CHARACTERIZATION AND EXPRESSION OF ABSCISIC ACID INDUCIBLE GENE(s) IN WILD LEGUMES

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Abscisic acid (ABA), a plant growth hormone, is synthesized through the carotenoid biosynthetic pathway (Xiong *et al.,* 2002). In 1963, the role of abscisic acid was first identified and characterized by Addicott *et al*. (1968). ABA plays a key role in many physiological and developmental processes such as water relations, seed dormancy and germination (Kermode, 2005). Environmental challenges such as freezing, water stress caused by either high osmotic, salt or drought and wounding increase endogenous ABA levels in plant cells (Thaler and Bostock, 2004). Content of endogenous abscisic acid (ABA) is increased in rice plants under salt stress (Seo *et al.,* 2005).

Vicia villosa is one of the *Vicia* sp. found in the north coastal region of Egypt, as well-known desert with its difficult climate. So, it is considered a good genetic pool for studying and isolating stress-related genes. This species is a weak-*stemmed* climbing annual and winter active legume (Aly and Hassan, 1993).

Differential display (DD), first described by Liang & Pardee (1992), is one of the methods for analyzing gene expression in eukaryotic cells and tissues. DD has been widely applied to study changes in mRNA expression induced by temporal developments, biotic and abiotic factors (Eissa *et al.,* 2007) and with ABA treatment (Bethke *et al.,* 2006; Mochida *et al.,* 2006). Aghoram *et al.* (2000) isolated a novel gene (*abg1*) by differential display RT-PCR from guard cells of *Vicia faba* (L.) incubated with 5 µM S(+)-ABA for 1 h. This technique simultaneously screens for both up- and down-regulated transcripts in multiple cell populations under different developmental and environmental conditions.

The aim of the present work was to examine the differential expression of *Vicia villosa* shoots transcripts under abscisic acid treatment. It focused on isolating and cloning down- or up- regulated ESTs and determining their functions using bioinformatics approaches.

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MATERIALS AND METHODS

Plant material and abscisic acid treatment

Vicia villosa seeds were sterilized and then germinated on MS medium (Duchefa Biochemie, Netherlands) for 7-8 days. For ABA treatment, seedlings were transferred into solution containing 100 µM abscisic acid (Sigma, USA) for 3 and 24 hrs (Rabbani *et al.,* 2003). The control seedlings were placed into 0.1% ethanol solution. The treated and control seedlings were harvested and immerged in liquid nitrogen and stored at -80°C until RNA extraction.

RNA isolation and differential display-polymerase chain reaction (DD-PCR)

The total RNA was extracted using the TriPure isolation reagent (Roche Molecular Biochemicals, Germany) from the shoots of treated plants (3 and 24 hrs) as well as shoots of control plant. The protocol for DD-PCR reactions was described in RNAmap kit (GenHunter Corporation, USA). For cDNA first strand synthesis, each reaction contained 1.0 µl of 10 mM dNTP mix, 2 µM anchor primer ($T_{11}A$ or $T_{11}G$), 2 µg total RNA and 200 U/µl MMLV reverse transcriptase. RT reactions were performed at 42°C for 50 min followed by incubation at 70°C for 15 min.

PCR amplification of cDNA

The reactions were done according to Liang and Pardee (1992). One tenth of each cDNA reaction was used for PCR amplification in the presence of 1.0 µl of 25 µM dNTPs, 1.0 µl anchored primer (2 µM), the same as in the RT reaction, 1.0 µl arbitrary primer (2 µM namely AP1, AP2, AP3, AP4 or AP5), 0.3 µl Taq DNA polymerase (5 U/ µl) (Fermantas, USA) and 1 µl $MgCl_2$ (25 mM). The reactions of PCR were carried out in a thermohybaid PCR instrument with the following program: 94°C for 4 min (1 cycle); 94°C for 1 min, 42°C for 2 min, 72ºC for 1 min (40 cycles); 72°C for 10 min (1 cycle), then held at 4°C.

PCR products were loaded and analysed on a 6% acrylamide gel after denaturation of PCR products for 5 min at 90°C. The gels were visualized using silver staining system (Promega, USA).

