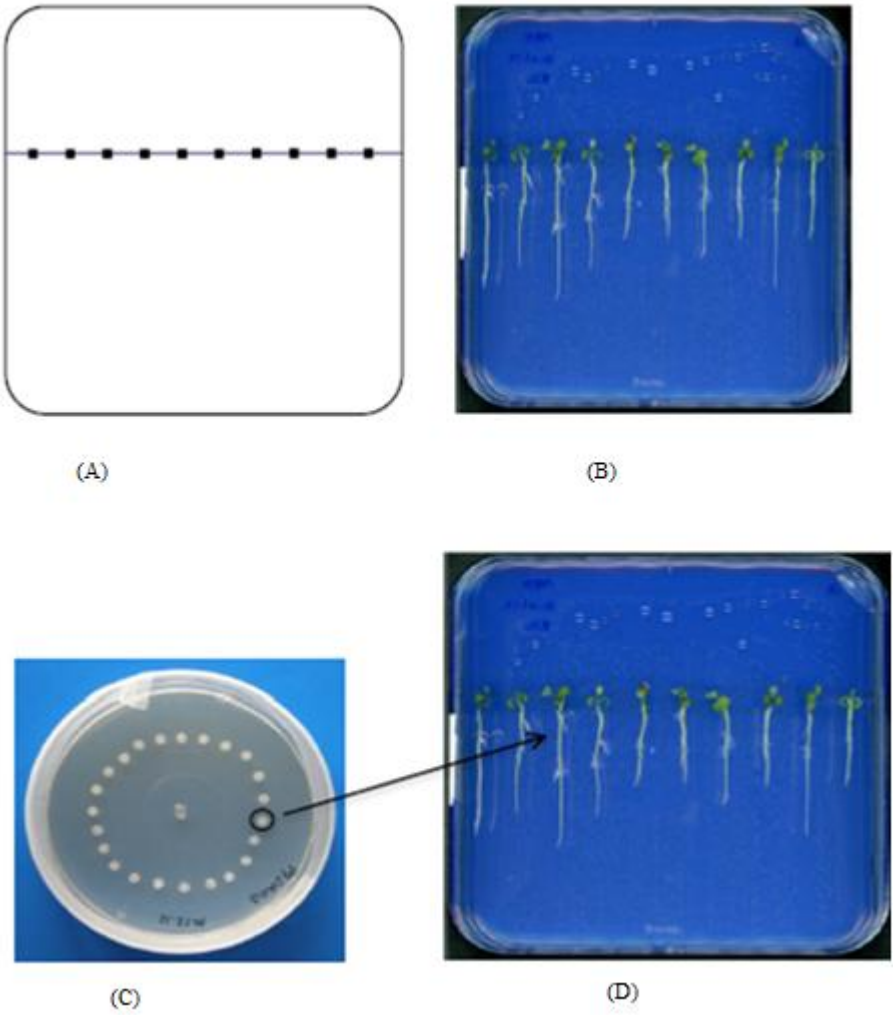

Figure 3.2 Layout of seedling placement on the casted plates and how to apply fungal infection on the cotyledon of plants.(A) Template of desired seedlings placement, (B) Placement of seedling on the plate, (C) Fungal culture on disc paper in Potato dextrose media, (D) Infecting the plantlets with Fungi by paper disc.

3.2.2 Experimental Plan

For fungal responses the experiments conducted are: MG1 with 26 accessions, MG2 with 18 accessions and MG3 with 16 accessions. While for Bacterial (Rhizobial) responses the experiments performed are: MGr1 with 28 accessions, MGr2 with 18 accessions and MGr3 with 15 accessions. MG20 and gifu wildtype are used in each experiment as control. For bacterial experiments the plantlets are inoculated with bacterial culture on day 04 of germination also 2 plates having MG20 and gifu are not inoculated with bacteria to remain as control of the experiments.

Table 3.9 Association mapping Experimental plan.

Day	**Experiment**	**Materials**
0	Seed Treatment’	Sulfuric Acid + HypoChloride 1/30 mixed water + Water
1	Germination	Plates with wet filter paper , foreceps,
4	Transfer to plates	¼ B&D Media plates(6.4 g of Agar Noble in 800 ml water)
Day 5 - 15	Every Day scanning the plates	Scanner
Day 17 – 25	Every second day scanning	
Day 25- 35	Putting in Cold chamber	Cold chamber with Pink light
Day 25	Fungi Culture from Scleritioa	PDA plates, forecpes, scalpel, EtOH(70% for flambé, 96 % Disinfection of foreceps), Water, Hypochloride 5%, empty sterile plate
Day30	Putting mycelium on Disc paper plates	Disc paper, PDA plates, foreceps, scalpel, parafilm
Day 34	Mycelium plates to Cold room	In dark
Day 35	Scanning before inoculation	
Day 35	Inoculation	Foreceps, after putting in the fungal incubator
1 Dpi- 8 dpi	Scanning the plates daily	
10- 32 dpi	Every second day scanning	

3.3. Bioinformatics

All the information about Lotus genome was obtain from KAZUSA and CARB Institutes webpages: http://www.kazusa.or.jp/lotus/,.http://users-mb.au.dk/pmgrp/, For Blast search we use: http://blast.ncbi.nlm.nih.gov/Blast.cgi, R Software, EMMAX, PLINK and Sequence analyses were done in CLC bio: Sequence Viewer Software.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 FORWARD SCREENING OF LORE1 LINE

From the forward screens that have been done by Niels and other fellows of CARB, Department of Molecular Biology and Genetics in the department green house, the R_2 of the lines shows the Mendalian phenotypic ratio of 3:1, the parents lines were subjected to insertion of LORE1 retro element with the help of agent *Agrobacterium tumefecian* in a process called as Insertional mutagenesis. The lines which are showing promising mutant phenotypes are: 30005017, 30005088, 30004049, 30005078, 30005403 and 30006602. The 30005017 has symbiotic mutant phenotype which could be either Nod- or Fix-. The line 30005078 mutants have light green leaves and also showing Fix- phenotype. The line 30005088 mutants have a clear phenotype of short bushy roots. Also the line 30005403 have probably symbiotic mutant showing the Nod- phenotype. While the mutants of line 30006602 have visible white sectors on leaves.

To demonstrate the phenotypes of mutants along with their wild-types, we have taken photos for some of our lines. The Fig. 4.2 shows the phenotypic expression of line 30005078: the mutants have light green leaves with fairly less amount of nodules on the roots also the plant size is short while on the other hand the wild types are dark green, healthy nodules and the plant length is longer. The line 30005088 mutants have clear distinction over the wild-types with short bushy root phenotype (Fig. 4.3). While the Fig. 4.4 demonstrate that the mutants belongs to the line 30006602 have white sectors on their leaves, the phenotypes of wild-types have clearly no white sectors on the leaves so it shows that this mutation is might due to the activity of retrotransposon element.

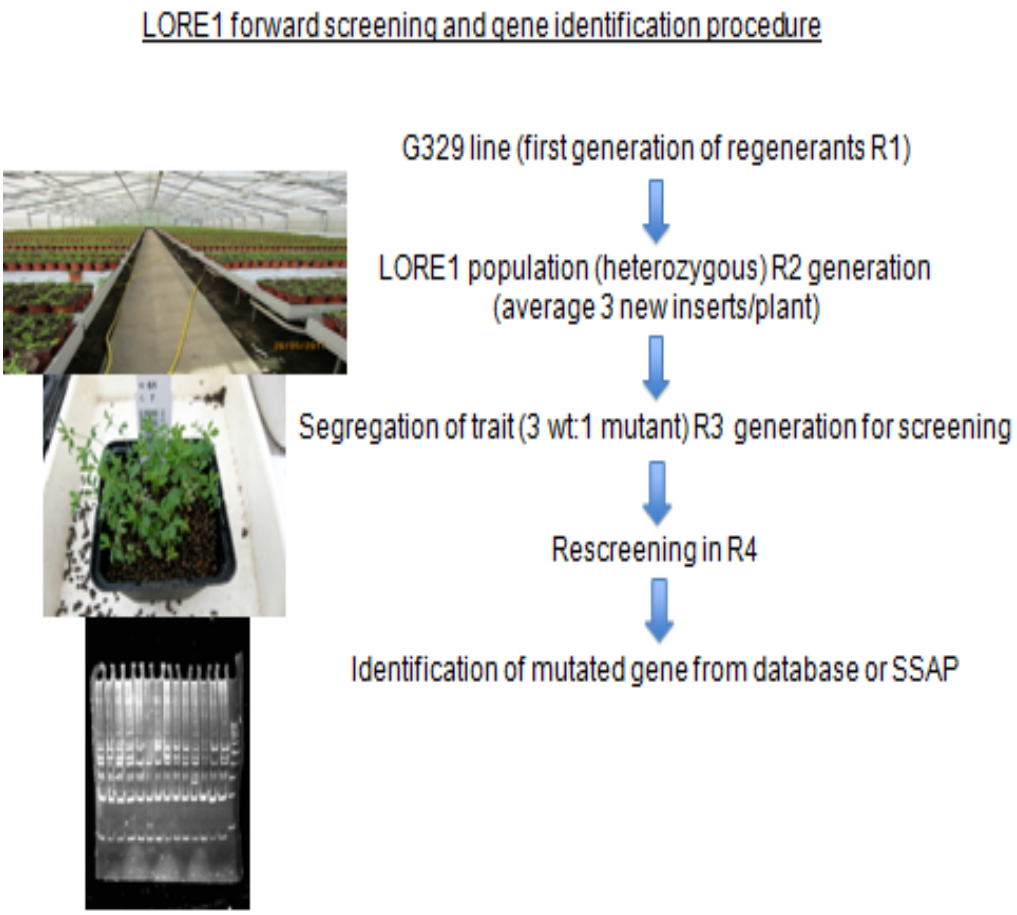

Figure. 4.1 LORE1 forward screening and gene identification procedure (Courtesy: Niels N. SANDAL).

Figure 4.2 Phenotypic expression of line 30005078, wildtypes on right side while the mutants are on left side.

Figure 4.3 Phenotypic expression of line 30005088, mutants have distinction with short roots plants.

Figure 4.4 Phenotypic expression of line 30006602, mutants have white sectors on the leaves.

So to check that whether any of these lines may have the gene of interest which may involve in any of these expressions we decided to perform Sequence Specific Amplified Polymorphism for all these lines. Because SSAP has the advantage of having the higher levels of polymorphism and also it needs less experimental procedure for the generation of desired numbers of Markers. Using the 5'LTR specific amplification approach and MboI, MspI and Csp6I restriction enzymes we aim to identify a band, which is present in each mutant with that phenotype, but should not be present in the wild-type plants. The results of SSAP for different lines are described below:

30005017: In this analysis we use 5 wild-type, 8 mutant plants and G-329 line as control. MboI and Csp6I are used as restriction enzyme. But the Gel shows no significant result for both the restriction enzymes (Fig. 4.5). So there is need to do with the 3'LTR specific amplification procedure or other restriction enzymes so that may be the cause of mutation can be found.

