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Expressed Sequence Tags Identification for Fusarium Wilt Resistance in Chick Pea (*Cicer arietinum*)

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ABSTRACT

Expressed sequence tags(EST)s have wide spread use viz., as a molecular marker and base material for gene expression studies. For the present analysis two subtracted libraries one from the resistant chickpea cultivar (WR315) and the other from the susceptible chickpea cultivar (JG62) to the *Fusarium* wilt pathogen were being used. Genes with altered expression upon pathogen infection were enriched by the subtraction procedure and were cloned in pGEMT Easy vector. Subtracted libraries after the insert screening were stored as glycerol stocks in 96 well microtitre plates and further analyzed by sequencing the inserts using automatic sequencing machine. The sequences were processed further to remove vector sequences like blast search against the existing databases available at the NCBI site. A cut off score of 100 was put to find the best matches and the top match was used for all the down stream work. Initially all the sequences were analysed using Blast-X Programme followed by Blast-N and TBlast-x. Sequences showing no significant match after the Blast search were further analysed using Stand alone Blast to remove redundant clones. Some of the interesting genes fall in the class of regulatory proteins, signaling proteins and defense response proteins as for example, bZIP, bHLH, Zn-finger transcriptional activators, kinases, phospahatases, G-protein coupled receptor and this study paved way to identify EST's upon Fusarium infection in Chickpea.

Key words : Chickpea, EST, Fusarium wilt.

Plants have evolved a broad array of defense mechanisms to diseases resistance and the resistance appears to depend on the ability of the host to recognize the pathogen rapidly and induce these defense responses in order to limit pathogen spread. Expressed sequence tags (ESTs) provide a very useful means of quickly accessing gene sequence information and bridge the gap between the genome sequence and gene function (Luo *et. al.,* 2003). ESTs provide an invaluable resource for analysis of gene expression associated with specific organs, growth conditions, developmental processes and responses to various environmental stresses White *et. al.,* 2000. EST have been useful for intra and intergenomic comparisons, gene discovery and molecular marker generation, for example, SSRs and SNPs that can be further used for creating genetic maps (Rafalski 2002). In the present study We used genomic approach specially EST's in studying molecular mechanism of *Fusarium* wilt in chickpea so to analyze the genes involved in resistance against the *Fusarium* wilt. Chickpea (*Cicer arietinum*) is one of the world's most important leguminous food crop, grown in over 40 countries representing all the continents, but is susceptible to this fungus (Nene and Reddy, 1987). In order to identify the transcripts induced upon *Fusarium* infection we used SSH libraries prepared from both the resistant and susceptible genotypes of Chickpea. Sequence analysis of transcripts (EST's) taking few representative clones revealed differences in the nature of transcripts between the genotypes. Some of the interesting genes fall in the class of regulatory proteins, signaling proteins and defense response proteins. In total the present study provided the basis in identifying functional markers i.e., EST's against *Fusarium* wilt and in future will aid in isolation and characterization of resistance genes for accelerated molecular breeding of Chickpea.

MATERIALS AND METHODS

Suppression Subtractive Hybridization

Clontech PCR-Select cDNA subtraction kit was used to carry out suppression subtractive hybridization (Over view in Fig.1.1).

Two subtractive libraries were made one each for the susceptible and resistant genotypes. For susceptible genotype library RNA isolated from *Fusarium* infected JG 62 plants was used as tester

and RNA isolated from uninfected, water inoculated plants was used as driver. Similarly for resistant library RNA isolated from WR-315 plants infected with *Fusarium* was used as a tester against WR-315 uninfected plants as driver.

First hybridization

In this procedure an excess of driver cDNA was added to each tester cDNA. Thereafter the sample was heat denatured and allowed to hybridize. During hybridization the concentration of high and low abundance sequences got equalized as re-annealing happened faster for the abundant molecules due to second order kinetics of hybridization. As the tester and driver cDNAs formed hybrids the differentially expressed sequences which remained single stranded, got enriched and were available for the second hybridization.

