ISOLATION AND CHARACTERIZATION OF *Cab8* GENE FROM WILD *Vicia cinera* SPECIES

RANIA M. ABOU ALI[1], MENNATALLAH M. EL-HEFNAWY[2] AND A. M. K. NADA[1,2]

1. Agricultural Genetic Engineering Research Institute (AGERI), ARC, Giza, Egypt

2. October University for Modern Sciences and Arts (MSA), 6 October City, Egypt

Drought stress is a limiting factor to the agricultural productivity in tropical, semi-arid and arid regions. More than 95% of Egypt's land is desert, while less than 3% is confined to farming and agriculture. Drought stress causes cellular water deficits, which results in the loss of turgor, change in cell volume, change in membrane integrity, concentration of solutes, denaturation of proteins and several physiological and molecular components (Bartels and Souer, 2003; Griffiths and Parry, 2002; Lawlor and Cornic, 2002; Parry *et al.*, 2002; Raymond and Smirnoff, 2002). Under such severe conditions, cells need to induce gene(s) producing some products that may act to sustain the cellular functions through osmotic adjustments and cellular structure protection (Bray, 2002).

High drought conditions and high light intensity from the sun is very damaging to plants subjected to these conditions. The morphology, molecular and biochemical characteristics of the plant structure contribute to maximizing the photon capture and their use in CO_2 fixation (Larcher, 1995).

Photooxidative stress was known to be the cause of the oxidative stress, but it has been proven that it also results from drought and salinity stresses. Oxidative stress is characterized by the accumulation of harmful reactive oxygen species (ROS) in plant tissues, and it is one of the most deleterious stresses. Most of the environmental stresses result in the overproduction of ROS, which consequently causes an oxidative stress. The reaction of ROS with lipids and proteins results in the fast accumulation of toxic products, which brings severe damage to the plants. One of these toxic products is lipid peroxide which causes cellular damage (Chia *et al.*, 1984; Dhindsa *et al.*, 1981).

Cab gene family is a Light Harvesting Complex II type I *(LHCI),* which is responsible for capturing, transporting and distributing the excitation energy to photosystems that are closely related, organizing the photosynthetic system by keeping the tight compensation of the thylakoid membranes and protecting the plant against any damage resulting from high light intensity- photooxidative stress. *Cab6A, B (LHCI type I); Cab7, LHCZ-15 (LHCI type II); Cab8 (LHCI type III) and*

Cab11, 12 (LHCI type IV) are four types of LHCI genes that have been isolated from tomato and petunia (Wang, 1994). In 1991, Jansson and Gustafsson gave another nomenclature for the above mentioned genes as *IhaA, B, C* and *D*, respectively. The whole photosystem I antennas were isolated and identified in barley (Jurgen, 1992).

One of this *Cab* gene family is the *Cab8* gene; which can bind more than 14 chlorophylls. The protein expressed from this gene consists of chlorophyll a and b binding subunits. It interacts with calcineurin B-like interacting protein kinase (CIPK). It is involved in the stroma with adhesion of granal membranes and post-translational modifications which mediates the distribution of energy between both photosystems (I & II). Its threonine residues regulate the post translational modifications through reversible phosphorylation. The gene was first sequenced in *Pisum sativum*.

As mentioned earlier, all of those genes that function during photosystem I and II get affected mainly by photooxidative stress, and indirectly by other stresses, especially heat and drought stresses. This is because their function is involved in the photosynthesis process and energy utilization by the plant subjected to abiotic or even biotic stresses.

In this study, our aim is to isolate and characterize a stress related gene (*Cab8*) from *Vicia cinera* which is one of the *Vicia* species found in the Northwest Coastal region of Egypt. This area is char-

acterized by its severe and harsh environmental conditions such as unstable temperature, soil salinity and drought. Therefore, plants that grow in this region are very good candidates for studies aiming to fish for stress genes as they have the perfect genetic pool.