30005088: The mutants of this line have short bushy roots. We perform the SSAP procedure while using MboI and Csp6I as restriction enzymes. But the Gel shows no

promising co-segregating band with both the restriction enzymes (Fig. 4.6 (A)). As we perform only with 5'LTR end so there is need to perform SSAP with 3'LTR end which may results in finding of co-segregating band corresponding to the mutation.

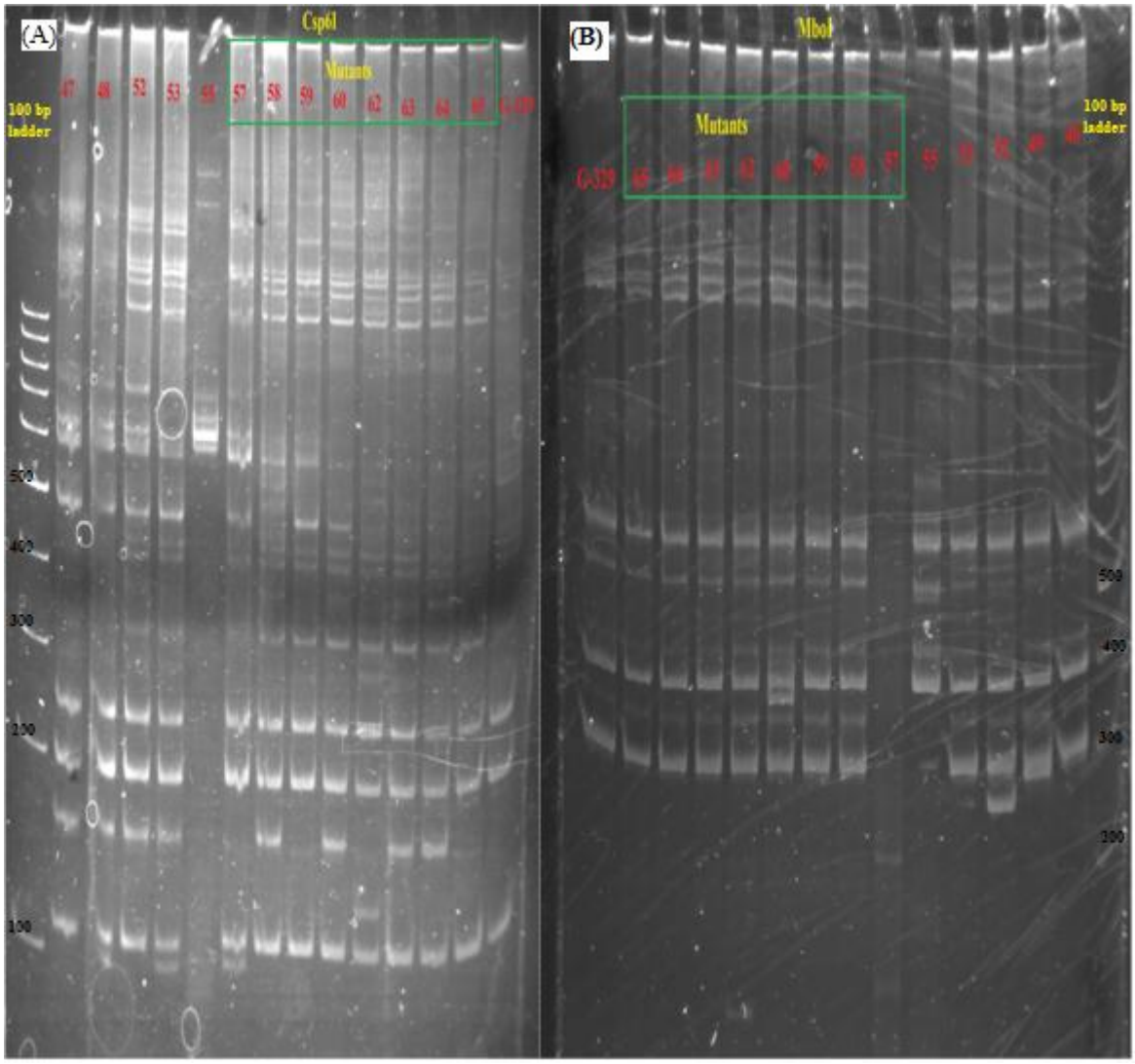

Figure 4.5 The polyacrylamide gel for LORE1 30005017 line after SSAP. With mutants under green label, 5' LTR specific amplification, Csp6I and MboI Restriction enzyme.

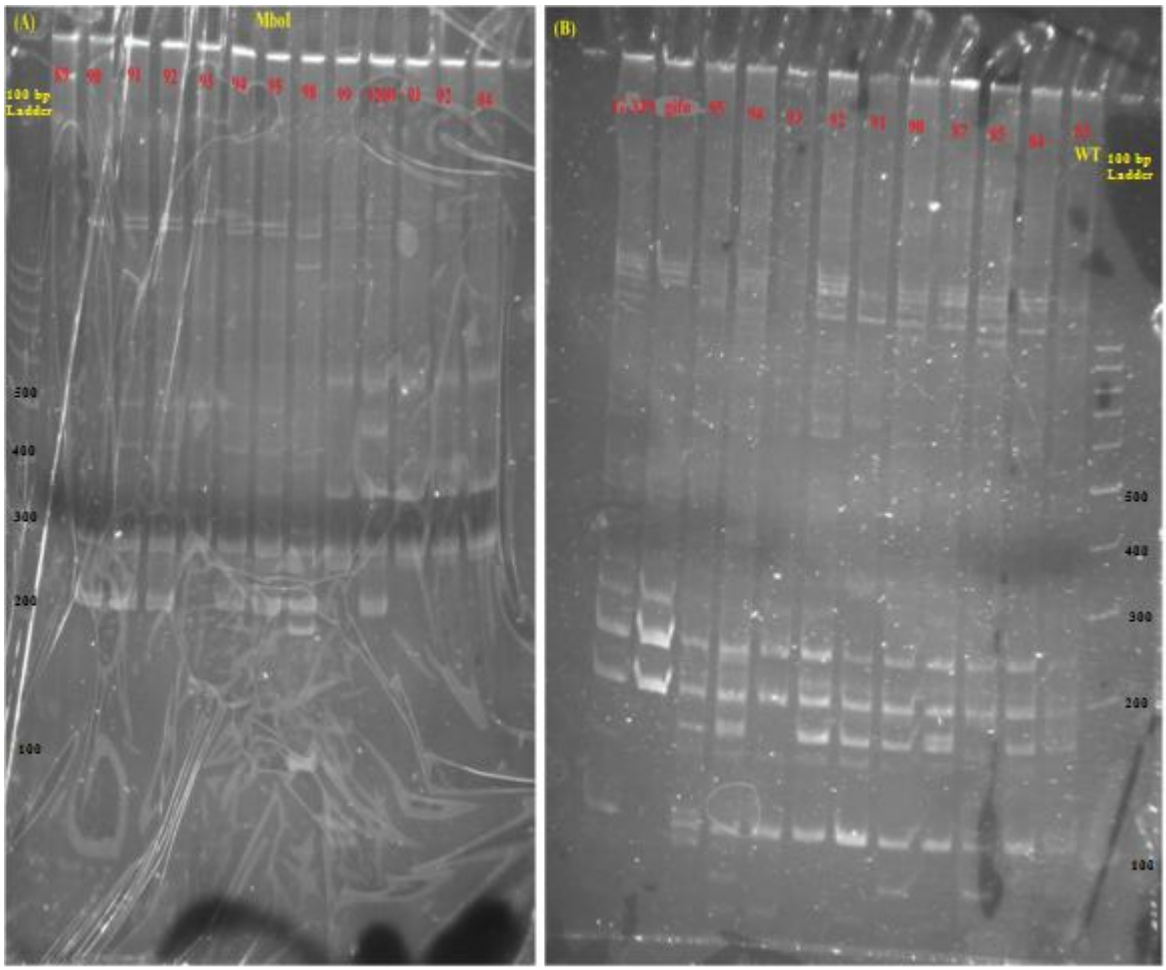

Figure 4.6 The polyacrylamide gel for: (A) LORE1 30005088 line (B) LORE1 30005403 line after SSAP. 5' LTR specific amplification. Csp6I and MboI Restriction enzyme respectively.

30005403: The mutants' phenotype of this line has characteristics of roots without nodules. We use Csp6I and MboI as restriction enzymes in SSAP. The Gel shows no promising co-segregating band with both the restriction enzymes (Fig. 4.6 (B)). So there is need to perform the SSAP with 3'LTR end which may be results in finding of co-segregating band corresponding to the Nod- mutation.

30005078: The forward shows that the mutants of this line have the phenotype of light green leaves with fix- type. Using the 5'LTR specific amplification approach and Csp6I restriction enzymes we identified a band around 100bp size, which was present in each mutant with the phenotype, but not in the rest of the plants or wild-types (Figure. 4.7). After sequencing performed by Niels we came to know that the suspected band would may be from Chromosome number 4. But there might need that there should be

investigation through 3'LTR end as well using other restriction enzymes that the band we find is only responsible for the mutation or there may other genes responsible for that.