Second Hybridization

The two samples from the first hybridization were mixed together and fresh denatured driver cDNA was added to further enrich for differentially expressed sequences. In the process of second hybridization only the remaining equalized and subtracted single-stranded tester which represented differentially expressed sequences re-associated and formed new hybrids. These hybrids were ds tester molecules with different ends. The primary hybridization samples were not denatured at this stage. The process was carried out without removing the hybridization samples from the thermal cycler for no longer than necessary to add fresh driver.

Amplification of subtracted cDNA population by PCR

As a result of second hybridization the differentially expressed ds cDNA with two different adaptor ligated to their ends got enriched. These enriched differential cDNA were selectively amplified by PCR amplification. Prior to PCR the missing strands of the adaptors were filled in by brief incubation at 75°C. During PCR due to suppression effect, the molecules which were not differentially expressed i.e., whose level of expression was same in the two populations, formed pan like structures and their amplification was prevented. The primary PCR was carried out using a primer that has a sequence common to both adaptor 1 and 2. The secondary PCR was carried out using adaptor specific nested primers which further enriched the subtracted population. For both the susceptible and resistant libraries the primary and secondary PCR was carried out for (1) the forward subtracted tester cDNA (2) the unsubtracted tester control for forward subtraction (3) the reverse subtracted tester cDNA (4) the unsubtracted tester control for reverse subtraction.

Analysis of PCR

5µl of the secondary PCR mix of forward and reverse subtracted and unsubtracted testers for each subtraction were analysed on 2% Agarose/ ETBr gel run in 1X TAE.

Cloning of subtracted population

The enriched differentially expressed cDNAs were cloned in pGEMT vector. 50 ng of the vector was ligated to 100ng and 150 ng of the PCR product separately.

Transformation of *E.coli* competent cells with ligation mix

For each transformation, a 100µl aliquot of the DH5a competent cells was used.

1. The competent cells were thawed on ice.
2. 5ìl of the ligation mix and 45 ìl of TE was added to the 100 ìl of competent cells and incubated for 45 min on ice with gentle tapping at regular intervals.
3. The cells were subjected to heat shock at 37°C in a water bath for 5 min and then quickly transferred to ice for 5 min.
4. The whole mix was added to 3.85 ml of 2XL broth containing 0.2% glucose and the cells were allowed to outgrow for 90min at 37ºC at 200 rpm.

A positive control of transformation was carried out with 1ng of intact pBluescript II vector and a negative control was kept without addition of DNA.

Plating of Transformation mix

LB agar plates (150mm diameter) containing 75 ìg/ ml ampicillin were smeared with 80 ìl of 2% X-Gal and 20ìl of 20% IPTG. The plates were dried in laminar air flow and then incubated agar side up in 37°C incubator. Whole of the 4ml -transformation mix, in 200 ìl aliquots, was spread plated on these LB agar plates and incubated at 37° C for 16hr. Afterwards the plates were kept in the cold room for 3-4 hr until blue and white coloured colonies became distinct. White

