Isolation of Tagged INUIT Gene by Establishment of TAIL-PCR in Tobacco

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ABSTRACT:

Chilling injury is one of the key factors restraining plant growth in tropical and subtropical crop species. It is recognized that stability of microtubules is directly linked to systemic cold resistance of entire organism. We used a mutant approach in order to examine a possible function of microtubules in cold resistance. For this reason we characterized a mutant called inuit generated by T-DNA activation tagging (Koncz et al. 1994; Ahad et al. 2003). Inuit mutants were directly screened for long term cold resistance. The analysis of these mutants is targeted to isolate new components of so far hardly known pathway that links cold sensing to microtubular response. A mutant called ATER was screened for resistance to anti microtubular herbicide EPC are altered in microtubular dynamics and are cross resistant to chilling stress (Ahad et al. 2002; 2003). From one of these mutant lines a novel member of cytochrome-P450 oxidase superfamily was identified by a tag. This gene might represent a central element of environmental signaling towards cytoskeleton. Although plasmid rescue technique works very well in Arabidopsis, it seems in tobacco does not work in same extent. Therefore we established a technique called TAIL-PCR in tobacco which is an efficient method to amplify unknown sequences adjacent to known insertion sites was applied here in this study to isolate INUIT gene.
BACKGROUND:

T-DNA activation tagging was initially used to isolate Arabidopsis mutants that could play important role in hormonal signaling pathway (Hiashi et al. 1992; Vigil et al. 2000). The same construct was used to generate different tobacco mutants expecting after insertion cytoskeleton dynamics alteration might take place. Cell cytoskeleton is highly dynamic and is an important step of any living cell especially while environmental change occurs. Protoplast- based transformation system was established in tobacco and several panel of tobacco (Nicotiana tabacum cv. Petit Havana SR1) mutants created by T-DNA activation tagging approach: the ATER mutants which are resistant to the antimicrotubular herbicide (EPC) and chilling stress are altered microtubular dynamics. Cloned tagged gene from one of the mutant (ATER2) is a novel member of the cytochrome-P450 oxidase superfamily possibly a central player in the signal network that link microtubules organization to environmental triggers (Ahad et al. 2002; 2003). The Ping Pong mutants were characterized as a faster cold acclimation with having very special tubulin plaques. Cloned tagged gene is a DREB transcription factor might play a role during the cold acclimation (manuscript in preparation). The Inuit mutants were directly screened for long term cold resistance and can survive for more than seven months at 3°C –might an expectation for cold sensing or transmitting mechanism could be related to cytoskeleton during cold signaling. Additionally, a panel of mutants is called EPC/Inuit which combine both EPC resistance and cold stability. Last panel of mutants were resistant to Al-toxicity by an active bundling of actin microfilaments (Ahad and Nick, 2007).

During the characterization work by Abdul and Nick [1] showed that microtubules persist in inuit mutant even after exposure to ice cold temperature (0°C) as shown in figure below. The result reveals a partial disassembly within the early hour (between 2-3 hours). Earlier all report shoed to be an indication of cold resistance phenotype. Whereas a longer expose until 12 hours studied cell showed to be very strong and bundle form of cold resistance microtubules reestablished in the cells (see figure below after 12 h study). [1] In contrast the wild type SR1 tobacco cells showed to be sensitive within 1 hour and disassembly of microtubules occurred therefore one could find a lot of fragmented form of microtubules in the cells. In addition, within 4 hour ice cold temperature exposed microtubules were disassembling completely (see below).

**Figure:** The “Eskimo” (later called Inuit) mutant can survive 7 months at 3°C, and regenerates vigorously after transfer to 25°C forming numerous plantlets.
Figure 1.1 (are showed with permission from Dr. Abdul Ahad), showing microtubular response to chilling. Figure A, B, and C are microtubular response of wild type SR1 with immunolocalization of microtubules at 0 h (figure A used as a control), after 1 h (figure B) and 6 h (figure C). Figure G and I shows microtubular response to ice cold water of inuit mutant either after 1 h or 8 h.

1 INTRODUCTION

Ecological scattering, growth, reproduction of a plant depends on its communication with the surrounding physical, chemical, and biological environments, so called physiological ecology [11]. There are a number of Environmental factors might influence the growth of plant. Among them the three foremost factors effecting growth of rangeland plants are light, temperature and water which would in turn modify the internal regulators of plants which are intended for plant growth as well as development [12].