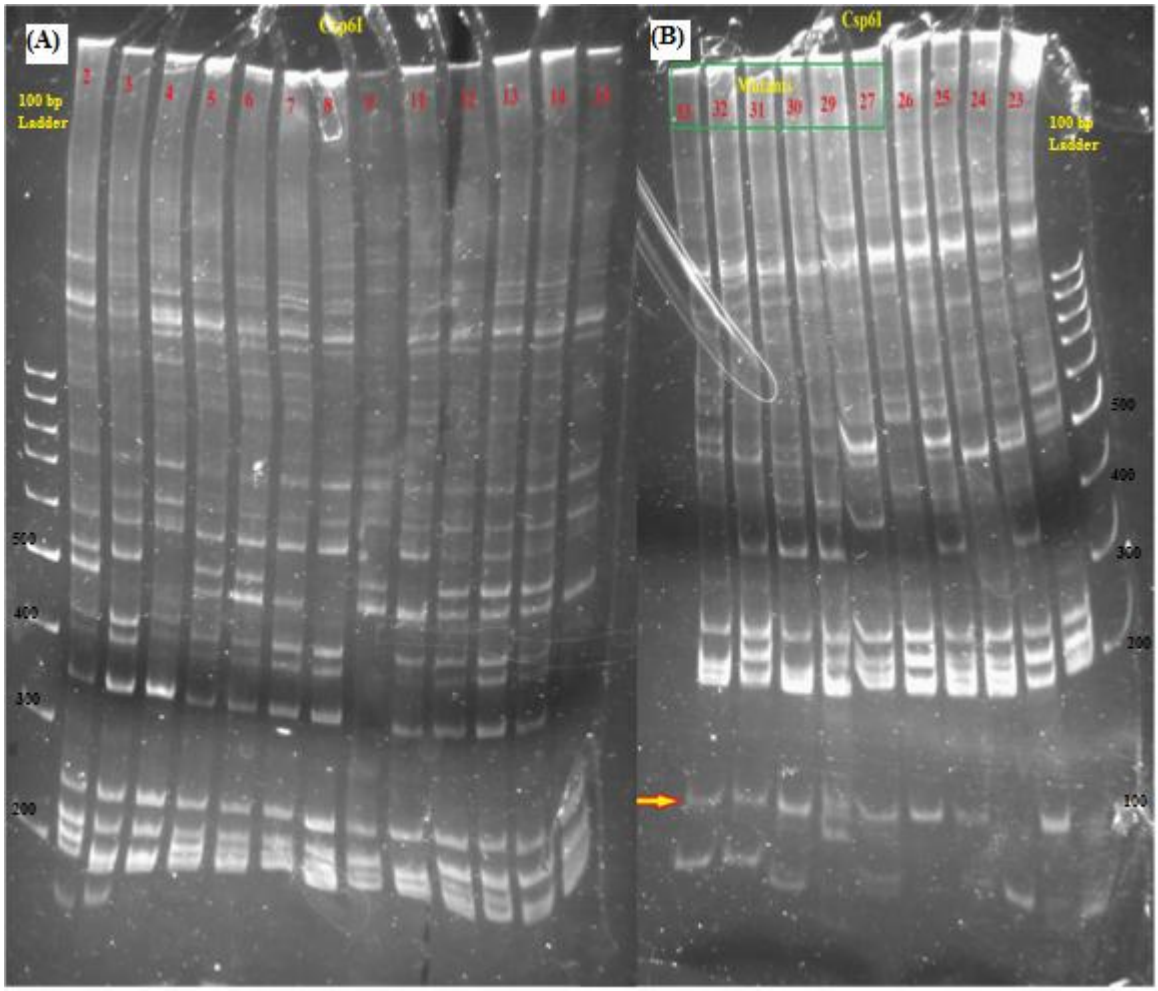

Figure 4.7 The polyacrylamide gel for LORE1 30005078 line after SSAP. The arrow indicates the band common for the plants with a phenotype, 5' LTR specific amplification, Csp6I Restriction enzyme.

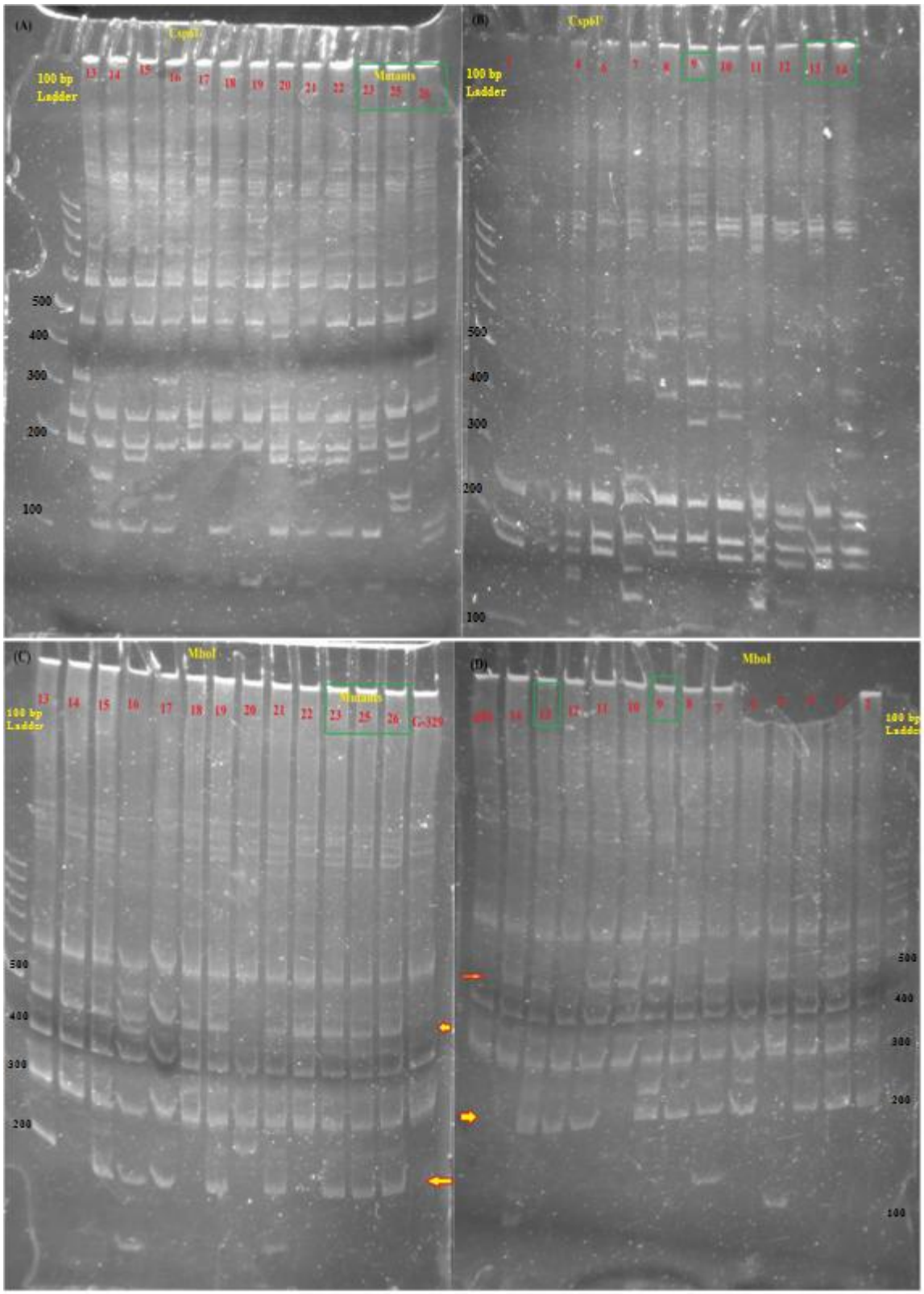

Figure 4.8 The polyacrylamide gel for LORE1 30006602 line after SSAP. The arrows indicates the band common for the plants with mutant phenotype, 5' LTR specific amplification, (A, B): with Csp6I Restriction enzyme, (C, D) with MboI Restriction enzyme.

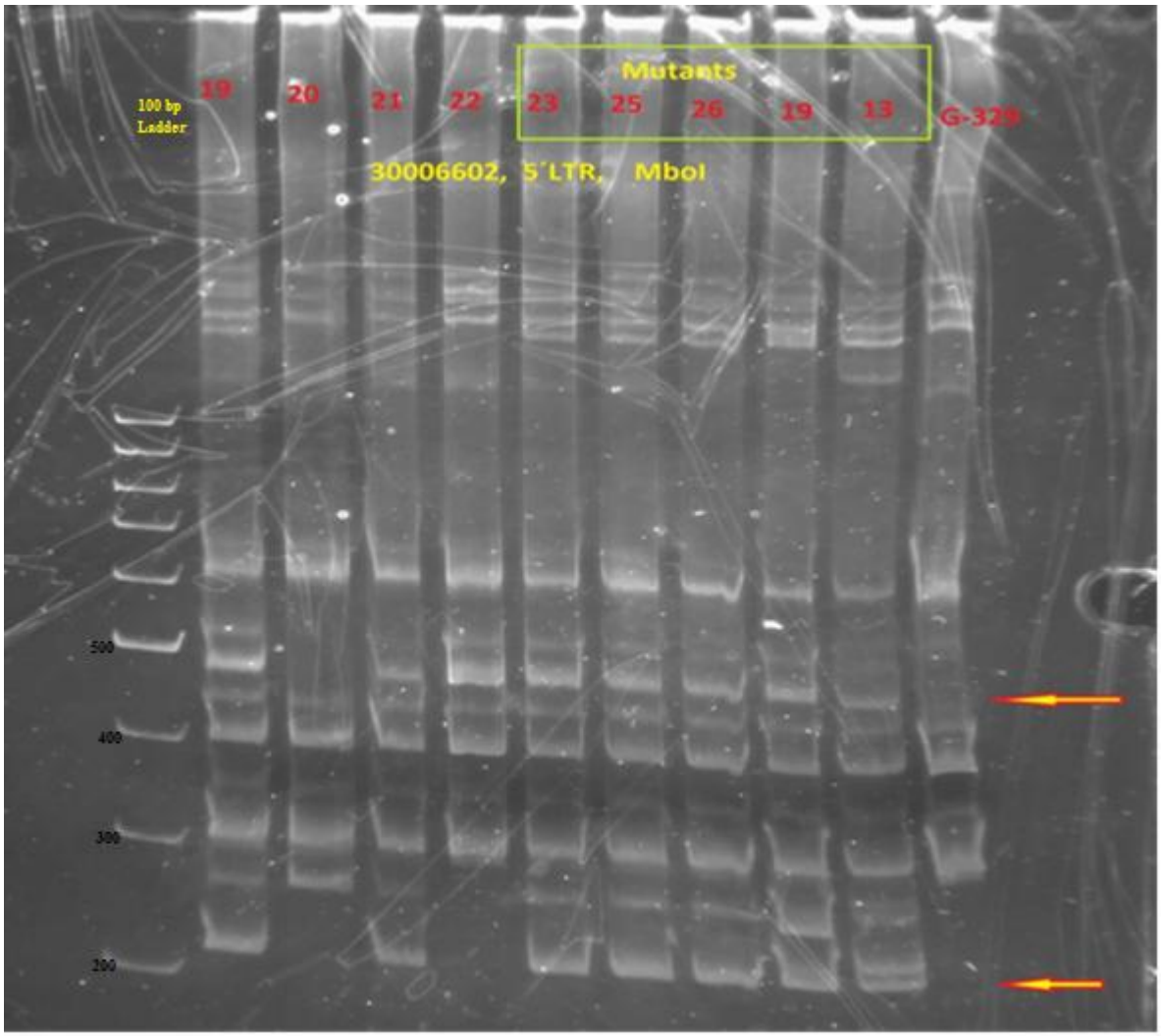

Figure 4.9 The polyacrylamide gel for LORE1 30006602 line mutants only after SSAP. The two arrows show the segregating bands.

30006602: The mutants of this line have white sectors on their leaves. Using the 5'LTR specific amplification approach and Csp6I and MboI restriction enzymes we are aiming to finding the mutant gene. First with CspI we were unable to find any gene of interest (Figure 4.8 A and B). Then we decided to do the same procedure with the restriction enzyme MboI, fortunately we are able to locating the band which were present in all mutants but are segregating in the wild-types (Fig. 4.8 C and D), as the mutants plants are scattered in the Gel so we decided to set up a polyacylamide gel for all the mutant plants with some wild-types and mother line G-329 to be sure that our results in the previous gel results were correct (Figure. 4.9). So we identified two bands around 220bp and 480bp size, which were present in each mutant with the phenotype, but not in

the rest of the plants or wild-types. Then cut the bands from gel with the help of sharp scalpel and subjected to the sequencing procedure.