MATERIALS AND METHODS

Plant material

Vicia cinera plants were used to screen for stress genes. Young seedlings of *Vicia cinera* were collected from the northwest coastal region of Egypt. Sampling was conducted in the salt marshes and coastal dunes located 25 km west of Marsa Matrouh (the coastal area of El Qasr). The green parts of the collected seedlings were immediately frozen in liquid nitrogen.

Primer design

The primers were designed after downloading the different sequences of *Cab8* genes from the Gene Bank (NCBI) and aligned together using CLUSTAL W (version 1.8) (Thompson *et al.*, 1994). Primers were designed to amplify the whole gene according to the beginning and the end sequences of these genes. Sequence of the designed *Cab8* primers:
Forward primer 5' ATG GCC GCT TCA TCC ATG GCT 3'
Reverse primer 5' TCA CTT TCC GGG AAC AAA GTT GGT 3'
Specific primers of the dehydrin gene were used as a positive control during this study.

RNA extraction from plant material

Total RNA was extracted from *Vicia cinera* leaves using the TriPure isolation reagent (Roche Molecular Biochemicals, Cat. No.1667165). A volume of 100 mg of each frozen tissue was ground in an autoclaved mortar to a fine powder. A volume of 1 ml TriPure isolation reagent was added to the resulting powder in a polypropylene tube at room temperature. Samples were homogenized and incubated for 5 min at room temperature to ensure the complete dissociation of nucleoproteins. After incubation, 0.2 ml chloroform was added and the tube was vigorously shaken for 15 sec. The tube was incubated at room temperature for 10 min and centrifuged at 12,000 xg for 15 min at 4°C. Then, 0.5 ml of isopropanol was added to the colourless upper aqueous phase in a new centrifuge tube. The tube was inverted several times to mix thoroughly. To precipitate RNA, samples were incubated for 10 min at 25°C and then centrifuged at 12,000 xg for 10 min at 4°C. The pellet was washed with 1 ml of 75% ethanol. The excess ethanol was removed from the RNA pellet by air-drying. The RNA pellet was resuspended in water treated with diethyl pyrocarbonate (DEPC). RNA was either used immediately or kept at -80°C until further use.

Reverse transcription-PCR

Reverse transcription using a One-Step RT-PCR Kit (Qiagen, Germany) was performed on 2 µg of total RNA ex-tracted from *Vicia cinera* as described by O'Rourke *et al.* (2000). Reaction products were submitted to electrophoresis in a 1.5% agarose gel in 1X TAE buffer as described by Sambrook *et al.* (1989).

The PCR program cycle consisted of: an initial denaturation step at 94°C for 2 min, followed by denaturation at 94°C for 2 min, annealing at 45°C for 45 sec, extension at 72°C for 2 min for 40 cycles then a final extension step at 72°C for 7 min.

Cloning of PCR product

The PCR products were cloned in pGEM-T Easy plasmid (Promega, USA) and transformed into *Escherichia coli* DH5α. White colonies were picked and screened for the presence of the cloned gene of interest through digestion with *EcoRI* (Sambrook *et al.*, 1989).

Sequencing

The automated DNA sequencing reactions were performed using ABI PRISM Big Dye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, USA), in conjunction with ABI PRISM (310 Genetic Analyzer). Cycle sequencing was performed using the Gene Amp 2400 thermal cycler, and the reaction was conducted in a total volume of 20 µl, containing 8 µl of terminator ready reaction mix, 1 µg of plasmid DNA, and 3.2 pmol of T7 universal forward primer or SP6 universal reverse primer. The cycle sequencing program was set at 96°C for 10 sec, 50°C for 5 sec, and 60°C

for 4 min, repeated for 25 cycles with rapid thermal ramping time.

Sequence analysis

Sequences were analyzed using Blast programs of the National Center for Biotechnology Information [www.ncbi.nlm.nih.gov/Blast] (NCBI), USA. Sequences of *Cab8* genes that showed similarities with the isolated genes were obtained from the NCBI non-redundant and dbEST data sets using BLASTn or BLASTx (version 2.0.10) (Altschul *et al.,* 1997). The full deduced amino acid sequences of the proteins were aligned using CLUSTAL W (version 1.8) (Thompson *et al.,* 1994) and subjected to phylogenetic analysis. Phylogenetic trees were constructed using the neighbor-joining (NJ) method (Saitou and Nei, 1987) with parsimony and heuristic search criteria and 1000 bootstrap replications to assess branching confidence .