It is vital to understand the essential mechanisms in cellular level that make biological processes in plants and how does the above mentioned environmental factors effect the growth of plants by altering the biological processes. This is vital to us since plants present our world, the main source of food material, wood for building and furniture purposes in addition to energy [13]. It is known that plants have developed plenty of temporary and enduring adaptive mechanisms to the varying environment, as they can not alter their location with the changing environmental conditions which may include changes in the light conditions or relative amount of water or may involve changes in temperature [13]. It is interesting to see what changes do occur at genetic level in plants while adapting to such environmental changes. The basic question arises is how does a plant sense any change in the environment, depending on which certain signals are transmitted making certain set of genes on or off. Many studies concerned the identification and isolation of genes which play a major role during stress conditions. For example genes involved in development, pathogen resistance and biochemical pathways [14].
Basically there are two important techniques which are widely used to isolate genes depending on their function in plants which involve the integration of T-DNA and Transposon tagging [14]. When a T-DNA, a part of tumor inducing plasmid from Agrobacterium tumifaciens or a part of root inducing plasmid from Agrobacterium rhizogenes or a transposon is inserted into a plant genome, it results in disruption or inactivation of that particular gene, so called insertional mutagens in plants, also known as recessive mutation [1, 2]. In case of diploid species such mutations seems to be manifested after frequent self crossing of mutagenised populations which is intricate in case of higherploidy species such as solanaceae which can be trounced by usage of activation tagging.

In present studies we are interested to isolate tagged gene INUIT in a tobacco mutan which can persist low temperatures for longer period. We established a technique known as TAIL-PCR, Thermal Asymmetric InterLaced-polymerase chain reaction, an efficient method to amplify unknown sequences adjacent to known insertion sites (T-DNA integration site) which is very well known to work in case of Arabidopsis [2] will be applied here in case of tobacco.

1.1 CHILLING TOLERANCE:

A low temperature instigates the production of crop yield as they are sessile and is the major setback in temperate regions. More than ever crop species like cucumber, watermelon, cotton, rice and tobacco (known as cold sensitive species) experience severe irreversible damage when exposed to low temperatures. This damage is called chilling damage or chilling injury which is quite different from freezing damage. The degree of chilling tolerance of a plant depends on its physiological state. For example fertility of rice drops down below 18°C where as in freezing tolerant species they are tolerable even when temperature falls below zero at certain times[1, 7]. It is also observed that the plants obtain more resistance nature to cold temperatures when exposed to or treated over and over again with low non freezing temperatures through a complicated adaptive process called cold acclimation [7, 9]. It would be interesting to know signaling processes by which plants get low temperature signals sent to nucleus and activate the genes required for cold tolerance [9]. Such genes can be identified and isolate by means of T-DNA activation tagging.

1.2 T-DNA ACTIVATION TAGGING:

It is known that an insertion element T-DNA fragment from soil growing bacterium Agrobacterium when transferred in to a plant genome causes gene fusions. This method has been used for induction of genes in genus Arabidopsis and tobacco which is clearly explained by Koncz et al [15]. It also explains that T-DNA is a well known mutator element that can cause inactivation of a gene in which it is inserted, called recessive mutation. The technique is totally engineered based on a T-DNA vector which contains four transcriptional enhancer elements which is originated from CaMV-35S promoter is linked to the left border where as right border of transformation plasmid contains a hygromycin resistance gene acting as a marker to select transformed plant/cells. The right border also contains an ampicilin resistance gene allows to select on bacteria and for Agrobacterium on carbenicillin. The right border also contains an E.coli origin of replication flanked by hygromycin resistance gene and multiple enhancer element site which allows selection of bacteria’s during plasmid rescue in E.coli. The idea is after integration of T-DNA into plant
genome it might cause a cis activation of genes in the neighboring area of tag i.e. multiple enhancer elements activates the genes flanking the insertion site. Therefore the over expression of flanking genes make dominant traits which makes a possible selection directly in primary transformants [1, 15].

Activation tagging has been successfully employed to identify a histidine kinase from Arabidopsis that can bypass the requirement for cytokinin during shoot regeneration or to isolate mutants with altered morphogenesis or constitutive pathogen resistance [1].

As plasmid rescue unable to give us a expecting result we tried to establish an efficient tool which can amplify the unknown sequences adjacent to insertion site in order to know what gene is it and its possible function. So we established a technique called TAIL-PCR which is found to work well in Arabidopsis now in tobacco as well.

1.3 TAIL-PCR:

Thermal asymmetric interlaced polymerase chain reaction is a very well known method to amplify the unknown sequences flanking the known T-DNA insertion site and was found to work very well in case of Arabidopsis. We use two different types of primers one is called nested primer which is insertion specific, long and have a higher annealing temperatures where as second set of primers are called arbitrary degenerate (AD) primers, which are shorter and have low melting temperatures possibly can randomly bind throughout the genome. This technique uses the advantage of high and low annealing temperatures to amplify the target sequences. It consists of three rounds reaction. First round starts with 5 cycles with high annealing temperatures where insertion specific primer binds and linear amplification occurs producing specific single stranded products. Second round consists of 1-2 cycles of low annealing temperature and producing primer specific target sites. Further tail cycling preferred target site is amplified which consists of 15 super cycles in which each single super cycle has two cycles with high annealing temperature and one cycle with low annealing temperature. We usually get three types of products called type-1, type-2 and type-3 products. Type-1 products called specific products primed by insertion specific primer on one side and a non specific AD primer on other side. Type two products are called non specific primers primed on both sides by insertion specific primers. Type-3 products are also called nonspecific products primed on both sides by non-specific AD primers on both sides. In order to increase the amount of specific target products, second round of TAIL-PCR is performed with insertion specific nested primers which are systematically explained by Singer and Burke [2]. In present study this method is established in tobacco.
2. MATERIALS:

2.1 GROWING TOBACCO PLANTS:

Seeds from T1 generation plants were used for cultivation of tobacco plants. Inuit mutants were generated by T-DNA activation tagging method as explained in [1] and seeds from T2 generation of such mutants were used in this study.