6602-MboI-480bp-Contig @08/01/13, 10:51
(Unsequenced Strand)

```
1       CCCAGCTAAC AGTAAACATC TGTAACAATC CATCACTTCC
41      TGATATAAAA AACAGCATAT ATGCCACAGA CGAGTTTCGC
81      ATGTTTTCAT TCAAGGTTCG CCCTTGCTCC CGTGCATACT
121     CTCATGATTG GACTGAGTGT CCTTTTGTTC ATCCTGGAGA
161     GAATGCCCGT AGGAGAGACC CCAGAAAGTT CCATTACAGC
201     TGTGTGCCAT GTCCTGAATT TAGAAAAGGG GCTTGTAGAC
241     GTGGGGATAT GTGTGAATAT GCCCATGGAG TATTTGAGTG
281     CTGGCTACAC CCTGCTCAGT ATCGGACAAG ACTCTGCAAA
321     GATGGCACTG ATTGCAACAG AAGGGTATGT TTTTTTGCTC
361     ACACTACTGA AGAGCTGCGT CCATTGTATG TGTCCACTGG
401     GTCTGC
```

Figure 4.10 The sequence of 480bp candidate band.

After sequencing we got the sequence of the band.

1. 6602 Lowest Band

The sequence is as follow:

CATCGAAGGCGCTTATCGATTTCTTCACGCGAAACAGGCGAGGAATCAGGGTGTGTCAT
GGGAAGCGGAAGAGGCTCAGCATCATCGACCAAGTAGCTCTCTCTATTCCGAGACCATT
TCTTCTCCTTCGCCTTCACCCTCACAC

When looking at the BLAST search it shows some similarities with SCO2 which may be the explanation of white sectors on the leaves. SCO2 gene the mutant phenotype in Arabidopsis is white cotyledons. The mature leaves are normal. So the phenotype is different from the 6602 phenotype but it has something to do about white leaves and it could therefore be an indication that it is the right gene.

2. 6602 480bp Band:

CCCAGCTAACAGTAAACATCTGTAACAATCCATCACTTCCTGATATAAAAAACAGCATA
TATGCCACAGACGAGTTTCGCATGTTTTCATTCAAGGTTCGCCCTTGCTCCCGTGCATA
CTCTCATGATTGGACTGAGTGTCCTTTTGTTCATCCTGGAGAGAATGCCCGTAGGAGAG
ACCCCAGAAAGTTCCATTACAGCTGTGTGCCATGTCCTGAATTTAGAAAAGGGGCTTGT
AGACGTGGGGATATGTGTGAATATGCCCATGGAGTATTTGAGTGCTGGCTACACCCTGC
TCAGTATCGGACAAGACTCTGCAAAGATGGCACTGATTGCAACAGAAGGGTATGTTTTT
TTGCTCACACTACTGAAGAGCTGCGTCCATTGTATGTGTCCACTGGGTCTGC

So when we look on Blast Search and Compare it with others related organism this sequence is very much related with Zinc Finger Protein family and as this protein has influence over the growth and development of the plants so may be this insert has something to do with the Growth.

30004049: Using the 5'LTR specific amplification approach and the two restriction enzymes: Csp6I and MspI. As in the Figure 4.11 shows that from both these two enzymes we got 1 candidate band each which was present in each mutant but is segregating in the wild-types. Then after sequencing the band we got the sequence of the band which is:

CGGCATTGCTGGATTGCTTAAATCAATCCATCCTGATTGGAGTGCTGCTGCTATCAAGT
CAGCACTAATGACCACAGGTAAATGGGTTTGTTTACACGTACACTTATAGCTATAAGAT
CATTGCATTGACATTCAATTTCTTATACCATTTATTATAAAAAATAACCTCTCCTTCTG
CAGCAAGTACAGAAGATAACTCTGGAGGGCCTATGTGGGAGTCAGATTATTCAGAATTG
GAAGAGGCAACTCCATTTGCTTGTGGCGCAGGCCATGTTACAGA

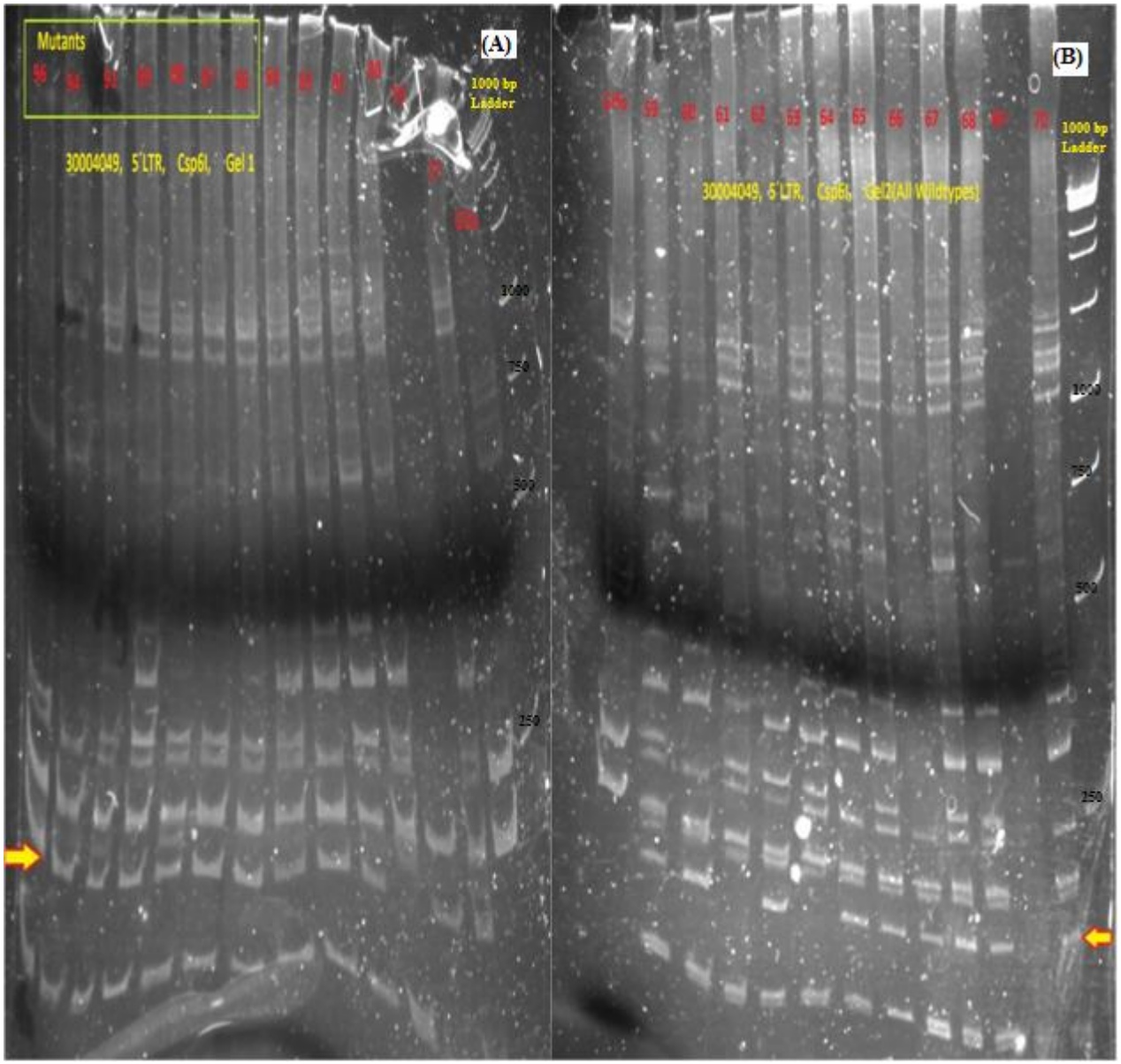

Figure 4.11(a) The polyacrylamide gel forLORE1 30004049 line after SSAP. The arrows indicates the band common for the plants with mutant phenotype, 5' LTR specific amplification, Csp6I and MspI restriction enzymes were used.

So after knowing the sequence of the co-segregating band we make the blast search for the sequence which shows its relation with the subtilase gene. So it might be the strong possibility that the mutation is due to the subtilase gene activity.

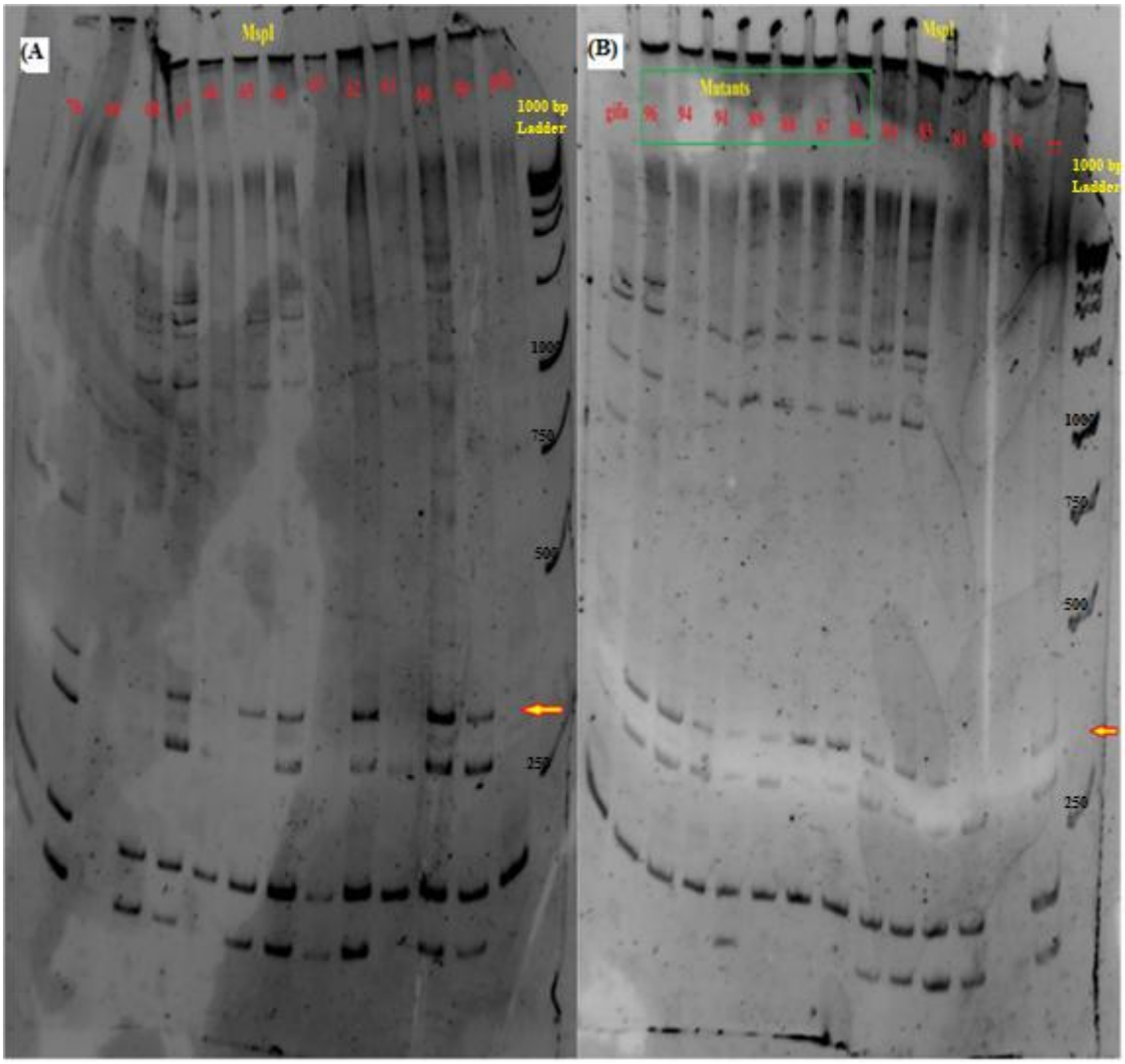

Figure 4.11(b) The polyacrylamide gel forLORE1 30004049 line after SSAP. The arrows indicates the band common for the plants with mutant phenotype, 5' LTR specific amplification, MspI restriction enzymes were used.