Isolation of DD fragments and cloning

Sterile scalpel blade was used to cut the desired bands from the gel. Gel slice was soaked in 400 µl dH_2O and held at 37°C for 1 hr followed by 15 min at 94°C. The cDNAs were recovered by ethanol precipitation, redissolved in 10 µl of dH_2O and 5 µl were re-amplified under the same PCR conditions described before. Three µl PCR products were cloned in pGEM-T easy vector (Promega, USA). The plasmid isolation was done using the modified boiling method (Callard *et al.,* 1996). Selected colonies were screened with *Eco*R1 digestions (Sambrook *et al.,* 1989).

Sequencing of the cDNA insert

The chain termination procedure

(Sanger *et al.,* 1977) was performed using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, USA) with T7 and SP6 primers in conjunction with ABI PRISM (310 Genetic Analyzer). The nucleotide sequence or the deduced amino acid sequence of each clone was compared with DNA and protein sequences from various databases by means of the basic local alignment search tool (BLAST) in GeneBank (http://www.ncbi.nlm.nih.gov) (Altschul *et al.,* 1997).

RESULTS AND DISCUSSION

Detection of abscisic acid-induced transcripts

Vicia villosa genes, whose expression was regulated by abscisic acid treatment, were studied by differential mRNA display. Reactions of different primer pair combinations were loaded on sequencing gels. These sequencing gels were used to fractionate the cDNA fragments and to visualize those fragments by silver staining. Figure (1) illustrates examples of DD-PCR results using total RNAs isolated from shoots of ABA-treated seedlings. A total of 60 cDNA fragments were found to be differentially displayed due to abscisic acid treatment across different time intervals.

Expression patterns of DD fragments

The gene expression patterns of the isolated 60 cDNA fragments were classified into four groups according to their expression patterns: group I included 24 cDNA fragments which were observed to be up-regulated in seedling shoots in response to ABA treatment across time, group II included 25 cDNA fragments which were up-regulated after 3 hrs and then down-regulated in shoots after 24 hrs of ABA treatment, group III included 8 cDNA fragments which were down-regulated in shoots of treated seedlings across time, whereas group IV included 3 cDNA fragments which were down-regulated after 3 hrs and then up-regulated after 24 hrs of ABA treatment.

The selected cDNA fragments were used in PCR reactions to re-amplify the products using the same primer sets and cloned into pGEM-T easy vector. After cloning, the recombinant plasmids were isolated and digested with *Eco*RI restriction enzymes to release the fragment.

Computer analysis of the cDNA fragments

Computer analysis was done using BLAST from National Center for Biotechnology Information (NCBI), USA http://www.ncbi.nlm.nih.gov/BLAST/ (Altschul *et al.,* 1997). As indicated earlier, the cDNA fragments were classified according to the expression patterns into up-regulated, up- & down-regulated, down-regulated and down- & up-regulated (Table 1).

1- Up-regulated cDNA fragments

RGP3-16 fragment

RGP3-4, RGP3-5, RGP3-8, RGP3-

10, RGP3-14, RGP3-15, RGP3-16 and RAP4-2 cDNA fragments gave the same alignment with *Arabidopsis thaliana* NADH dehydrogenase. So, they were considered as one fragment namely RGP3-16. The computer analysis of RGP3-16 cDNA fragment in the GeneBank (BLAST N and BLAST X) showed significant homology with *Medicago truncatula* chromosome 2 BAC clone mth2-13b10 and with *Arabidopsis thaliana* NADH dehydrogenase. Mito-chondrial energy-dissipating systems involving alternative oxidase (AOX), NAD(P)H dehydrogenases were expected to have a major role in stress tolerance by preventing over reduction of electron carriers that would lead to oxidative stress (Macherel *et al.,* 2007). NADH dehydro-genase affects the ability to induce CBF upon the accumulation of detoxified ROS during photo-inhibition at low tempera-ture (Houde *et al.,* 2006). Also, it was found to be induced under salt stress in barley treated plants (Eissa *et al.,* 2007).

RAP1-3 fragment

The computer analysis of RAP1-3 fragment in the GeneBank showed sig-nificant homology with phosphoenolpyru-vate synthase (*Clostridium acetobutyli-cum* ATCC 824). The phosphoenolpyru-vate synthase enzyme can catalyze the final step of the conversion of phos-phoenolpyruvate (PEP) to pyruvate (Imanaka *et al.,* 2006). In this work, it was induced after ABA treatment in agreement with the results obtained by Moons *et al.* (1998) who isolated this enzyme corresponded to the ABA-induced proteins from rice roots. This enzyme was also induced in guard cells in rice by ABA treatment (Leonhardt *et al.*, 2004).