2.2 COLLECTION OF PLANT MATERIAL:

Scalpel, Liquid nitrogen, gloves.

2.1 PLANT DNA PREPARATION:

Mortar and pestle, liquid nitrogen, falcon tubes, cetyltrimethylammonium bromide(CTAB), β-mercapito ethanol, water bath, chloroform: IAA (ISO AMYL ALCOHOL) mixture (24:1), isopropanol, distilled water, 2ml eppendorf tubes, lithium chloride (10 M), ice, 95% ethanol, 70% ethanol, nano drop meter (ND-1000 spectrophotometer)

2.2 OLGONUCLEOTIDES:

All the primers were ordered at In Vitrogen, Stockholm

2.5 TAIL-PCR:

PCR used is PTC-100™, programmable thermal controller

AD primer sequences: AD1 -5’ NGT CGA SWB ANA WGA A 3’, (183.993 pmol/μl)

AD2 -5’ TGW GNA GSA NCA SAG A 3’ (197.965 pmol/μl)

Where W=A or T, S=G or C, N=A or T or G or C.

T-DNA specific sequences:

LB1 - 5’ GCC TTT TCA GAA ATG GAT AAA TAG CCT TGC TTC C 3’ (79.263 pmol/μl)

Pst Tac Sequence - 5’ GCT GCA GAA TTA CTA TTT ACA A 3’ (120.197 pmol/μl)

Tac Sequence LB - 5’ ATT GAA TAT ATC CCG CCG CTG 3’

Where LB is left border
2.6 AGAROSE GEL ELECTROPHORESIS OF TAIL-PCR PRODUCTS:

TAE buffer, DNA gel loading buffer- bromophenol blue ordered from Bio-Rad laboratories, Stockholm and Agarose used is electrophoresis grade from invitrogen, Agarose gel apparatus, 16well comb, marker (100 bp ladder) used is from Bio-Rad, Stockholm.

2.7 GEL PURIFIED DNA OF TAIL-PCR PRODUCTS:

QIAquick PCR purification Kit is used to obtain pure DNA from agarose gel which can be used for sequencing. Purification kit was ordered from Qiagen

2.8 DNA SEQUENCING:

DNA sequencer used is model 377 DNA sequencer, ABI PRISM™,AB applied biosystems eppendorff tubes, double distilled water, sequencing primers (Taq LB).
3. METHODS:

3.1 GROWING TOBACCO PLANTS:

_inuit_ tobacco plants were grown in green house at temperature 25\(^{\circ}\)C and light intensity of 150 \(\mu\text{mol/m}^2/\text{s}\). Soil used to grow plants is Yrkes plant jord, korn mull, weibulls\(^{R}\). Seeds were sterilized with sterilizing solution (sodium hypochloride containing 0.1% Triton X-100) for 15 minutes and then washed with 95% ethanol for 5 times. Special care have been taken not to leave seeds for longer time in 95% ethanol and dried in hood. Seeds were first sowed in small pots and around 2 weeks old seedling were transferred to bigger pots for proper grown. Plants were grown up to flowering stage. We grew four different lines of _inuit_.

3.2 COLLECTION OF PLANT MATERIAL:

Leaves from plants were collected when plants were approximately of age 45 days; sampling was done when leaves were quite big enough. Leaves were cut out with a scalpel and frozen immediately in liquid nitrogen.

3.3 PLANT DNA PREPARATION:

3.3.1 ISOLATION OF DNA FROM _inuit_ LEAVES USING CTAB METHOD:

1. Take two 50 ml falcon tubes and add 10 ml of warm CTAB buffer in each of falcon tubes
2. Grind collected leaf material with mortar and pestle with liquid nitrogen and add 3-4 g of grinded leaf tissue to the falcon tubes (mentioned above in point 1).
3. Add 100\(\mu\)l of β-mercapito ethanol
4. Incubate in water bath at 65\(^{\circ}\)C for 20', keep shaking in between
5. Add 10 ml of chloroform: IAA mixture and place the tubes on shaker for 10'
6. Centrifugue the tubes for 7' at 4500 rpm at 20\(^{\circ}\)C
7. Transfer the supernatants to a fresh tube and add 5 ml of 10% CTAB warm and keep in water bath for at 65\(^{\circ}\)C for 5-10'
8. Add 10 ml of chloroform: IAA once more and keep on shaker for 10'
9. Centrifugue for 7' at 20\(^{\circ}\)C at 4500 rpm.
10. Transfer supernatant to fresh tube, add equal amounts of isopropanol was added and incubate on cold ice for 10'.
11. Centrifugue for 15' at 4500 rpm at 20\(^{\circ}\)C
12. Discard the supernatant, dissolve the pellet in 1 ml of distilled water and transfer the content in to a new eppendorf tube and add equal volume of (1 ml) 10 M lithium chloride, incubate on cold ice for 20'
13. Centrifugue for 30' in cold room at maximum speed
14. Transfer supernatant to a new tube and add equal volume of 95% ethanol, incubate on ice for 20'.
15. Centrifugue at room temperature for 20' at 14000 rpm
16. Discard supernatant and wash pellet with 70% ethanol (50-100 \(\mu\)l)
17. Centrifugue for 10' at room temperature at 14000 rpm
18. Discard supernatant and suspend pellet in required amounts of distilled water (50-100 µl).
19. Measure concentration of DNA using a Nano drop spectrophotometer.