The LORE1 project was started to get knockout mutations in most of the Lotus japonicus genes. People that are interested in a particular gene can then get knockout mutants in that particular gene and see if there is a mutant phenotype. Most other mutant collections are made with EMS mutagenesis giving single base mutations. Many of these mutants are silent mutants with no amino acid change or an amino acid change that is not important for the function of the protein. LORE1 inserts a 5kb retrotransposon which will give a knockout mutation with a protein without function.

From the results of line 30006602 we can say that from SSAP analysis these two bands give good candidates for the gene, that when mutated causes the mutant phenotype with white sectors on the leaves. To be a good candidate it should have the LORE1 insertion in an exon which is the case for these two bands and all mutants should have this SSAP band and it should not be present in the parent line G-329. So these two bands give good candidates for the mutated gene in the 30006602 line giving the white sectors leaf phenotype. Maybe one of these genes is the right gene. But there could also be other LORE1 insertions that could be candidates as one would not able to see the segregation of bands for all LORE1 insertion with a single (or two) enzyme(s) and only 5´ LTR SSAP. This is due to the fact the restriction fragment could be larger than we are able to amplify in this type of PCR.

For the SCO2 gene the mutant phenotype in Arabidopsis is white cotyledons. The mature leaves are normal. So apparently the phenotype is different from the 30006602 line phenotype but as in BLAST search it is related to our candidate insert that's why we have strong believe that it has something to do about the white sectors on leaves and it could therefore be an indication that it may be the right gene.

The next step is the specific PCR for the wild-type copy and the specific PCR for the copy with the LORE1 insertion. We have done this in the whole 6602 population. If it is a LORE1 insertion that is already known from the LORE1 database on the net http://users-mb.au.dk/pmgrp/, we can use the primer set suggested there. There have been problems with these PCRs for 30006602 (Suggested by Niels) and though he is not convinced that it is the right gene yet. As we know that all mutants should only show the PCR band for the copy with the LORE1 insertion and not the wild-type copy of the gene. For the PCR on the wild-type plants we should see the wild-type PCR band in all the wild-type plants and the PCR band for the LORE1 insertion in that gene in many of the plants with wild-type phenotype as both homozygous wild-type plants without a LORE1 insertion PCR band for this gene and heterozygous plants with both the wild-type PCR band and the LORE1 PCR band would have a wild-type phenotype. This is because it is recessive mutation where you only see the phenotype when both gene copies are non-functional (contain a LORE1 insertion). Another way of proving that it is the right gene is to find more LORE1 lines with insertion in that gene and see if these lines also segregate mutants with a white leaf sector phenotype. As far

as the line 30004049 is concern, we have done the SSAP procedure with 2 of restriction enzyme and with both these enzymes we are able to identify the candidate band with sequencing both these bands we are able to relate it with Subtilase gene family. We are very firm that the fix-/hist- phenotype is because of this LORE1 insert. But there is lot to do to finally confirm that whether this insert is responsible for the mutation, off course 3´ LTR strategy may also endorse if the results would be same as we have and also using more restriction enzymes can increase the precision of results. In the last we can say that our strategy of doing SSAP to find out the mutant gene was successful as we are able to find some good candidate gene and through this work we are able to contribute significantly in the LORE1 genome project in model plant *Lotus japonicus* started in CARB (Aarhus University Denmark) started in recent times.

4.2 ASSOCIATION MAPING OF LOTUS NATURAL LINES

The sclerotinia phenotype was scored as percentage of plants with visible fungi on the cotelydons (See A7). The association analysis was based on this phenotype at 14 dpi. Because we see a more Variation in response of plants against the fungus so there was high chances of finding a good variation. Then we compare and score this data against the genotypic data available from CARB and carried out further steps.

The genotypic data was filtered to remove SNPs with rare allele occurrences (allele observed in less than 10 of all accessions) and orphan alleles (with only one nucleotide observed in all accessions). The genotype file was then transposed and formatted using PLINK (Purcell et al., 2007). Afterward, associating mapping was subsequently performed according to the instructions [1] provided by the authors of Efficient Mixed-Model Association eXpeditied (EMMAX) (Kang et al., 2010). QQman [2] loaded in R (R Development Core Team, 2008) was used to generate Manhattan plot from the association mapping results obtained from EMMAX.

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[1] . Instructions for EMMAX beta usages are available from http://genetics.cs.ucla.edu/emmax/install.html (last updated on March 7, 2010)

[2] QQman is an open-source R plugin written by Stephen Turner and Daniel Capurso. It is available from https://github.com/stephenturner/qqman/blob/master/qqman.r (last updated on June 10, 2013)

The Manhattan plot shows the negative logarithm of the P-value as a function of genomic location. Thus the SNPs with the most significant association will stand out on the plot. Each dot represents a SNP, with the X-axis showing genomic location and Y-axis showing association level (P-value). The Figure 4.12 shows an illustration of a Manhattan plot for the 28 lines of Lotus japonicas which was infected by the Sclerotinia fungus, upon their response to the susceptibility to fungus disease. The most important thing while in our observation was the unusual behaviour of the control; MG20 and gifu. From these results we can say that there is no significant association among the samples. This also may be because of the reason that as we have 28 lines genetic data (This plot is based on association mapping among 28 lines), and for association mapping analysis it is too much less. But the peoples in CARB are working for developing the genetic data of all other lines and since we have done the experiments with fungal infection to lots of *Lotus Japonicus* ecotypes so eventually in near future we would see more significantly associated results.

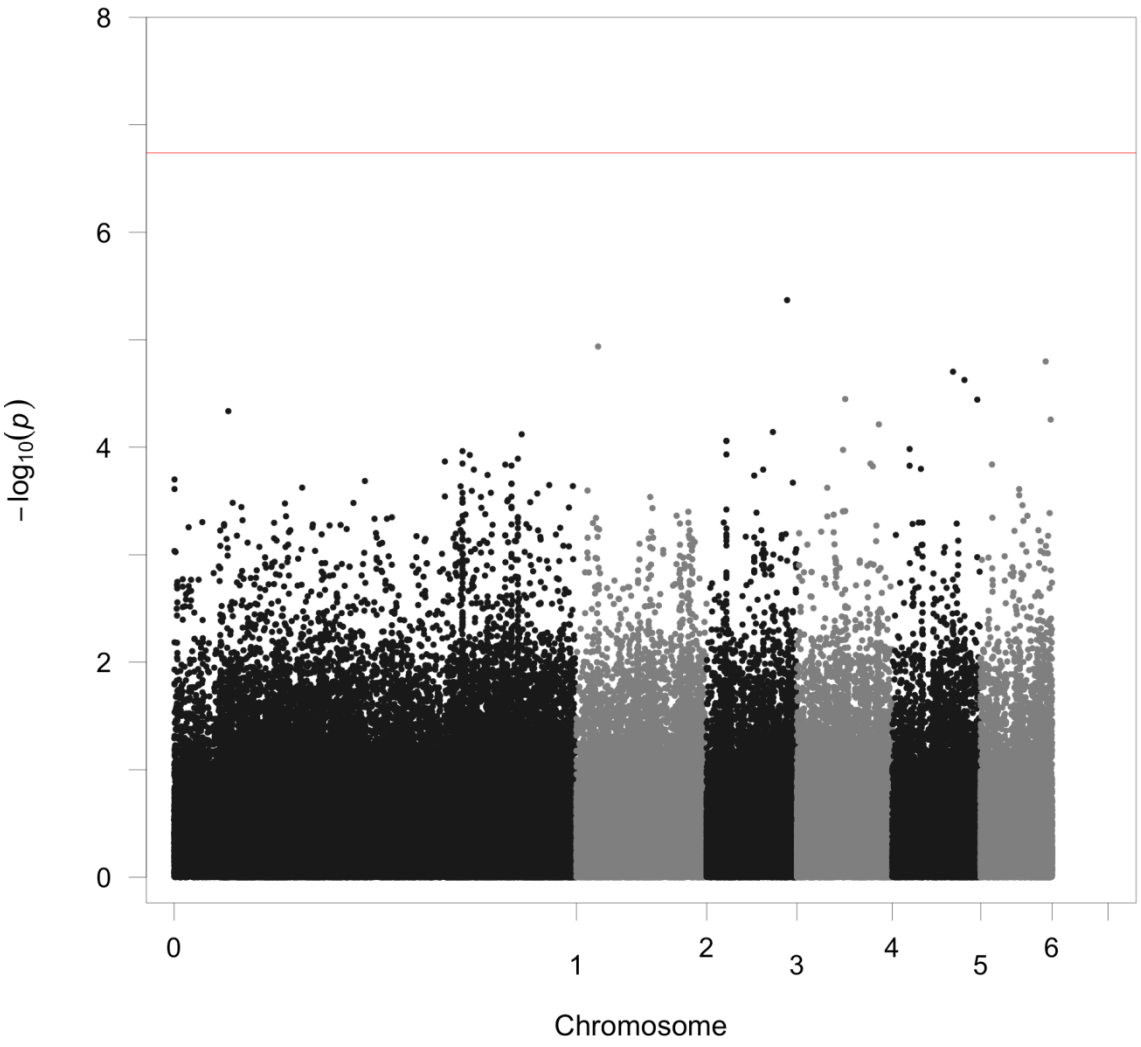

Figure 4.12 The Manhattan plot from the association mapping results obtained from EMMAX, X-axis shows the distribution of SNPs in different chromosomes and the Y-axis shows the level of association.