Up- and down-regulated fragments

RGP3-7 fragment

The four fragments, RAP4-4, RGP3-6, RGP3-7 and RGP3-9 shared the same sequence, so considered as one fragment namely RGP3-7. The computer analysis of this fragment in the GeneBank showed significant homology with Zinc finger, CCHC-type (*Medicago trunca-tula*). The CCHC-zinc finger is one of the zinc finger transcription factors, also known as a "zinc knuckle" (Krishna *et al.,* 2003), which occurs in a number of mRNA-associated proteins, including the splicing factors SLU7, h9G8 and hSF1. CCHC zinc finger depends upon the cysteine/histidine as the zinc-chelating residues. An *Arabidopsis* zinc-finger gene, a mediator of ABA-regulated dor-mancy 1 (MARD1) expression, was up-regulated by ABA. The results suggested that the novel zinc-finger protein with a proline-rich N-terminus is an important downstream component of the ABA signaling pathway that mediates ABA-regulated seed dormancy in *Arabidopsis* (He and Gan, 2004). Also, AZF2 (*Arabi-dopsis* zinc-finger protein 2) was strongly induced by ABA treatment (Sakamoto *et al.,* 2000). SCOF-1 (zinc finger protein gene), from soybean was induced by low temperature and abscisic acid (ABA) (Kim *et al.,* 2001). CAZFP1 (a pepper

zinc-finger protein gene) transcript was induced in response to abscisic acid (Kim *et al.,* 2004). Also, zinc finger protein gene (*OsRING-1*) was induced at different degrees by pathogen infections, SA, ABA and JA treatments (Meng *et al*., 2006).

RGP3-12, RGP4-1, RGP4-2, RGP5-3 and RAP4-3 fragments gave no similarity in Genebank (blast X and blast N), while the RAP2-3 fragment showed significant similarity with hypothetical protein PPA2404 (*Propionibacterium acnes*) and RGP5-11 fragment gave significant similarity with an unknown protein (*Arabidopsis thaliana*).

RAP2-1 fragment

The RAP2-1 fragment, whose expression was up-regulated after 3 hrs treatment and then down-regulated after 24 hrs treatment when analyzed by NCBI, showed significant homology with DNAJ heat shock N-terminal domain-containing protein (*Arabidopsis thaliana*). A protein DnaK (J) class was found to be essential for the HSP70-mediated refolding/repair mechanism. This protein reacts specifically with a particular cytosolic isoform of HSP70 and not with other forms of HSP70 which appear in drought stress (Grene, 2002). Also, Eissa *et al*. (2007) found that DNAJ was induced under salt stress in barley.

RAP2-2 fragment

The RAP2-2 fragment showed significant similarity with *Homo sapiens* chromosome 15 clone RP11-295H24 maps with unknown function.

RAP4-5 fragment

This fragment showed significant homology with ATP binding/ kinase/ uracil phosphoribosyltransferase-uridine kinase (*Arabidopsis thaliana*). Uracil phosphoribosyltransferase and one of the house-keeping enzymes involved in nucleotide metabolism, is often thought to be expressed at a consistent level in all cells. However, it is now evident that activities of constitutively expressed gene are very dynamic so as to meet the metabolic demands of plant cells. Since these changes can have a profound effect on plant development, there is a new appreciation of the importance of house-keeping activities in plant survival such as in response to abiotic stress (Moffatt & Ashihara, 2002). Uracil is converted directly into uridine 5'-monophosphate (UMP) by the action of uracil phosphoribosyltransferase (UPRT) which transfers the phosphoribosyl moiety from 5'-phosphoribosyl--1-pyrophosphate (PRPP) to uracil (Bressan *et al.,* 1978). UPRT was isolated from *A. thaliana* (Islam *et al.,* 2007). Identification of the mechanisms by which plant cells monitor and respond to their basic metabolic requirements which rely on the integration of transcript, protein and metabolite profiles of different cell types (Fiehn *et al.,* 2001). Unfortunately, the modest expression levels of house-keeping genes complicate their analysis since small differences in activity (2-3 folds), that may have large metabolic impacts, are difficult to be reproducibly

detected.