3.4 OLIGONUCLEOTIDES:
3.4.1 PREPARATION OF AD POOL:

AD1- 15 µl + AD2 -15 µl + Nuclease free water 15 µl = 45 µl

3.4.2 Dilution of stocks to bring it to concentration of working stock:

\[ \text{LB1} - 1:7 - 5 \text{ µl} + 35 \text{ µl nuclease free water} \]
\[ \text{Taq 7 LB1} - 1:13 - 3 \text{ µl} + 39 \text{ µl nuclease free water} \]
\[ \text{Taq 7 LB2} - 1:17 - 3 \text{ µl} + 51 \text{ µl nuclease free water} \]
\[ \text{Taq 7 Pst} - 1:12 - 3 \text{ µl} + 36 \text{ µl nuclease free water} \]

3.5 TAIL-PCR:
3.5.1 SINGLE REACTION SET UP FOR PRIMARY PCR:

Nuclease free water – 4 µl

10X PCR buffer containing MgCl\(_2\) - 1 µl

d NTP solution – 0.5 µl

LB\(_1\) primer – 0.3 µl

AD pool – 2.5 µl

Taq Poly – 0.2 µl

Genomic DNA (diluted 100X) – 1.5 µl

Total- 10 µl

PCR SET UP:

1. 94\(^\circ\)C - 3'
2. 94\(^\circ\)C - 30 sec
3. 62\(^\circ\)C - 1'
4. 72\(^\circ\)C - 2.3'
5. 5 cycles of step (3-5)
6. 94\(^\circ\)C - 30 sec
7. 25\(^\circ\)C - 3'
8. 72°C - 2.3'
9. 2 cycles of step (7-9)
10. 94°C – 10 sec
11. 68°C - 1'
12. 72°C – 2.3'
13. 94°C – 10 sec
14. 68°C - 1'
15. 72°C – 2.3'
16. 94°C – 10 sec
17. 44°C – 1'
18. 72°C - 2.3'
19. 15 cycles of step (11-19)
20. 72°C - 5'
21. Holding temperature - 4°C

3.5.2 SINGLE REACTION SET UP FOR SECONDARY PCR:

Nuclease free water – 5 µl

10X PCR buffer containing MgCl₂ - 1 µl
d NTP solution – 0.5 µl

Pst Taq7 primer – 0.2 µl

AD pool – 2.5 µl

Taq Poly – 0.2 µl

Genomic DNA from primary PCR (diluted 100X) – 1.5 µl

Total- 10 µl

PCR SET UP:

1. 94°C - 3'
2. 94°C - 10 sec
3. 64°C - 1'
4. 72°C - 2.3'
5. 5 cycles of step (2-4)
6. 94°C - 10 sec
7. 64°C - 1'
8. 72°C - 2.3'
9. 94°C - 10 sec
10. 64°C - 1'
11. 72°C - 2.3'
12. 94\(^0\)C - 10 sec
13. 64\(^0\)C - 1'
14. 72\(^0\)C - 2.3'
15. 15 cycles of step (6-9)
16. 94\(^0\)C - 10 sec
17. 44\(^0\)C - 1'
18. 72\(^0\)C - 3'
19. 5 cycles of step (16-18)
20. 72\(^0\)C - 5'
21. Holding temperature - 4\(^0\)C

3.5.3 SINGLE REACTION SET UP FOR TERTIARY PCR:

Nuclease free water – 9.5 µl
10X PCR buffer containing MgCl\(_2\) - 2 µl
d NTP solution – 2 µl
Taq LB primer – 0.6 µl
AD pool – 5 µl
Taq Poly – 2.5 µl
Genomic DNA from secondary PCR (diluted 100X) – 1.5 µl

Total- 10 µl

PCR SET UP:

1. 94\(^0\)C - 3'
2. 94\(^0\)C - 10 sec
3. 44\(^0\)C - 1'
4. 72\(^0\)C - 2'
5. 20 cycles of step (2-4)
6. 72\(^0\)C - 5'
7. Holding temperature - 4\(^0\)C

3.6 AGAROSE GEL ELECTROPHORESIS OF TAIL-PCR PRODUCTS:

Protocol to prepare 1.5% agarose gel:
1. Take 100 ml of TAE 1X buffer and add it to a beaker containing 1.5% electrophoretic buffer, warm it until the buffer melts appropriately
2. Set up a gel chamber with comb, pour the above prepared gel and wait for half an hour at least so that gel can solidify.
3. Remove the comb, immerse gel with TAE buffer, load samples 3μl of each in the lanes with loading buffer, in one lane add a marker (4μl)
4. Run the gel for 35-40 minutes at 100-120 volts and stain by shaking it in EtBr solution, scan and save the picture.