CHAPTER 5

CONCLUSION

Lotus Japonicus is an important model legume plant has the characteristic feature of fixing nitrogen with the help of rhizobia through the process of symiosis. Identification of LORE1 transposable element results in rapid research on insertional mutagenesis in *Lotus japonicus* (Madsen et al., 2005). The present study is about the identification of genes using different forward genetic approaches in *Lotus japonicus.*

The first technique was the phenotypic screening of LORE1-tagged mutant populations for aberrant nodulation phenotypes followed by identification of causal mutations through sequence specific ampliphied polymorphism (SSAP). The second approach was image-based quantitative phenotyping of plant responses to microbial inoculation followed by the association analysis based on available genotypic information from *L. japonicus* natural accessions. The main aim of this study was to contribute in the research of model legume *Lotus japonicus.*

Through SSAP we are able to find some promising candidate genes in different lines which would be confirmed after the completion of LORE1 project. The LORE1 project was started to get knockout mutations in most of the *Lotus japonicus* genes. LORE1 inserts a 5kb retrotransposon which will give a knockout mutation with a protein without function. The SSAP analysis of line 30006602 revealed that the gene involved in the white sectors leaf phenotype, may have close relationship with the SCO2 in Arabidopsis. But as in Arabidopsis only the cotyledon has albino phenotype and the mature leaves were normal leaves, so it requires further studies. As far as the line 30004049 is concern, we have done the SSAP procedure with 2 of restriction enzyme and with both these enzymes we were able to identify the candidate band with sequencing both these bands we were able to relate it with Subtilase gene family. We

are very firm that the fix-/hist- phenotype is because of this LORE1 insert. By these promising results we can say that our strategy of doing SSAP to find out the mutant gene was successful as we are able to find some good candidate gene and through this work we are able to contribute significantly in the LORE1 genome project in model plant *Lotus japonicus* started in CARB (Aarhus University Denmark) started in recent times.

Using Lotus natural accessions, mainly collected from Japan, we have generated Image-base phenotypic data of plants after microbial inoculation for future use in Genetic mapping.

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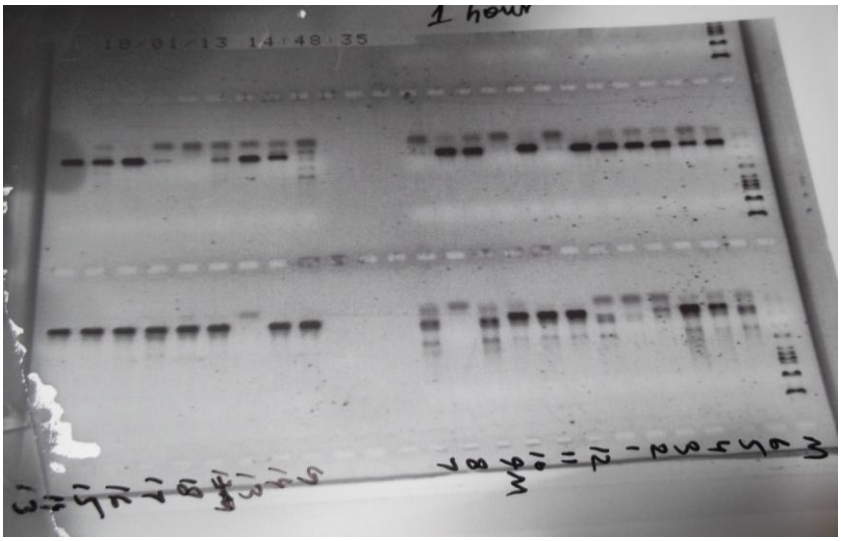
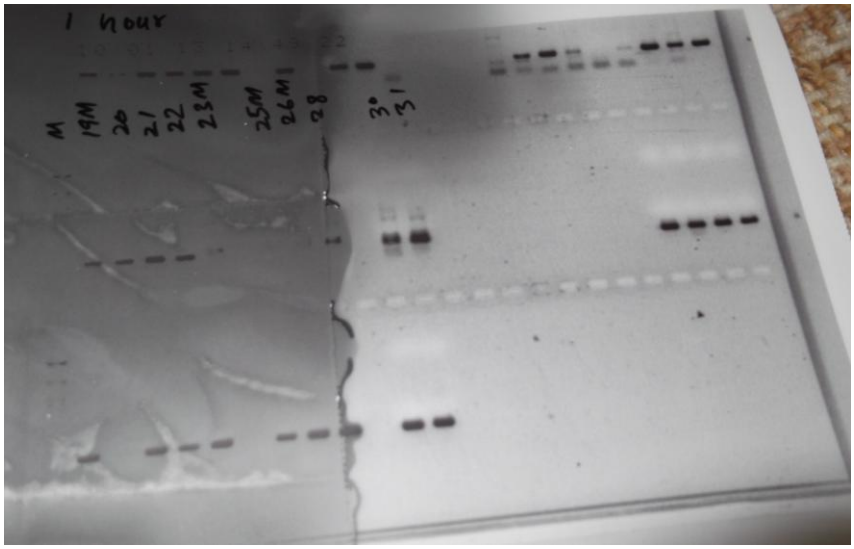
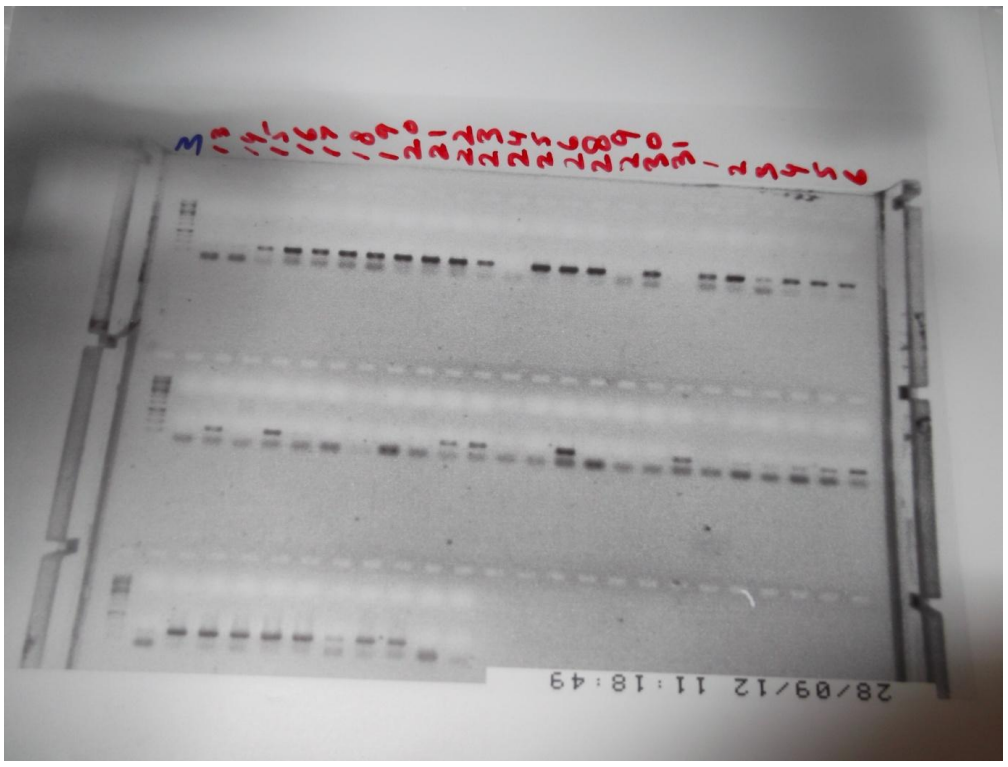
APPENDIX

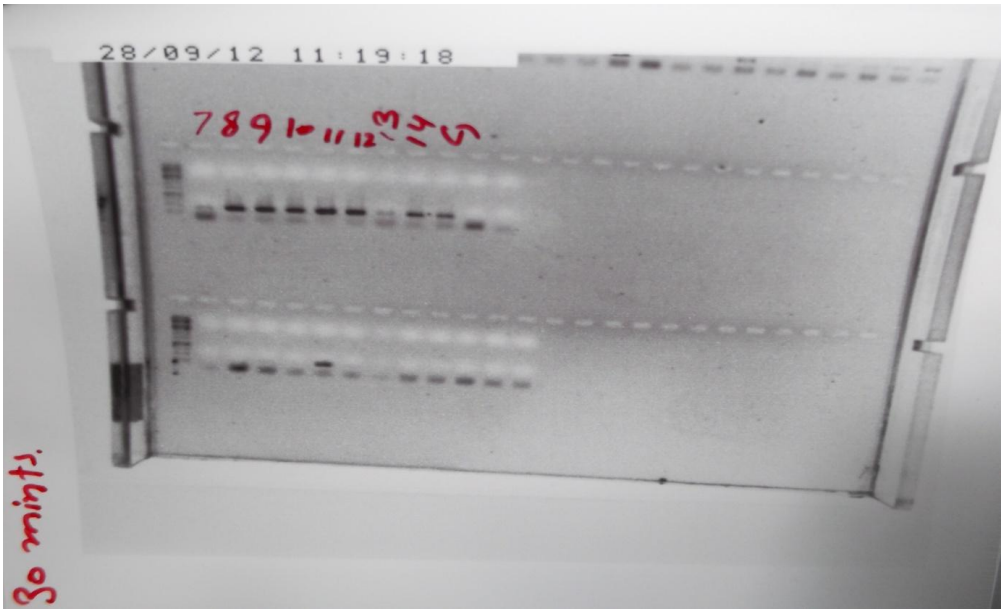
A1. Quality Check PCR for the line 30006602