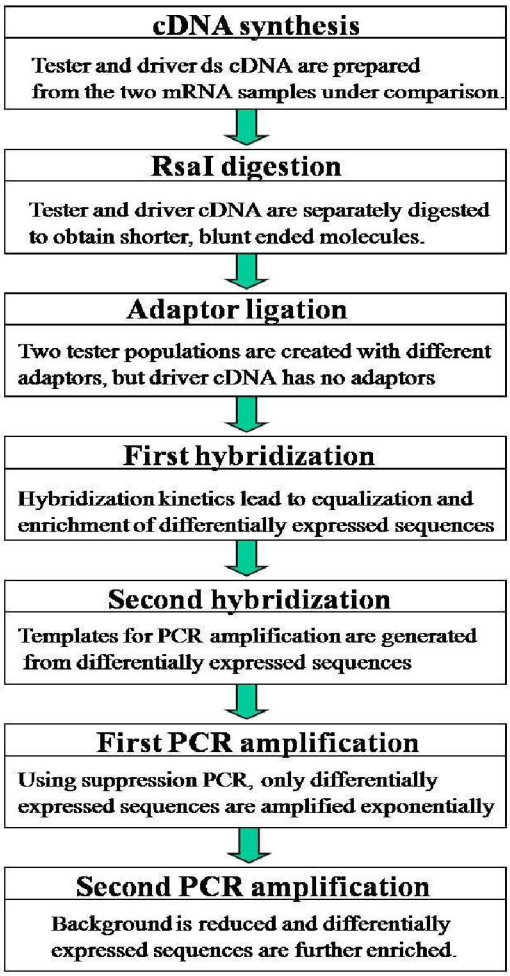

Fig.1.1 Overview of the PCR-Select procedure

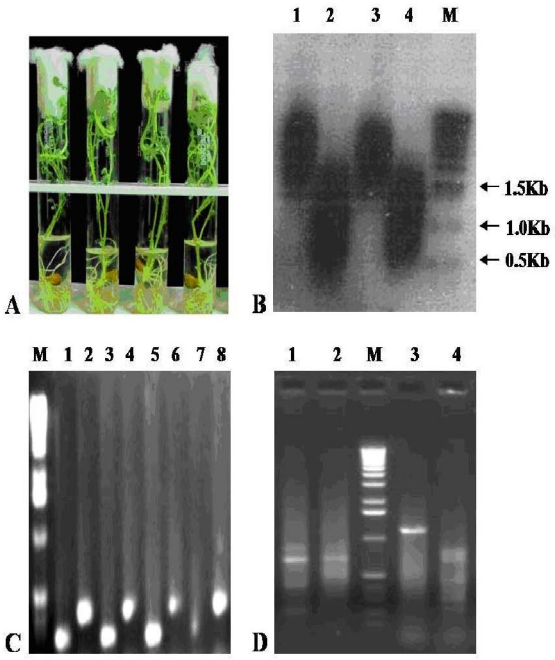

Figure 1.2. Construction of SSH library from WR-315 resistant variety of chickpea. (A) 4 week old plants of resistant variety 5 days after infection with *Fusarium;* (B) Characterization of SSH library; Lanes 1, 2, 3 & 4 represents Undigested ds tester, *RsaI* digested tester, Undigested ds driver and *RsaI* digested driver, respectively; (C) Ligation efficiency; Lanes 1-4 represents tester and lanes 5-8 represents, driver, lane 1 & 5: adaptor 1 ligated tester/driver amplified with actin 3' primer and PCR primer; lane 2 & 6: adaptor 1 ligated tester/driver amplified with Actin 3' and 5' primer, Lane 4 & 8: adaptor 2 ligated tester/driver amplified with Actin 3' and 5' primer; (D) Subtraction efficiency; Lanes 1, 2, 3 & 4 represent unsubtracted tester, subtracted tester, unsubtracted driver and subtracted driver, respectively.

colonies were picked with sterile toothpicks and patched on LB ampilcillin plate with X-Gal and IPTG. Next day after distinct colour had developed white colonies were picked and re-patched on fresh LB ampicillin plates. These white colonies represent different cDNAs constituting the library and were used for making the glycerol stocks.

Preservation of clones as glycerol stocks on microtiter plates

1. 1.2ml of 2xYT medium containing ampicillin (75µg/ml) was dispensed into each well of 96 well deepwell plate.
2. Using sterile toothpick the colonies were picked and inoculated into media already dispensed into deepwell plate.
3. The deepwell plate was sealed with airpore sheet and incubated at 37ÚC for 16-18hr under shaking at 250rpm.
4. For preparing the glycerol stocks 30µl of 80% glycerol was dispensed in all the rows of 500µl capacity 96 well U bottom microtiter plate using a multichannel pipette in the laminar.
5. 220µl of each bacterial culture was picked in each row with multichannel pipette and mixed with already aliquoted glycerol in the U bottom microtiter plate by pipetting up and down few times.
6. The U bottom microtiter plate was capped, sealed and stored at -80Ú C.

Plasmid DNA isolation for Sequencing

For high throughput plasmid isolation Millipore 96 Miniprep Plasmid kit was used. The protocol followed is as follows:

1. 1.25 ml of 2X LB was dispensed into sterile 96 deep well blocks (2.2ml capacity). The wells were inoculated with 20µl of glycerol stock microtitre plate using a multi channel-pipette.