3.7 GEL PURIFIED DNA OF TAIL-PCR PRODUCTS:

Protocol for precipitating tertiary Tail-PCR products before loading on agarose gel to be used for sequencing:

As we need a sufficient amount of DNA from tertiary Tail-PCR products, we run several rounds of Tertiary PCR in order to have sufficient amount. In order to get some extent of pure DNA we precipitate tertiary PCR products by following method in order to get rid from excess primers, nucleotides etc.

1. Measure the volume of all Tertiary Tail-PCR products put together in to a eppendorff tube
2. Add double the volume of ethanol to the above tube
3. Add 10% percent of 5M sodium acetate (according to the total volume of PCR products)
4. Incubate the tube for twenty minutes at -80\degree C
5. Centrifuge for twenty minutes at 4\degree C
6. Wash pellet with seventy percent ethanol and centrifuge at room temperature for five minutes at 14000 rpm.
7. Dry and dissolve pellet in required volume of nuclease free water.

Protocol to get purified DNA from agarose gel to be used for sequencing:

1. When Tertiary PCR products are run after precipitation on agarose gel, we get a band which should be purified (here we used QIAquick PCR purification Kit) in order to use that DNA for sequencing.
2. Cut out the gel where you have interested band, weigh it and add three volumes (three times to amount of band) of QG buffer to eppendorff tube containing the gel with band.
3. Incubate the tube at 60\degree C for 10', keep vortexing in between
4. Add one volume of isopropanol mix thoroughly and load the sample in to the column provided with kit.
5. Centrifuge for 30 seconds at maximum speed at room temperature.
6. Wash column with required amount of PE buffer and centrifuge for 30 seconds.
7. Centrifuge for one more minute so that column is completely dry and transfer column to a new eppendorff tube.
8. Add required volume of nuclease free water and incubate for one minute at room temperature, centrifuge for two minutes and finally we will have DNA in eppendorf tube

9. Measure concentration of DNA using a Nano Drop. (Nano Drop technologies, Saveeno Werner, Sweden)

3.8 DNA SEQUENCING OF TAIL-PCR PRODUCTS:

Sequencing was performed in Genomics lab of Umeå Plant Science Centre according to their preferred protocol where the sequencing reactions are performed using DYEnamic ET terminator cycle sequencing kit from ABP. Cycle sequencing reaction program is

95°C – 20 seconds (denaturation)

50°C – 15 seconds (annealing)

60°C – 1 minute (extension)

28 cycles

For further details to prepare sequencing samples refer http://www.upsc.se/samples.htm

3.9 SEQUENCE STUDY:

Sequence homology was studied in

1. NCBI (National Center for Biotechnology Information) BLAST (Basic Local Alignment Search Tool)
2. Expacy Blast Program
4. CELL BIOLOGY WORK:

4.1 Materials: Ice box, MS medium, seeds, forceps, scalpel, slides, cover slips, and microscope.

4.2 Method: seeds were sterilized as mentioned above in section 3.1 and plated on MS medium with out antibiotic for wild type and with (50mg/l) hygromycin antibiotic (Duchefa, Netherlands) for inuit and were allowed to grow until the seedlings reached an age of 15 days. At this stage some of seedlings of wild type and mutant were exposed to cold conditions by incubating the MS plate containing seedlings, covered with aluminum foil in an ice box. After three days root and hypocotyls cells were observed under Axio vision microscope (Axioplan 2 imaging microscope). Another set of seedlings of wild type and mutant on MS plate were incubated at low temperatures in an ice box, root and hypocotyls cells were observed after 5 days of exposure to low temperatures. This method was followed similar for wild type as well as inuit. A control was maintained in parallel for wild type and mutant which are unexposed to low temperature to which exposed were tallied. After exposure to low temperatures, a recovery stage was also observed for wild type and mutant by placing the exposed seedlings to temperature where controls were grown i.e. at 22\(^0\)C. We also measured length of hypocotyls and root at particular intervals after exposure to low temperatures and compared with control which are un exposed to low temperature and recover phase is also observed. We collected data of recovery at intervals up to 15 days and for each data an average of three seedlings were collected.
5 RESULTS:

5.1 Growing Tobacco Plants:

We observed that *inuit* is dwarf when compared to the wild type and is a male sterile. We observed a severe inflorescence fall in *inuit* and blackend anther was observed where as in wild type anthers were healthy. We stained pollen grains with 1mM DAPI and observed with Axioscope, Zeiss, Giessen, Germany. Dwarfism of *inuit* is because of reduced internodal length.