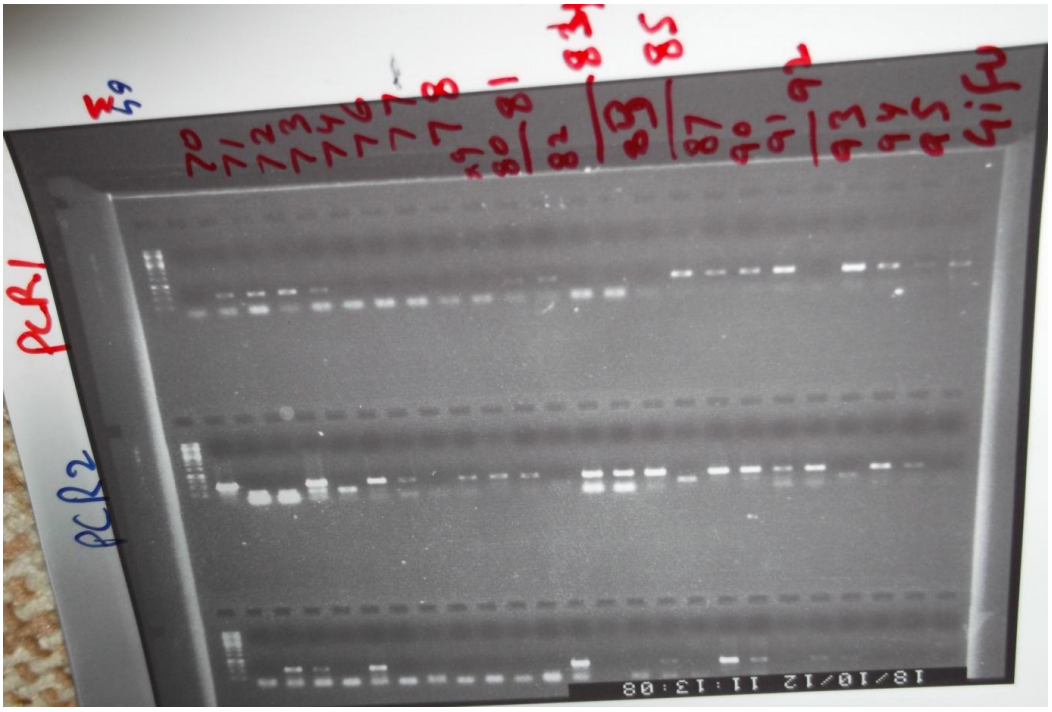


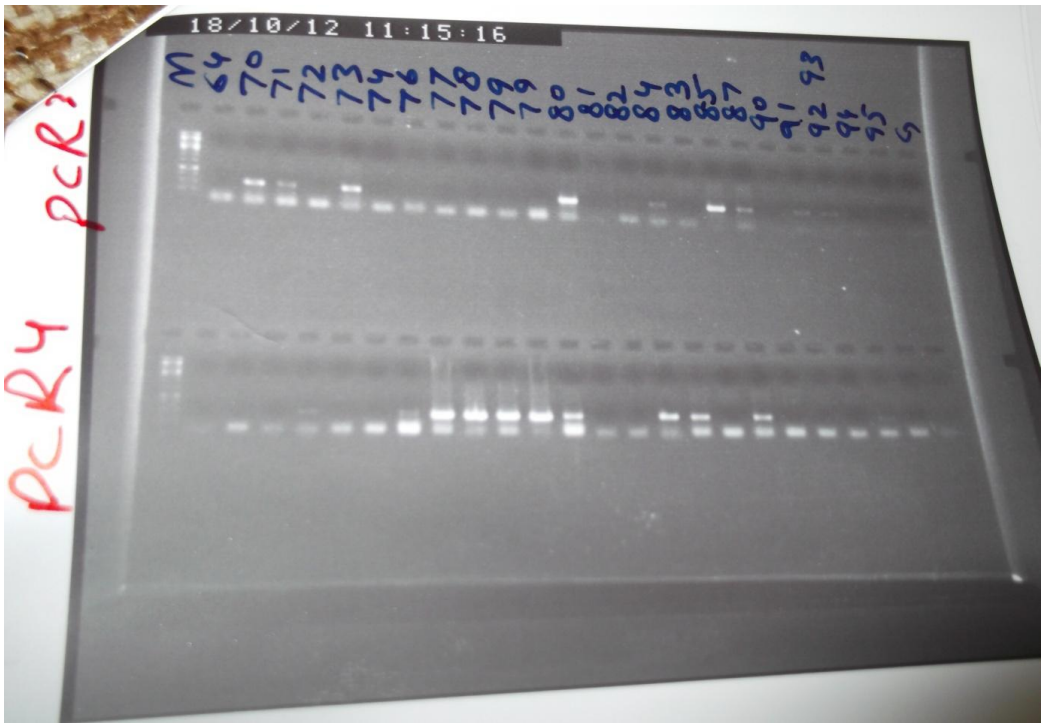
A2. Quality Check PCR for the line 300050



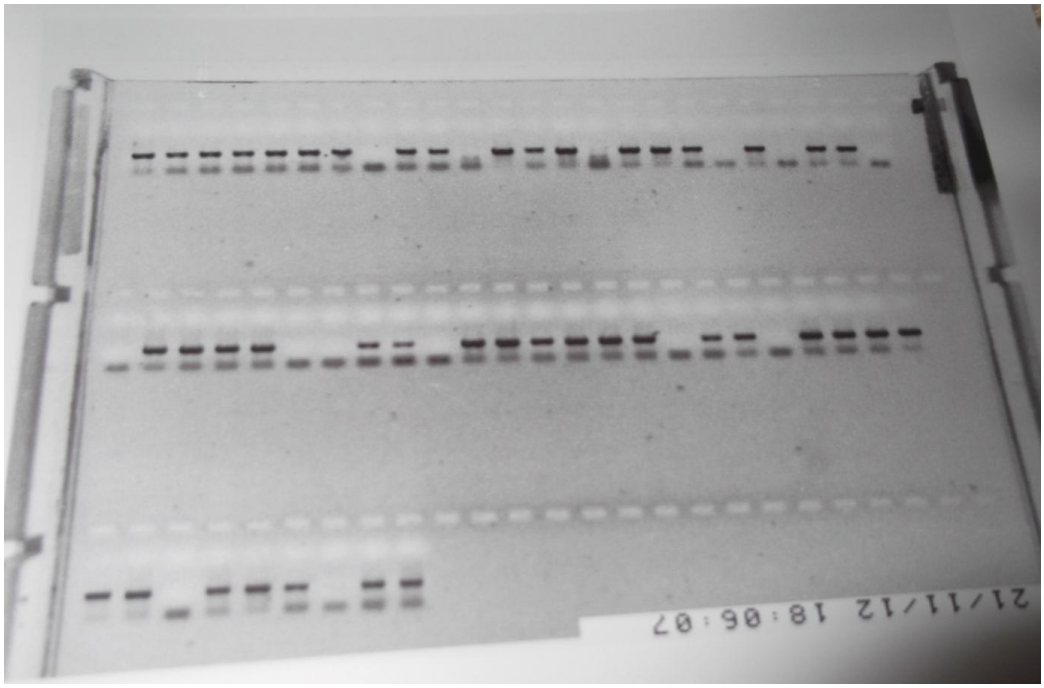


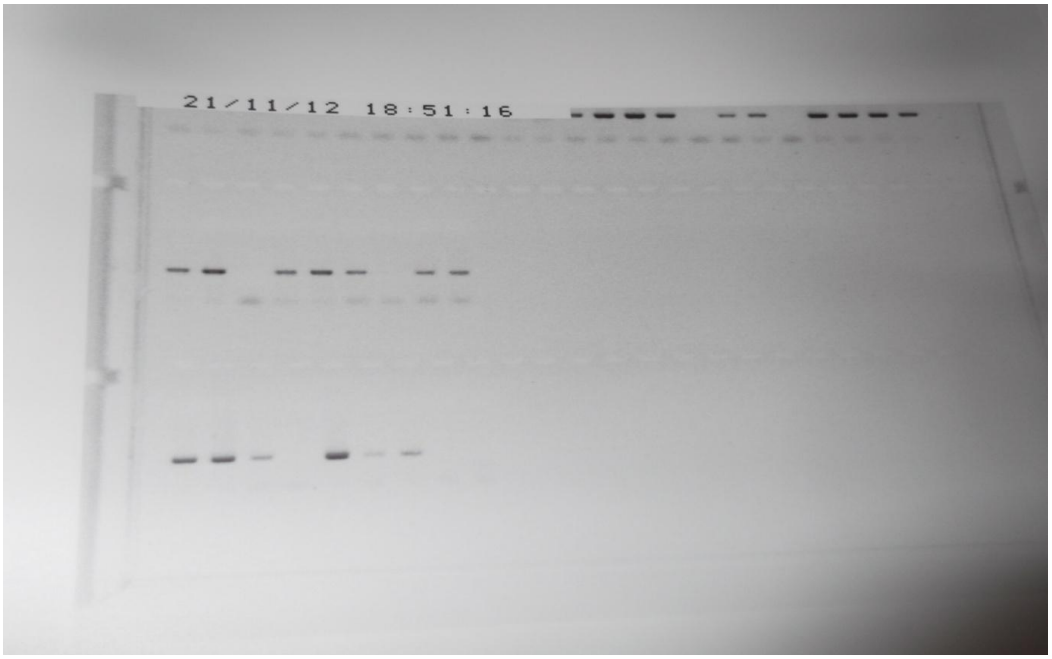
A3. Quality Check PCR for the line 30005403.





A4. Quality Check PCR for the line 30005078.





A5. **Primers used for LORE1 genotyping**

Primers used for LORE1 genotyping

Oligo ID	Oligo Name	
PM-06549	030006602-fw	TAGCATCACTAACAGTAGGTAGGCCC
PM-06550	030006602-rv	GGGATGACTGCATCTCCAGTTAAGTCG
PM-06699	6602-3-fw	TTTCTGGGGTCTCTCCTACGGGCA
PM-06700	6602-3-rv	GCCTGGGGGTAAAGCAATGTCGAA
PM-07122	6602MboI-band3	GGCGAGGAATCAGGGTGTGTC
PM-07123	6602MboI-band3-rv	GCTCTCCGTCTTCCGCTG
PM-07185	6602MboI-band3-rv2	GGGTTGCCATCTCTCCTGTAGC
PM-4152	LORE1 Primer P2	CCATGGCGGTTCCGTGAATCTTAGG
PM-4153	LORE1 Primer P3	CCAACTAACAACCCAGCTAACAGT
PM-6687	5403-fw-SGA	TGGATCCATTTTTCTGCTGCATATGTTT
PM-6688	5403-rv-SGA	TTCATCAATGATCCACAGATGACATGC
PM-6689	5403-fw-chr5-31737270	TGGGCACTTTCCAATGGCCCTTCT
PM-6690	5403-rv-chr5-31737270	TTGTCATTCCAAAGCTGATGCCCA
PM-6461	030005017-fw	AGCTGCATGTCCTGATGCTGTGCC
PM-6462	030005017-rv	ACCCGTGCCTCCAAGTACCAGTCC
PM-6463	030005078-fw-chr5	GAGCCAGAATCATCCAGGACCGCA
PM-6464	030005078-rv-chr5	TCACCCACCATGCCAGGGTCTACA
PM-4399	Splink1	CGAAGAGTAACCGTTGCTAGGAGAGACC
PM-4400	Splink2	GTGGCTGAATGAGACTGGTGTCGAC

A6. Seed Treatment with sulfuric acid of Lotus natural accessions

RIL	**Collection date**	**Germination**	**Sulfuric acid treatment (Min)**
gifu	24/01/2006	+	35
MG004	05/08/2010	+	18
MG010		+	21
MG012	27/04/2010	+	21
MG019	12/01/2011	+	18
MG020	05/09/2011	+	18
MG023	03/06/2010	+	18
MG036	03/01/2011	+	18
MG049	25/05/2010	+	21
MG051	06/01/2011	+	18
MG062	17/05/2010	+	21
MG072	19/08/2010	-	14
MG073	27/04/2010	+	15
MG077	29/04/2010	+	15
MG080	26/05/2010	+	18
MG082	26/05/2010	+	20
MG083	26/05/2010	+	20
MG086	19/05/2010	+	20
MG089	17/05/2010	+	20
MG093	30/08/2010	+	20
MG095	26/05/2010	+	18
MG097	14/04/2010	+	15
MG101	19/05/2010	+	20
MG107	?	-	17
MG109	14/04/2010	+	15
MG113	12/11/2010	+	15
MG118	03/08/2010	+	18
MG122	05/08/2010	+	15
MG123	17/05/2010	+	20
MG128	14/04/2010	+	15