2. The block was sealed with an air pore sheet and incubated at 37°C for 18-20hrs with shaking at 300 rpm.
3. The bacterial cells were pelleted in the deep well blocks by centrifuging 1250g for 5min in a centrifuge with rotor for microtiter plates.
4. The media was removed by decanting the culture supernatant into proper disposal container. The deep well block was inverted and tapped firmly on several layers of paper towels many a times to remove residual culture supernatant.
5. The bacterial pellets were resuspended by adding 100ìl of Solution 1 (containing RNase A) to each well and then vortexing on the plate vortex till every pellet was totally resuspended.
6. 100 ìl of Solution 2 was added to each well. The deepwell block was sealed with a tape and inverted 6 times and then incubated for additional 2 min at room temperature to ensure lysis.
7. !00 ìl of Solution 3 was added to each well and mixed by inverting the sealed block 6 times.
8. The deep well block was centrifuged at 2000 rpm for 20 min to pellet the debris.
9. The Multisceen $_{96}$ PLASMID plate was kept inside the vaccum manifold.
10. The entire lysate volume from each well of deep well plate, without taking debris, was dispensed into the corresponding wells of MultiSceen lysate clearing plate.
11. The lysate clearing plate was placed on the top of the manifold and vaccum was created to draw the lysate through the clearing plate into Multisceen $_{96}$ PLASMID plate. The vaccum was adjusted to 8 inches of Hg (0.27 bar) and applied for 3min. The lysate clearing plate was discarded.
12. The Multisceen $_{96}$ PLASMID plate was placed on the top of the empty manifold. Vaccum at 24 inches of Hg (812.7 millibar) was applied for 5-7 min or until the wells were empty. The filterate was directed to waste. The empty wells were shiny in appearance.
13. Into each well of Multisceen $_{96}$ PLASMID plate 200µl of Solution 4 was dispensed.There after vaccum at 24 inches of Hg (812.7 millibar) for 5-7 min or until the wells were empty. The filterate was directed to waste.
14. The plasmids were recovered by adding 50 µl of Solution 5 into each well of Multisceen $_{96}$ PLASMID plate. To get maximum yield the plates were allowed to sit at room temperature for 30 min after addition of Solution 5.
15. The plasmid retained on the wells of Multisceen $_{96}$ PLASMID plate were pipetted into the V-bottom plate for storage. It was sealed and stored in -20ºC.
16. The quality and quantity of plasmids was checked by running 2 µl of each plasmid on 0.8% agarose gel with a reference plasmid of known concentration

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DNA Sequencing using ABI automated sequencer

Sequencing was done using ABI sequencer. The isolated plasmids were sequenced using M13 reverse primer.

Sequence Analysis

For all sequences obtained, the vector sequence was removed using Vec Screen available at NCBI site. The vector removed sequences were subjected to BLASTN and BLASTX.

RESULTS AND DISCUSSION

Construction of SSH library from WR-315 resistant variety of chickpea:

The SSH library constructed as per Clontech Protocol and was shown in the Figure1.2

Metabolic Clustering of genes

BLASTX search identified a number of genes that associated with plant biosynthetic pathway, intermediates of signal transduction networks or participate in defense responses. There were several genes which were uniquely identified in susceptible library and others in resistant library. Few common clones were found in both the libraries and genes were grouped as shown in the Fig1.4

Genes involved in cell architecture

The study identified genes coding for structural components in the subtractive library. Actin, alpha tubulin and kinetin like proteins sequences were also obtained. Kinetin proteins transport diverse cellular materials in a directional manner along microtubules. Kinetin-like proteins are characterized by a highly conserved “head” region that comprises the motor domain, and a nonconserved “tail” region that is thought to participate in recognition and binding of the appropriate cargo. Besides these glycine rich cell wall proteins which are involved in defense was also found to be differentially expressed. Upon fungal invasion soon after perception of pathogen