For *in silico* mapping, the isolated sequences were compared to barrel medic BAC/PAC sequences using BLAST (with an e-value threshold of $1e^{-1000}$). The barrel medic BAC/PAC that matches the query was used to identify anchored barrel medic markers from the barrel medic genetic linkage map (http://www.tigr.org). The results obtained from this stage were used to construct a comparative map between pea, soybean and barrel medic to identify the tentative chromosomal location of *Cab8* in the previously mentioned plants using comparative mapping strategy (Diab

et al., 2007).

IPTG induction

Cab8 protein was induced in *E. coli* harbouring the transformed pGEM plasmid by IPTG. An overnight culture of *Cab8* clone was grown in LB broth media for 3 hrs to reach an absorbance value of 0.4 OD_{600}. Then IPTG was added to a final concentration of 1 mM to the sample (positive sample), while there was a sample containing *E. coli* DH5α cells without IPTG (negative control), then pellets were collected at different time intervals (1, 2 & 3 hrs), by centrifuging at 8000 rpm at 4°C. An aliquot of 30 μl of the extraction buffer and 3 μl of PMSF were added to the cell pellets and then boiled for 5 min. The mixtures were centrifuged for 1 min at 14000 rpm. The samples generated were run on 12% SDS-PAGE gels (Sambrook *et al.,* 1989) to verify that the new protein of the predicted size is produced on the IPTG induction.

RESULTS AND DISCUSSION

RNA isolation from Vicia cinera and RT-PCR reactions

Isolated RNA from *Vicia cinera* was used to perform RT-PCR reactions to detect and isolate *Cab8* related sequence. The results of the RT-PCR reactions revealed that there was one band representing the *Cab8* gene $\cong$ 800 bp, while the negative control gave no bands (Fig. 1). Primers specific for dehydrin (DHN) gene were used in the same reaction as positive control.

Cloning and sequencing of Cab8 fragment

The *Cab8* PCR fragment was ligated into pGEM-T easy vector and transformed into *E. coli* DH5α competent cells. The recombinant plasmids were isolated from the selected colonies and digested with *Eco*RI (Fig. 2). The released cDNA insert was migrated to about 800 bp when subjected to electrophoresis on % agarose gel at ampere.

Sequencing of the isolated *Cab8* gene revealed that the length of *Cab8* was 801 bp as shown in Fig. (3). The obtained sequence was subjected to the BLASTx analysis which proves that the sequence has a high homology (89%) with the *Cab8* gene isolated from *Pisum sativum.* This is the first time for a *Cab8* gene to be isolated from a *Vicia* species. The isolated sequence was submitted to GeneBank under the accession numbers AB505862 for DNA sequence and BAH70299.1 for expressed protein sequence.

Phylogenetic analysis

To determine the evolutionary relatedness of the isolated *Cab8* expressed protein to other *Cab8* proteins, the neighbor-joining method (NJ) was used to generate a phylogenetic tree based on the deduced amino acid sequence homology (Fig. 4). The tree showed that isolated *Cab8* proteins form a distinct clade with *Cab8* sequence isolated from *Pisum sativum*. This is supported with Gepts *et al.* findings in 2005, as they proved that *Vicia*, *Pisum*, *Medicago* and *Cicer* are de-

scendants of Hologalegina. Among the cool-season legumes, phylogenetic relationships within the legume family are reflected in relatively high similarity or synteny at the genome level (Wojciechowski *et al.*, 2004), including barrel and pea (Kalo *et al.*, 2004) or between the warm-season legumes common bean and soybean (Lee *et al.,* 2001).

Even though *Pisum sativum* is closer in distance to *Vicia cinera* than *Medicago sativa* and *Cicer arietinum,* however they all have an equal percentage of protein homology of 84% in the BLASTx to *Vicia cinera*. This tree helps in supporting the homology and linkage obtained in the genomic comparative analysis which was performed in the current study.