MG002	31/05/2010	+	15
MG003	03/06/2010	+	15
MG005	26/05/2010	+	17
MG008	19/05/2010	+	15
MG020	05/09/2011	+	15
MG014	03/06/2010	+	15
MG015	31/05/2010	+	15
MG016	03/06/2010	+	15
MG029	05/06/2010	+	15
MG034	29/04/2010	+	15
MG035	06/01/2010	-	15
MG038	17/05/2010	-	15
MG039	17/05/2010	+	17
MG040	12/01/2011	-	15
MG046	16/08/2010	+	15
MG052	29/04/2010	+	15
MG053	06/01/2011	+	15
MG055	06/01/2011	-	15
MG057	14/04/2010	+	15
MG058	14/04/2010	+	15
MG061	06/01/2010	+	15
MG066	18/11/2009	+	15
MG071	11/01/2011	-	15
MG076	06/01/2011	-	15
MG074	03/05/2010	+	15
MG075	03/05/2010	+	15
MG079	27/04/2010	+	15
MG081	25/05/2010	-	15
MG084	08/04/2010	+	15
MG085	05/08/2010	+	15
MG088	18/11/2010	-	15
MG090	19/05/2010	+	17
MG094	14/04/2010	+	15
MG096	05/08/2010	+	15
MG098	11/01/2011	+	15
MG099	----------	-	15
MG100	14/04/2010	+	15
MG111	05/08/2010	+	15
MG115	29/04/2010	+	15
MG117	11/01/2011	+	15
MG121	14/04/2010	+	15
MG124	17/05/2010	+	17
MG126	17/05/2010	+	17
MG127	17/05/2010	-	15

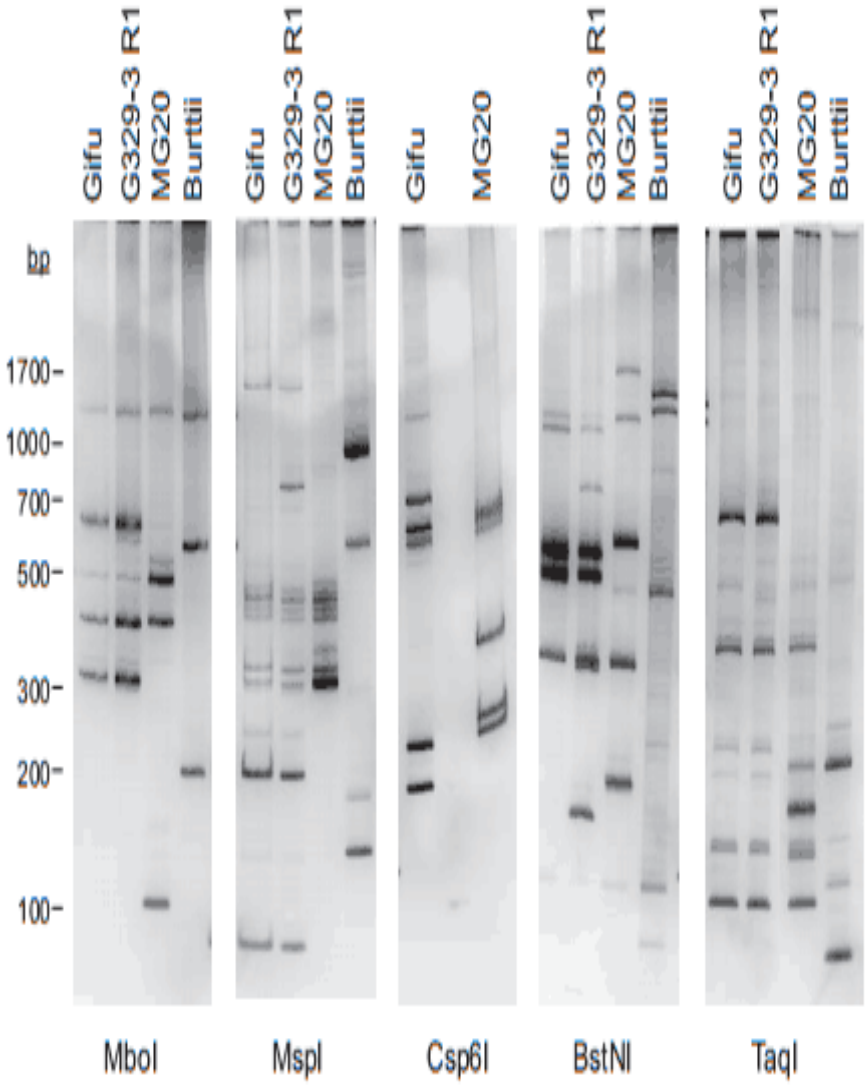
A7. Percentage of plants with visible fungi on their cotelydons scored on 14 dpi.

MG	01dpi	02dpi	03dpi	04dpi	05dpi	06dpi	07dpi	08dpi	10dpi	12dpi	14dpi	16dpi	18dpi	20dpi	22dpi	24dpi	26dpi	28dpi	30dpi	32dpi
gfíu	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.33	8.33	16.67	16.67	16.67	16.67	16.67	16.67	16.67	16.67	16.67	16.67
MG004	0.00	0.00	0.00	0.00	0.00	16.67	16.67	16.67	25.00	25.00	25.00	25.00	33.33	41.67	41.67	41.67	41.67	41.67	41.67	41.67
MG010	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.33	8.33	16.67	16.67	25.00	25.00	25.00	33.33	33.33	33.33	33.33	33.33
MG012	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	16.67	25.00	25.00	25.00	25.00	25.00	25.00	25.00	33.33	33.33	33.33	33.33
MG019	0.00	0.00	0.00	8.33	8.33	8.33	8.33	8.33	8.33	16.67	16.67	33.33	33.33	33.33	33.33	33.33	33.33	33.33	33.33	33.33
MG020	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.33	16.67	16.67	33.33	33.33	33.33	33.33	33.33	33.33	33.33	33.33	33.33
MG023	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.33	8.33	8.33	8.33	8.33
MG036	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.33	16.67	16.67	25.00	25.00	25.00	25.00
MG049	0.00	0.00	0.00	0.00	0.00	0.00	8.33	8.33	8.33	16.67	16.67	16.67	16.67	16.67	33.33	33.33	33.33	33.33	33.33	33.33
MG051	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.33	8.33	25.00	25.00	25.00	25.00	25.00	25.00	25.00
MG062	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.33	8.33	25.00	33.33	41.67	41.67	41.67	41.67	41.67	41.67	41.67	41.67
MG073	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	7.69	23.08	23.08	23.08	23.08	23.08	23.08	23.08	23.08	23.08	23.08
MG077	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	7.69	7.69	23.08	23.08	23.08	23.08	23.08	23.08	23.08	38.46	38.46
MG080	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.33	16.67	16.67	25.00	25.00	25.00	25.00	25.00	25.00	25.00
MG082	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.33	8.33	8.33	8.33	8.33	16.67	16.67	16.67	25.00	25.00
MG083	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.33	8.33	16.67	16.67	25.00	33.33	33.33	41.67	41.67	58.33	58.33	58.33
MG086	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.33	8.33	8.33	16.67	16.67	16.67	16.67
MG089	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	25.00	29.17	33.33	50.00	50.00	50.00	50.00
MG093	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.33	8.33	8.33	8.33	8.33	8.33	8.33	8.33	8.33
MG095	0.00	0.00	0.00	4.17	8.33	8.33	8.33	8.33	8.33	8.33	8.33	8.33	16.67	16.67	16.67	16.67	25.00	25.00	33.33	33.33
MG097	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
MG101	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
MG109	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.33	8.33	8.33	8.33	8.33	8.33	8.33	16.67	16.67	16.67	16.67
MG113	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.33	8.33	16.67	16.67	16.67	16.67	16.67	16.67	16.67	16.67	16.67	16.67
MG118	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.33	8.33	16.67	25.00	25.00	33.33	33.33	33.33	33.33	33.33	33.33	33.33	33.33
MG122	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
MG123	0.00	0.00	0.00	0.00	0.00	0.00	8.33	8.33	8.33	8.33	8.33	8.33	16.67	33.33	33.33	33.33	33.33	33.33	33.33	33.33
MG128	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	8.33	25.00	25.00	25.00	25.00	25.00	25.00	25.00	25.00

A8. Root nodule count after inoculation with rhizobium scored on 35 dpi.

Ecotype	1	2	3	4	5	6	7	8	9	10	11	12
gifu	4	3	2	0	3	2	2	5	1			
MG004	0	0	0	1	0	1	1	0	1	1	3	1
MG010	0	0	0	0	0	0	0	0	0	0	0	0
MG012	1	0	1	0	0	0	1	0	0	1	0	1
MG019	0	0	0	1	0	0	1	0	1	1	1	1
MG020	0	0	0	0	0	0	0	0	0	0	0	0
MG023	0	0	0	1	0	0	0	1	1	1		
MG036	1	0	0	1	0	1	1	0	1	3		
MG049	2	7	4	4	2	2	4	1	1			
MG051	1	3	1	2	1	0	1	0	1	0		
MG062	3	2	2	1	2	2	2	2	0	2		
MG073	3	0	0	0	1	1	3	2	0	0	0	2
MG077	3	2	3	4	1	1	4	0	0	0	1	0
MG080	0	3	1	1	2	2	3	2	1	0	0	0
MG082	2	1	0	0	0	1	0	0	1	1	1	3
MG083	4	0	2	2	0	0	0	2	0	0		
MG086	0	1	3	1	0	3	1	0	0	0	0	
MG089	0	0	0	3	0	0	0	1	0	0	0	0
MG093	0	1	1	0	0	0	0					
MG095	1	1	0	2	0	1	1	0	0	0		
MG097	0	0	1	1	0	0	2	0	1	0		
MG101	0	0	0	0	0	0	0	0	0	0	0	0
MG107	0	0	0	0	0	0	2	1	1	0	1	1
MG109	1	0	0	1	0	1	1	2	0	0	0	1
MG113	1	2	1	2	4	1	2	2	2	2	1	2
MG118	0	0	0	0	0	0	0	0	0	0	1	1
MG122	0	0	1	1	2	1	1	1	0	0	0	1
MG123	0	3	2	0	1	0	0	0	0	0	1	1
MG128	1	1	0	1	0	2	0	0	0	0	0	1

A9. Wild-types standard SSAP bands with specific restriction enzymes



CARB Centre for Carbohydrate Recognition and Signalling
Danish National Research Foundation's Centre of Excellence

To whom it may concern

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Umair Ahsan has carried out the experiments for his Master of Science thesis at the CARB Centre, Department of Molecular Biology and Genetics, Aarhus University. This work was carried out in collaboration with other researchers at the centre. He has permission the present the resulting data in his thesis. The ownership of and right to publish the data remains with the CARB Centre.

Yours sincerely
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