Down- regulated fragments

RAP1-2 fragment

Screening of RAP1-2 fragment in the GeneBank showed significant homology with *Psychrobacter cryohalolentis* K5, arginine biosynthesis bifunctional protein ArgJ. In prokaryotes, lower eukaryotes and plants, N -Acetylglutamate (NAG), is the first intermediate in the biosynthesis of arginine (Caldovic & Tuchman, 2003). The argJ gene codes for N2-acetyl-L-ornithine: L-glutamate N-acetyltransferase, the key enzyme involved in the acetyl cycle of L-arginine biosynthesis. ArgJ also catalyzes the conversion of glutamate to N-acetylglutamate using acetylCoA as the acetyl donor (Marc *et al.,* 2000). The wild watermelon glutamate *N*-acetyltransferase (CLGAT) is catalysing the transacetylation reaction between acetylornithine and glutamate to form acetylglutamate and ornithine. They are functioning in citrulline biosynthesis, which increases during drought stress (Takahara *et al.* 2005). N-acetylornithine: glutamate acetyltransferase (NAOGAcT) facilitates recycling of the acetyl moiety during ornithine formation (Slocum, 2005). Both glutamate and ornithine are recognized as possible precursors of proline. In higher plants, proline accumulation during osmotic stress appears to be essential (Verbruggen *et al.,* 1996). Accumulation of more proline was observed in transformed tobacco under osmotic stress conditions (Roosens *et al.,* 2002).

RGP5-12 fragment

Two fragments (i.e. RGP5-5 and RGP5-12) gave the same alignment with *Medicago truncatula*, *Phaseolus vulgaris* and *Glycine max* NBS-LRR plant resistance protein. The expression of RGP5-12 was found to be down-regulated. One of the mechanisms involved in plant defenses is the induction of specific defense and pathogenesis-related (PR) genes. Those stress-responsive genes are regulated by multiple signaling networks (Knight and Knight, 2001) with significant overlap between the patterns of gene expression that are induced in response to different stresses (Chen *et al.,* 2002). Many plant disease resistance (R) genes encode proteins predicted to have an N-terminal coiled-coil (CC) domain, a central nucleotide-binding site (NBS) domain and a C-terminal leucine-rich repeat (LRR) domain. NBS was found in many characterized plant disease resistance genes. With few exceptions, abscisic acid has been considered as a negative regulator of disease resistance. This negative effect appears to be due to the interference of abscisic acid with biotic stress signaling that is regulated by salicylic acid, jasmonic acid and ethylene, and to an additional effect of ABA on shared components of stress signaling (Mauch-Mani and Mauch, 2005). There are very few reviews cover the link between ABA and the nucleotide-binding site-leucine-rich repeat resistance plant disease proteins (NBS-LRR resistance plant disease proteins). NBS-LRR protein was suppressed due to the ABA treat-

ment. Anderson *et al.* (2004) obtained evidence that a complex interplay between ABA and JA-ethylene signaling pathways regulate plant defense gene expression and disease resistance. In *Arabidopsis*, ABA treatment, or stimulated drought stress that resulted in a large increase in ABA concentrations, increased susceptibility to avirulent bacteria (Mohr and Cahill, 2003). Suggesting that, ABA interferes indirectly with disease resistance by interacting with biotic stress signaling.

RGP5-13 fragment

Fragment RGP5-13 showed significant homology with *Medicago truncatula* clone mth2-23d7. Table 2 summarizes the results from computer analysis of the DD fragments.

SUMMARY

The present investigation examined differential gene expression in *Vicia villosa* under ABA treatment for 3 and 24 hrs. Sixty differentially expressed fragments resulted from DD-PCR were displayed and accordingly classified into 4 groups according to their expression patterns (up- or down- regulated by ABA treatment). DNA sequences and deduced amino acids analysis showed that 7 fragment sequences had significant homology to the following genes and/or proteins: *Arabidopsis thaliana* NADH dehydrogenase; *Medicago truncatula* zinc finger CCHC type; *Medicago truncatula*, *Phaseolus vulgaris*, *Glycine max* NBS-LRR plant resistance, *Psychrobacter sp.* arginine acetyl transferase, *Clostridium acetobutylicum* phosphoenolpyruvate synthase, *Arabidopsis thaliana* ATP binding/ uracil phosphoribosyl transferase and *Arabidopsis thaliana* heat shock protein binding. The significance of the function of the identified differentially expressed cDNAs was discussed in relation to their possible roles as stress-related genes. Results of this work were a first step towards understanding the molecular mechanism of ABA in response to abiotic stresses in *Vicia villosa* plant.