Comparative Mapping

The fact that *Vicia cinera* and *Pisum sativum* descend from the same ancestor made the choice of *Pisum sativum* supportive to be used in the comparative mapping due to the unavailability of data regarding *Vicia cinera.*

Based on the phylogenetic tree, the high homologies found between the *Vicia cinera* (beans) and *Pisum sativum* (pea) indicates that pea represents the organism under study in the comparative mapping with the legume model plant, barrel medic, *Medicago truncatula* (Fig. 5). The *Cab8* gene, designated as h2_20m4a marker on the barrel medic chromosome 6, is positioned between 2 other markers (CrS & DK229L) in which only 1 cM

separates then from each other. The CrS is positioned 1 cM downstream from our marker on the barrel medic, and is mapped on chromosome 5 of the pea, which means that both markers are physically linked and move together in cross recombinations. So it is predicted that the h2_20m4a marker would have a match on chromosome 5 in the pea. This prediction could be supported if the fact that chromosome 5 in the pea is equivalent to chromosome 6 in the barrel medic can be proven. On the other hand, DK229L on chromosome 6 in the barrel medic is mapped as the marker A235_1 on the G chromosome/clone of the soybean (*Glycine max*). The close proximity between the DK229L marker and the h2_20m4a marker on the barrel medic gives a similar prediction to that of the CrS marker in the pea.

IPTG induction

As shown in Fig. (6), a band of molecular weight 28 KDa started to be induced after 2 and 3 hours. This result is compatible with computation analysis results revealing that the isolated *Cab8* protein has a molecular weight of 28117.8 Daltons and theoretical pI is 5.29 (as computed on the Expasy ProtParam tool). In future, the induction experiment needs to be optimized to produce more protein products for protein analysis purposes.

The *Cab8* gene, which is responsible for capturing and transporting the excitation energy, also holds a long cascade in the chloroplast during photosynthesis or any other physiological process that would

need energy which means that it triggers the expression of other genes as well. Studying this gene is like studying the head or initiative to any process that would require energy to survive or resist any kind of stress. Therefore, this gene would not just help in photooxidative stress or oxidative stress but in all the stresses that would strip the plant from its energy and cause its weakness and death.

SUMMARY

Vicia cinera is one of the common plants grown in the North West Coastal region of Egypt. Plants there are subjected to different abiotic stresses due to the harsh conditions. For that reason, these plants serve well to fish for stress related genes. Total RNA was isolated from *Vicia cinera* leaves and screened for the presence of *Cab8* gene. *Cab8* gene was successfully isolated, cloned, sequenced and submitted to gene bank with the accession no. AB505862 for DNA sequence and BAH70299.1 for expressed protein sequence. Taking in account that this is the first time to isolate the *Cab8* gene from *Vicia* species, it showed 89% homology in the BLAST search with the *Cab8* gene from *Pisum sativum*. The expression of the isolated sequence was tested in *E. coli* with an IPTG induction experiment. The isolated sequences were subjected to different computer analysis tools such as the comparative genome analysis and phylogenetic analysis in order to complete its characterization and see its relationship and ancestral origin to the other plant species. Even though the original organism

where the *Cab8* gene was isolated from, *Vicia cinera,* was not used in the comparative mapping, instead *Pisum sativum* data served well in the comparison to the legume model plant *Medicago truncatula.* The markers that were linked and mapped to *Pisum sativum* and *Glycine max* helped in the predictions, and their close proximity to our marker, the h2_20m4a (*Cab8* gene) support the fact that it could be present near the CrS marker on chromosome 5 in the *Pisum sativum*, and near A235_1 marker on chromosome/clone G in *Glycine max.*