Figure 5.1.1 A-G, A-*inuit* and SR1 plants grown in green house, from the figure we see that *inuit* is an dwarf mutant compared to that of wild type. B- Representative pollen samples stained with DAPI for nuclei of *inuit* are shown are male sterile accompanied by production of nuclear pollen. C- Nodal flowers were observed in *inuit* and anthers were male sterile shown in figure, D- falling of nodal flowers in *inuit* compared tot hat of wild type which were healthy as shown in figure E, F- Flower of a wild type showing normal and healthy anthers. G- Representative pollen samples stained with DAPI showing healthy pollen.
Figure 5.1.2 A, B showing Hand cross section of flower stock from SR1(A) and Inuit (B)

Figure 5.1.2 shows us cross section of flower stock which was incubated with phloroglucinoethyl (serva) and a few drops of HCl for 15 min, the sections were then washed three times with distilled water and observed with bright field microscope, Axioskop, images were recorded digitally, Axiovision [5]. Comparative stock section result reveals that there are more layers of cortex when compared to that of wild type which make the stem thicker in case of inuit.

We observed that there are expanded layers of cortex when compared with wild type, which might be one of the case to have a thicker stem in inuit.

5.2 TAIL-PCR:

After extraction of DNA from inuit, TAIL PCR was performed. TAIL PCR products are run on agarose gel in order to detect specific Type-1 products. As we know that in TAIL PCR, after first round multiple bands commonly representing non specific type-2 products are noticeable on agarose gel where type-1 specific products are too low to be detected. So specific type-1 products are clearly detectable after third round of Tail Per where unspecific products are too low and no longer detectable as shown in Figure 4.2.2 and specific type-1 products are sufficient to be visible but disturbed by unspecific products after second round of Tail Pcr as shown in Figure 4.2.1. As explained in figure 4.2.1 we tried different insertion specific primers. As Taq7 Pst was found to work well in our study and we used the same primer for further Tail pcrs and it shows that inuit has a same integration prototype in all four lanes.
Figure-4.2.1 One percent agarose gel showing secondary Tail Pcr products stained with ethidium bromide. Wells starting from left to right first four wells were loaded with secondary Tail Pcr products of *inuit* subjected to pcr using different primers following from left to right are LB₁, Taq7 LB₁, Taq7 LB₂, and Taq7 Pst. As we clearly see that Taq7 Pst primer gave us a good visible band, this primer was used for further Tail Pcr experiments.

Figure-4.2.2 One percent agarose gel showing Tertiary Pcr products stained with ethidium bromide where specific type-1 products can be clearly visible. Multiple bands may be visible after secondary or tertiary Tail Pcr which may represent multiple insertion sites of T-DNA in to plant genome or may be a single insertion site primed with nested AD primers. Figure shows four different lines of *inuit* starting from left to right, and a marker (100 bp ladder)
Figure 4.2.3 1.8% Agarose gel showing Tertiary Tail Pcr products in a bulk amount which were precipitated before running as explained in methods, on agarose gel and stained with ethidium bromide. The bands marked are of 700bp and 600bp which were used for sequencing. Second well shows us a 1kb ladder, Marker.

5.3 SEQUENCE STUDY:

We had a gene sequence of 829 bp which showed homology to putative Leucine-rich repeats LRR protein kinase and a ATP binding/kinase/protein serine/threonine kinase mRNA of Arabidopsis thaliana.
6 CELL BIOLOGICAL WORKS:

We could collect the data only for mutant as wild type showed abnormal behavior on MS plates.

Table 6.1

<table>
<thead>
<tr>
<th>Number of days</th>
<th>Inuit Exposed to low temp</th>
<th>CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HYPOCHOTYL</td>
<td>ROOT</td>
</tr>
<tr>
<td>3 DAYS</td>
<td>0.2 CM</td>
<td>0.9 CM</td>
</tr>
<tr>
<td>5 DAYS</td>
<td>0.4 CM</td>
<td>1.0 CM</td>
</tr>
</tbody>
</table>

Table 6.1 shows us data of *Inuit* exposed to low temperature. Data of hypocotyls length and root length were obtained after three and five days of exposure.

Table 6.2

<table>
<thead>
<tr>
<th>Days of recovery</th>
<th>Inuit</th>
<th>CONTROL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HYPOCHOTYL</td>
<td>ROOT</td>
</tr>
<tr>
<td>AFTER 1 DAY</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>2 DAY</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>3 DAY</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>6 DAY</td>
<td>0.8</td>
<td>0.7</td>
</tr>
<tr>
<td>15 DAY</td>
<td>0.9</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Table 6.2 shows us data of recovery phase of seedlings exposed to low temperature. Recovery phase has been observed up to 15 days and hypocotyls length, root length was obtained in CM.
7. DISCUSSION:

Plants growing in moderate climates build up a better aptitude to endure freezing in retort to low but non freezing temperatures by a process called cold acclimation. There is insufficient information if how plants get low temperature stimulus and transduce it to nucleus to turn on genes needed for amplifying freezing tolerance [9]. Characterization of eskimo1 mutants and other mutants showed that there are different signaling pathways which activate many aspects of cold acclimation. Activation of a single path way can bring in significant freezing tolerance with out activation of other path ways. Proline was found to play a major role in plant freezing tolerance i.e. mutation at esk1 locus causes an increase in level of proline and which would induce AtPOX gene and results in breakdown of proline so this is one of the examples out of many signaling path ways [9].