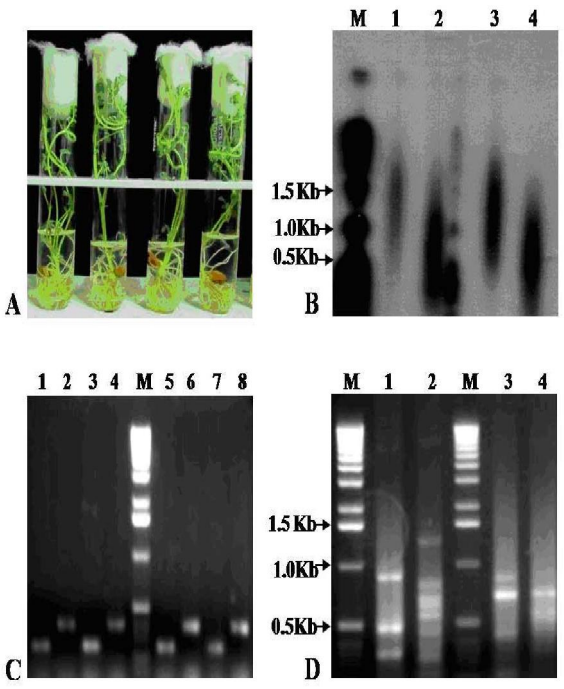

Figure 1.3. Construction of SSH library from JG-62 susceptible variety of chickpea. (A) 4 week old plants of susceptible variety 5 days after infection with *Fusarium;* (B) Characterization of SSH library; Lanes 1, 2, 3 & 4 represents Undigested ds tester, *RsaI* digested tester, Undigested ds driver and *RsaI* digested driver, respectively; (C) Ligation efficiency; Lanes 1-4 represents tester and lanes 5-8 represents, driver, lane 1 & 5: adaptor 1 ligated tester/driver amplified with actin 3' primer and PCR primer; lane 2 & 6: adaptor 1 ligated tester/driver amplified with Actin 3' and 5' primer, Lane 4 & 8: adaptor 2 ligated tester/driver amplified with Actin 3' and 5' primer; (D) Subtraction efficiency; Lanes 1, 2, 3 & 4 represent unsubtracted tester, subtracted tester, unsubtracted driver and subtracted driver, respectively.

the plant puts forward several protective structural barriers.

Genes involved in metabolic pathways

Several genes involved in primary metabolism were found in the subtractive library. Genes like glucose 6 phosphate isomerase, pyruvate kinase, aconitase, transaldolase are few to name. A vacuolar ATPase related gene has also been found. This leads to conclusion that *Fusarium* infection effects the host metabolism including different biosynthetic pathways which might be responsible for the symptoms incurred upon infection. Many genes with links to the protein synthesis and turn over have been found. The list includes branched chain amino transferase, cytoplasmic ribosomal protein, eukaryotic translation initiation factor, mitochondrial elongation factor G (with similarity to *Oryza*), ubiquitin conjugating enzymes, 26 S proteasome regulatory particle triple-A-ATPase etc.

Defense related genes

The sequencing of differentially expressed proteins revealed that PR 10, PR 5 and PR 4 are expressed in chickpea upon *Fusarium* infection. Chitinases were also isolated from the subtracted library. These hydrolytic enzymes cleave b- 1,4 glycosidic bonds between N- acetyl glucosamine residue of chitin. This polysaccharide is a primary structural component of all fungi. So chitinases are important in plant fungal interaction. A â glucosidase was also found to be differentially expressed.

Several genes involved in the biosynthetic pathway of phytoalexins were found to be up regulated upon *Fusarium* infection. These include chalcone synthase, chalcone isomerase, trans cinnamate 4-monooxygenase, isoflavone reductase and vestitone reductase. Phytoalexins are low molecular weight antibiotic compounds that are inducible in plants in response to infection. Phytochemical studies of *Cicer* have already shown that both roots and foliage express phytoalexins dominated by the pterocarpan isoflavonoids maackiain and medicarpin. These two pterocarpans exhibit potent antifungal activity towards *Fusarium* spores. Both germination and hyphal growth of the spores is inhibited in their presence. The constitutive levels of these pterocarpans in the roots of susceptible variety has been reported to be significantly lower than the resistant cultivars. Upon infection with *Fusarium* in resistant variety the levels of medicarpin has been found to be induced 10 folds whereas in susceptible one accumulation of pterocarpans was found to be slow and less. Thus high constitutive levels of maackiain and medicarpin in the roots and increased production in the presence of pathogen are both strongly associate with resistance (Stevenson *et al.,* 1997).