ACKNOWLEDGEMENT

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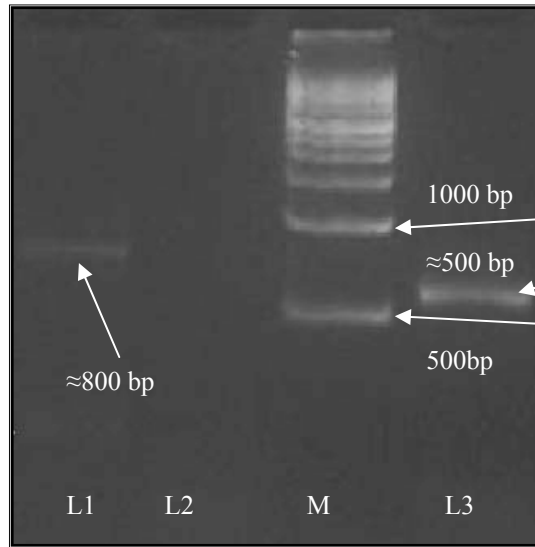
Fig. (1): RT-PCR products from *Vicia cinera*, lane 1: *Cab8*, lane 2: negative control, 1 Kb marker (Fermentas), lane 3: positive control (DHN).
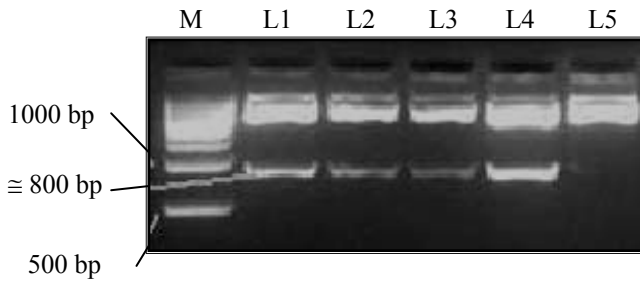

Fig. (2): Transformed white colonies digested with EcoRI. Lane 1 represents 1Kb marker (Fermentas), lanes from 1 to 4 show the vector and insert (around 800 bp), and lane 5 is the vector without any insert (negative control).

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Cab8   ATGGCCGCTTCATCCATGGCTCTCTCTTCCCCAACCTTGGC
Cab8   TGGCAAGCCAGTCAAGCTGACCCCATCAAGCCAAGAATTGG
Cab8   GAGCTTCAAGGTTCACCACGAGGAAGTCTGCTACCACCAAG
Cab8   AAAGTAGCTTCCTCTGGCAGCCCATGGTACGGACCAGACCG
Cab8   TGTTAAGTACTTAGGCCCATTCTCTGGTGAGCCCCCGTCCT
Cab8   ACTTGACTGGAGAGTTCCCAGGTGACTACGGTTGGGACACT
Cab8   GCTGGACTTTCTGCTGATCCAGAGACATTTGCCAAGAACGT
Cab8   GAGCTTGAGGTCATCCACTCCAGATGGGCCATGTTGGGTGC
Cab8   CTTGGGATGTGTCTTCCCAGAGCTTCTGTCCCGTAACGGTG
Cab8   TTAAATTCGGTGAAACTGTGTGGGTTCAAGGCCGGATCTCA
Cab8   AATCTTTAGCGAGGGTGGACTTGACTACTTGGGTAACCCAA
Cab8   GCTTGGGTTCACGCCAAAGCATCCTTGCCATCTGGGCCACT
Cab8   CAGGTTATCTTGATGGGAGCTGTTGAAGGTTACCGTATTGC
Cab8   TGGTGGTCCTCTTGGTGAGGTGGTTGACCCACTTTACCCAG
Cab8   GTGGTAGTTTTGATCCATTAGGCTTAGCTGAAGACCCAGAA
Cab8   GCATTCGCAGAATTGAAGGTGAAGGAACTCAAGAACGGTAG
Cab8   ATTAGCCATGTTCTCTATGTTTGGATTCTTTGTTCAAGCTA
Cab8   TTGTGACAGGAAAGGGTCCTTTGGAGAACCTTGCTGATCAT
Cab8   CTTTCCGACCCAGTCAACAACAACGCCTGGTCATATGCTAC
Cab8   CAACTTTGTTCCCGGAAAGTGA
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Fig. (3): *Cab8* gene sequence submitted to the DNA Database of Japan and assigned the accession number AB505862.
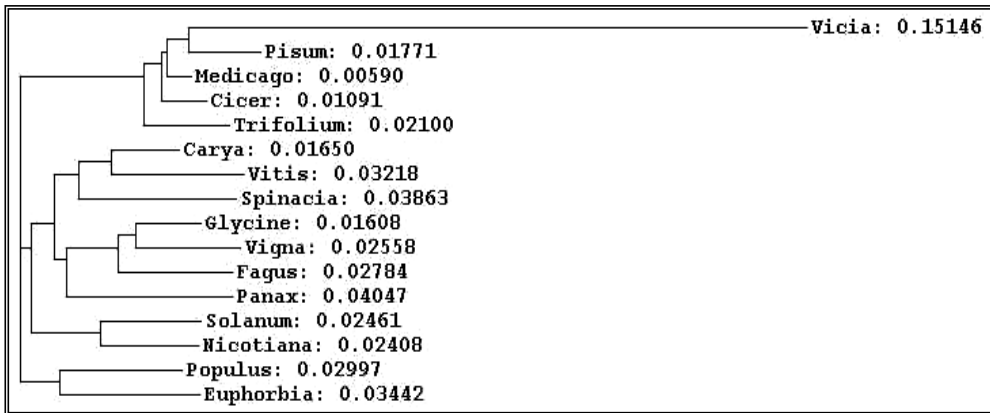