It is clearly known that there are several signaling pathways which are supposed to increase freezing tolerance; even activation of single pathway has a significant increase in amplifying freezing tolerance [9]. Results from Zhanguo and Browse suggests that cold acclimation is not a straightforward and linear signaling pathway turning on full set of processes vital for improved freezing tolerance as an alternative they anticipated a model for cold acclimation in which parallel or branches signaling pathways activate discrete sets of cold-acclimation responses. It was found that there are 12-15 different genes which when mutated, results in constitutive increases in freezing tolerance [9]. On other hand studies by Nick showed that microtubules disassemble in response to low temperature and are key targets of chilling stress. Treatment with blockers of microtubule assembly was observed to boost chilling sensitivity [1]. When microtubule disassembly in response to low temperature was suppressed by taxol, long-term acclimatization to low temperature was impaired signifying microtubule disassembly is necessary to induce proficient cold-acclimation. Nick in 2000 projected a model where chilling induced microtubule disassembly capacitates calcium influx through cold sensitive ion channels which triggers cold hardening and arrangement of cold resistant micro tubular cytoskeleton. So one can make out from that is genes which are accountable for microtubule assembly or disassembly are supposed to control chilling tolerance. To confirm which genes played a role one can over express the genes and study the change in chilling tolerance; nevertheless those genes are not known [1].

To detach unidentified genes based on their function mutant approaches are extensively used frequently in permutation with gene tag. Insertional mutagenesis is an attractive tool to establish gene function through loss of function alleles. In plants T-DNA is used as an insertional mutagen in many cases. T-DNA (transferred DNA) is a part of tumor inducing plasmid from Agrobacterium a soil growing bacteria. T-DNA is transformed in to plants along with plasmid replicon and a selectable hygromycin resistance gene [15]. Up on integration in to plant genome this insert causes cis activation of genes in surrounding area of insert. Thus insertion of this tag activates flanking genes with help of multiple enhancer elements; this over expression of flanking genes would create dominant character such that selection would be feasible in primary transformants itself. For instance Activation tagging has been productively engaged to identify a histidin kinase from Arabidopsis that can evade the requirement for cytokinin during shoot regeneration or to isolate mutants with changed morphogenesis or constitutive pathogen resistance [1]. It has been known that dicistronic gene fusions are translated in tobacco [15].
Several mutants were generated for analysis of genes related to chilling tolerance in *Arabidopsis* and *Tobacco* which was thoroughly explained by Koncz et al. In order to explore possible role of microtubules in cold resistance several panels of *Tobacco* mutants were generated using T-DNA activation tagging [1]. *Inuit* mutants were directly screened for long term cold resistance in this advance. The phenotype of *Inuit* was characterized by exceedingly slow development accompanied by semi-dwarfism caused by condensed internode length and by male sterility as shown in figure 5.1.1. Findings with *Inuit1* did not show any indications for altered abundance of tyrosinilated α-tubulin compared to other mutants suggesting that in these mutants chilling tolerance is based on different mechanism [1]. Extensive functional genomics methods which aim to inundate whole genome with insertions, thousands of insertion sites have to be determined. Success of such projects depends on ability to amplify sequences flanking insertion sites.

Even though plasmid rescue technique works very well in Arabidopsis it seems in Tobacco does not work in same extent. We established usage of TAIL-PCR in Tobacco to isolate INUIT gene in this approach. TAIL-PCR was found to work very efficient in case of Arabidopsis. The disadvantage of using T-DNA as an insertional mutagen is it may insert tandem arrays or more complex insertion pattern, resulting in adjacent T-DNA border hampering isolation of plant sequences flanking T-DNA inserts. It seems that 62% of adjacent T-DNA arrays were found in right border of insert whereas its 25% incase of left border [2]. So in our study we used insertion specific primers binding to left border of T-DNA as followed by Singer and Burke. Reason behind using AD pool in our study is because it was found to be the perfect combination out of many combinations tested yielding most specific products and also a finest way to evade different TAIL-PCRs with individual AD primers. AD primers are pooled such that their final concentration in reaction mixture for primary and secondary TAIL-PCR is relative to their level of degeneracy [2]. Figure 4.2.2 in our studies is a result after tertiary TAIL-PCR which shows similar integration pattern of INUIT gene in all four mutant lines. Smears were observed after secondary as well as tertiary TAIL-PCR it may be due to many reasons, dNTPs may have been misaliquoted or little genomic DNA or inequity in primer concentrations and there might be many other reasons for smear formation which are methodically explained by Singer and Burke. Tertiary TAIL-PCR products are generally used directly for sequencing, but we preferred to purify tertiary TAIL-PCR products to be certain that our products are free from excess nucleotides and primers.