Genes involved in cell signaling

Several genes to which putative roles in cell signaling can be ascribed were found. Clones showing homology with Calcium dependent kinase from Arabidopsis was found. BLASTX revealed a phospho-protein phosphatases and a receptor like kinase homolog. A putative transcription initiation factor resembling transcription initiation factor IIE from rice was also isolated from the library. These genes might be involved in transduction of signal in the plant cell upon perception of *Fusarium*. Though the precise role of each component needs to be experimentally proved.

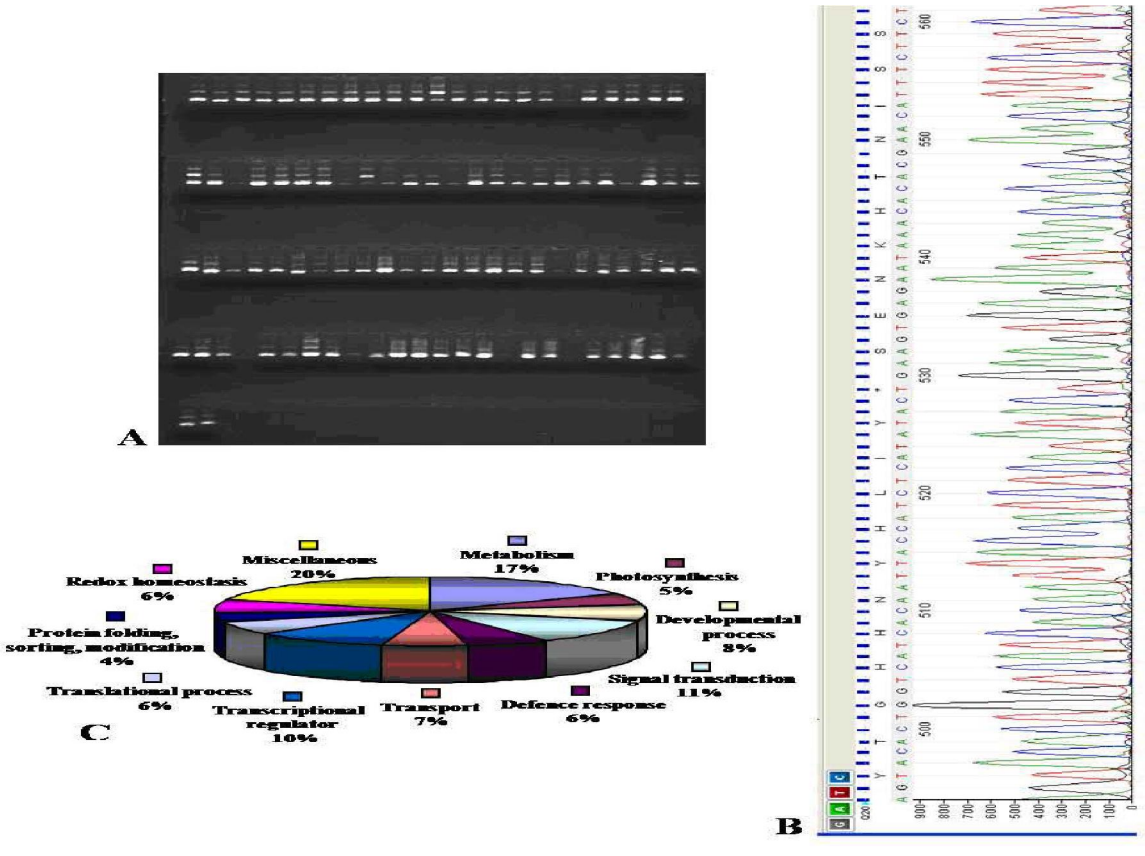

Figure 1.4. Metabolic grouping of ESTs. (A) 0.8% Agarose gel showing plasmid DNA isolated for sequencing; (B) Representative Electropherogram showing the quality of DNA sequence; (C) Pie Chart showing metabolic grouping of sequences obtained from both the libraries.