Fig. (4): Phylogenetic tree based on the amino acid sequence homology annotating *Vicia cinera* to other species constructed using the ClustalW: (www.ebi.ac.uk/clustalw/). *Vicia cinerea, Pisum sativum, Medicago sativa, Cicer arietinum, Trifolium pretense, Carya cathayensis, Vitis vinifera, Spinacia oleracea, Glycine max, Vigna radiate, Fagus crenata, Panax ginseng, Solanum tuberosum, Nicotiana sylvestris, Populus trichocarpa and Euphorbia esula.*
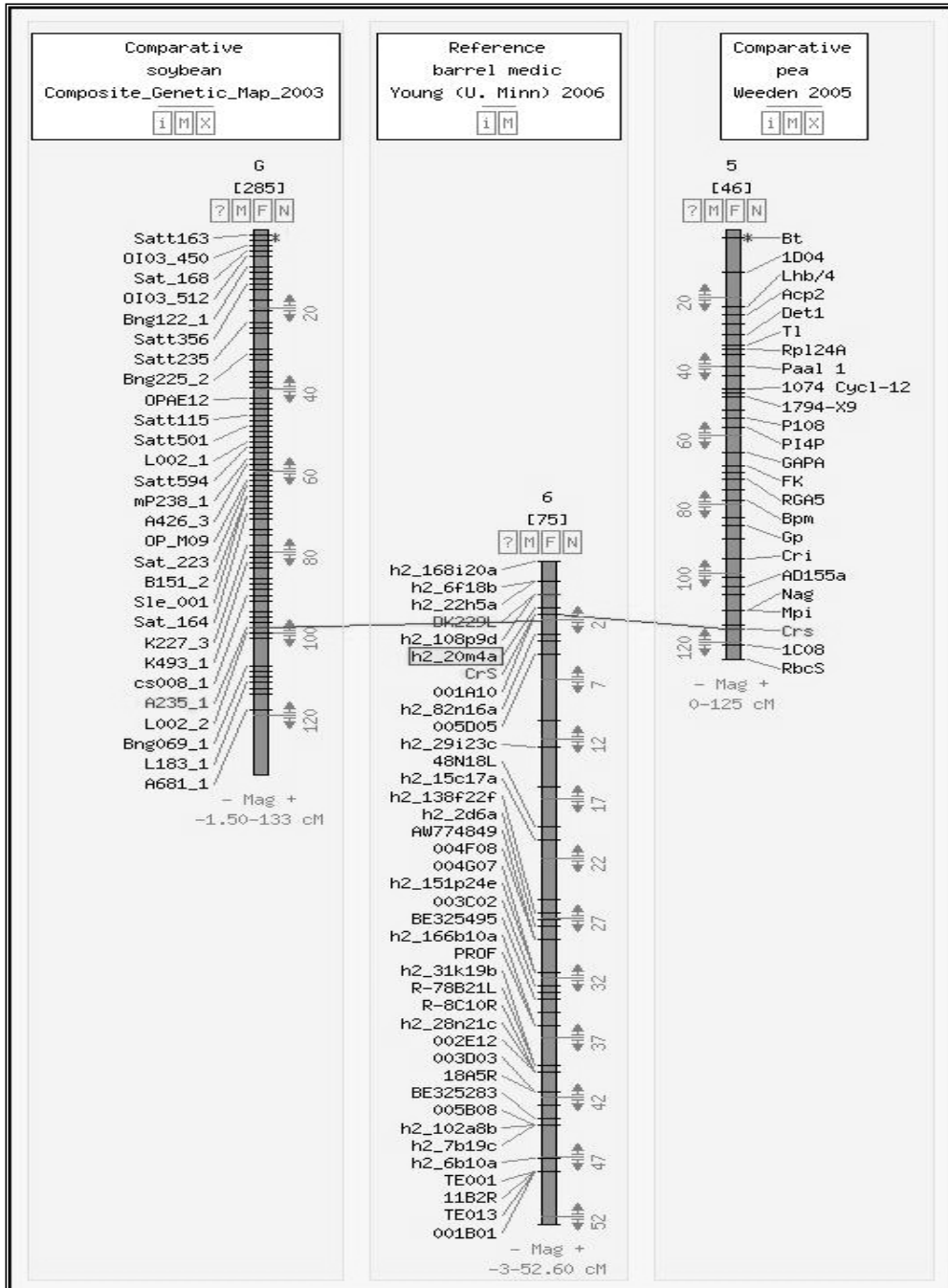
Fig. (5): Comparative Mapping reveals that *Cab8* gene is located on barrel medic chromsome 6, and predictably on Soybean chromosome/clone G and Pea chromosomes 5.
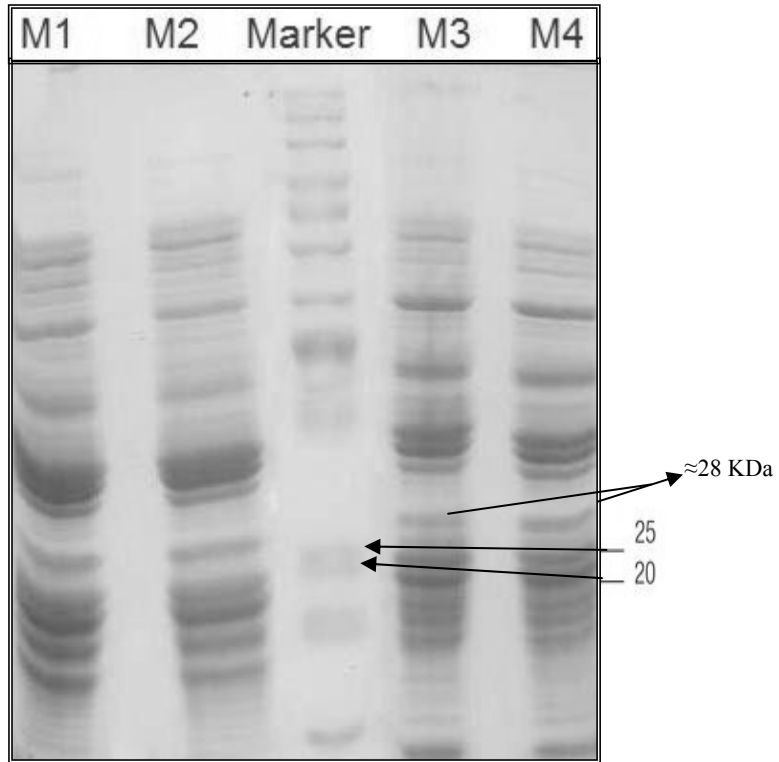
Fig. (6): Protein expression of *Cab8* gene; (M1) control (without IPTG), (M2) after one hour induction, (M3) after two hours induction, (M4) after three hours induction.