Sequencing results, as mentioned above showed homology to putative Leucine-rich repeats LRR protein kinase and a ATP binding/ kinase/ protein serine/ threonine kinase mRNA of *Arabidopsis thaliana*. Gene sequence obtained after sequencing had 829 bp. Receptor kinases play significant role in cellular signaling processes in animals. A receptor kinase is commonly known to have an extracellular domain, a transmembrane domain and an intracellular catalytic kinase domain. Studies with animal receptor kinases suggested a model for receptor kinase mediated cellular signaling processes, the model suggests that when a ligand binds to extracellular domain, receptor dimerization occur which triggers following activation of intracellular kinase domain. Activated kinases then phosphorylate substrate proteins with in cell resulting in transduction of signals [4]. Lately it was set up that a number of gene products with this feature design have been identified in plants referred to as receptor like kinases (RLKs). Plant RLKs were found to have similar biological mechanism of action as of animals. It seems
that categorized plant RLKs are categorized into three groups based on protein motif in the supposed extracellular domain. The major group is leucine rich repeat (LRR) RLKs which contain imperfect repeats of 24 amino acid leucine rich motif in extracellular domain [4].

Plant LRR-RLKs were established to play a vital role in mediating a diversity of cellular processes which includes morphogenesis, embryogenesis, meristamatic growth and pollen self incompatibility. Some were found to regulate environmental signals such as light, hormones, and pathogen and some were found to which no cellular functions has been assigned [4]. Xiaorong Zhang identified plant LRR-RLKs from databases using computer programs. In database searches it was found that there were over forty sequences of LRR-RLKs in plants, most of them were isolated from *Arabidopsis thaliana* genome and were found on all five chromosomes of *Arabidopsis thaliana*. Biological functions of them were mostly unknown. Xiaorong Zhang attempted to compare amino acid sequences among all available LRR-RLKs of plants from databases and to investigate evolutionary and functional relationships among LRR-RLKs which are clearly explained in [4].

Our results in this study suggest that there are genes encoding putative LRR-RLKs in *in situ* close to T-DNA insertion site i.e. close to left border and hence might be over expressed because of enhancer elements in the insert which were supposed to over express genes in its vicinity or could be some kind of mutation in the gene might have occurred making the mutant a long term cold resistant also inducing changes from wild type like male sterility and dwarfism due to reduced internodal length. Apart from these *in situ* was found to be resistant to toxic metal Aluminium in soil [5].

A similar case was found in *Arabidosis*, where a gene *STRUBBELIG (SUB)* defines a receptor mediated signaling pathway in plants. *SUB* was found to encode a putative leucine rich repeat transmembrane receptor like kinase. Mutant *sub* phenotype suggested that *SUB* effects formation and shape of several organs such as carpels, petals and is necessary for shape and height of stem, influencing cell morphogenesis, orientation of division plane and cell proliferation. Mutational analysis was found to suggest that kinase domain is important for *SUB* function. Some biochemical assays were performed by David and Martine using bacterial expressed fusion proteins which indicated that *SUB* kinase domain lacks enzymatic phosphotransfer activity [8]. Signaling involving RLKs constitutes an important aspect of plant cell communication and contributes to plant pathogen interactions, hormone signaling and development [8]. It seems that particular mutations in *α*-tubulin genes and defects in a number of genes encoding microtubule interacting proteins result in alterations in cortical microtubule organization and helical growth of various plant organs [8]. It is known that cold stability of microtubules is actively maintained by a pathway that might involve protein kinase [7].

Cell biological work in this study mainly targeted to study cell division against cell growth suggest that hypocotyls length was increased in *in situ* from 0.2 cm to 0.9 cm when exposed to low temperature for five days and to a temperature of 22°C for 15 days when compared to that of control which is exposed to low temperature suggesting that exposure to low temperature caused an increase in length of hypocotyls where as in seedlings which were not exposed to low temperatures, length of hypocotyls was constant showing 0.3 cm as shown above in results. In case of roots there was an increase in length from 0.2 to 0.9 cm as shown above where as in
control root length was much higher, it grew from 1.0 cm to 5.0 cm as shown in results. Owing to some technical reasons we could not collect the data for number and size of root and hypocotyl cells microscopically but could measure the length of hypocotyl and root manually.

We speculate from the results that there might be some leucine rich repeat mediated signaling pathway is induced in *inuit* which bring it special features like long term cold resistance, male sterility with blackening of anthers as shown in figure 5.1.1, semi dwarfism due to reduced internodal length and thickening of bark which makes the floral stalk thick as shown in figure 5.2.2 when compared to that of wild type as well as we observed a reduced length in root when exposed to low temperatures and an increased hypocotyl length. It was also found that plant RLKs are known to function in development, hormone perception and pathogen response. RLKs are also involved in diverse array of developmental and defense functions that include gametophyte development, pollen-pistil interactions, shoot apical meristem equilibrium and cell morphogenesis. So we speculate that LRR-RLKs play a major role in activating a signaling pathway which induces cold resistance in *inuit*.

REFERENCES:

1. Ahad, Abdul; Wolf, Jochen; Nick, Peter (2003) -Activation-tagged tobacco mutants that are tolerant to antimicrotubular herbicides are cross-resistant to chilling stress (Transgenic Res. 2003 Oct; 12(5):615-29
7. Activation tagged rapid cold acclimatized Ping Pong tobacco mutants- Abdul Ahad and Peter Nick (submitted).
11. Plant physiological ecology by –Thijs L Pons, Hans Lambers, F Stuart Chaplin II


17. Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways-Shinozaki K, Yamaguchi-Shinozaki K.

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