Other genes

The study identified clone showing high homology with nodulin from *Arabidopsis*. Nodulin genes are considered to have essential roles in the Rhizobium infection process. Some nodulin genes which encode products homologous to plant defense proteins have also been reported (Gamas et al., 1998).

One of the clones also showed significant homology with Arabidopsis cyclophilin. Cyclophilins are a growing family of ubiquitous, often abundant, and highly conserved proteins. Originally described as the intracellular target for the immuno suppressive drug cyclosporin A, they were subsequently shown to have peptidylprolyl cis-trans isomerase activity in vitro (Fisher *et al*., 1989). The natural function of cyclophilins in the cell remains unknown. These enzymes are believed to facilitate the folding of proteins, and to act as chaperones for protein trafficking in vitro. Cyclophilin genes have been described in several organisms, ranging from bacteria to mammals. In higher plants cDNAs encoding proteins homologous to cyclophilins have been isolated. Distinct isoforms are present in many compartments of eucaryotic cells, including cytosol, mitochondria, chloroplasts, endoplasmic reticulum and nucleus (Galat, 1993).

A homolog of Tonneau 2 protein homolog of Arabidopsis was found with BLASTX. This protein codes for protein phosphatases 2a regulatory subunit essential for the control of the cortical cytoskeleton (Camilleri et. al., 2002)

Germination and post-germination events constitute critical transitions in the life cycle of higher plants. During this period, dramatic physiological changes occur in concert with establishment of the basic architecture of the mature plant from the pattern formed during embryogenesis. Starch and protein reserves are mobilized in the endosperm of germinating seed to provide carbon and nitrogen for the seedling prior to the initiation of photosynthesis. Several lines of evidence support the conclusion that thioredoxin promotes the mobilization of carbon and nitrogen in the early period of seedling development during germination. One of the clones wie1 11 shows significant homology with thioredoxins.

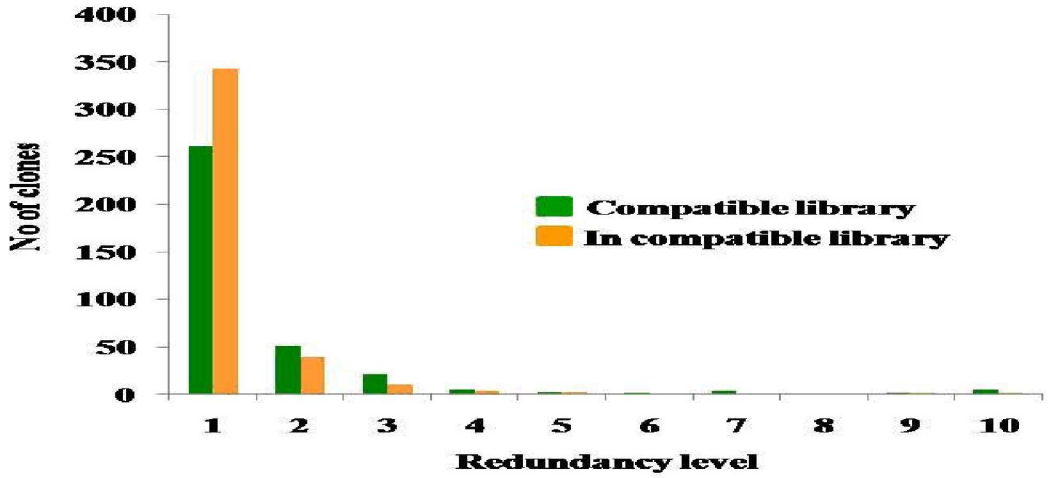

Figure 1.5. Redundancy distribution of subtracted clones in compatible (susceptible) and incompatible (resistant) libraries.

Senescence is an important symptom of wilt. In this regard it is note worthy to mention that several senescence associated proteins were obtained when BLASTX of sequences were carried out.

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