REFERENCES

Addicott, F. T., J. L. Lyon, K. Ohkuma, W. E. Thiessen, H. R. Carns, O. E. Smith, J. W. Cornforth, B. V. Milborrow, G. Ryback and P. F. Wareing (1968). Abscisic acid: A new name for abscisin II (dormin). Science, 159: 1493.

Aghoram, K., W. H., Jr. Outlaw, G. W. Bates, J. Cairney, A. O. Pineda, C.M. Bacot, L. M. Epstein and C. W. Levenson (2000). Abg1: a novel gene up-regulated by abscisic acid in guard cells of *Vicia faba* L. Journal of Experimental Botany, 51: 1479-1480.

Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller and D. J. Lipman (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res., 25: 3389-3402.

Aly, A. and M. TH. Hassan (1993). Atlas of legume plants of the north-west coast region of Egypt.

Anderson, J. P., E. Badruzsaufari, P. M. Schenk, J. M. Manners, O. J. Desmond, C. Ehlert, D. J. Maclean, P. R. Ebert and K. Kazan (2004). Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in *Arabidopsis*. Plant Cell, 16: 3460-3479.

Bethke, P. C., Y. S. Hwang, T. Zhu and R. L. Jones (2006). Global patterns of gene expression in the aleurone of wild-type and dwarf1 mutant rice. Plant Physiol., 140: 484-498.

Bressan, R. A., M. G. Murray, J. M. Galeand and C. W. Ross (1978). Properties of pea seedling uracil phosphoribosyltransferase and its distribution in other plants [pinto beans, soybeans, cereals]. Plant Physiol., 61: 442-446.

Caldovic, L. and M. Tuchman (2003). N-acetylglutamate and its changing role through evolution. Biochem. J., 372: 279-290.

Callard, D., B. Lescure and L. Mazzolini (1996). A method or the elimination of false positives generated by mRNA differentially display technique. Biotechniques, 16: 1096-1103.

Chen, W., N. J. Provart, J. Glazebrook, F. Katagiri, H.-S. Chang, T. Eulgem, F. Mauch, S. Luan, et al. (2002). Expression profile matrix of *Arabidopsis* transcription factor genes suggests their putative functions in response to environmental stresses. Plant Cell, 14: 559-574.

Eissa, H. F. O. M. Saleh and W. E. Dyer (2007). Genomic characterization of stress-related genes from wild barley. Egypt. J. Genet. Cytol., 36: 1-23.

Fiehn, O., S. Kloska and T. Altmann (2001). Integrated studies on plant biology using multiparallel techniques. Current Opinion in Biotechnology, 12: 82-86.

Grene, R. (2002). Oxidative stress and acclimation mechanisms in plants. In The Arabidopsis Book, C.R. Somerville and E.M. Meyerowitz, eds (Rockville, MD: American Society of Plant Biologists).

He, Y. and S. Gan, (2004). A novel zinc-finger protein with a proline-rich domain mediates ABA-regulated seed dormancy in *Arabidopsis*. Plant Mol. Biol., 54: 1-9.

Houde, M., M. Belcaid, F. Ouellet, J. Danyluk, A. F. Monroy, A. Dyranova, P. Gulick, A. Bergeron, *et al.* (2006). Wheat EST resources for functional genomics of abiotic stress. BMC Genomics, 7: 149.

Imanaka, H., A. Yamatsu, T. Fukui, H. Atomi and T. Imanaka (2006). Phosphoenolpyruvate synthase plays an essential role for glycolysis in the modified Embden-Meyerhof pathway in *Thermococcus kodakarensis.* Molecular Microbiology, 61: 898-909.

Islam, M., H. Kim, S. W. Kang, J. S. Kim, Y. M. Jeong, H. J. Hwang, S. Y. Lee, J. C. Woo and S. G. Kim (2007). Functional characterization of a gene encoding a dual domain for uridine kinase and uracil phosphoribosyltransferase in *Arabidopsis thaliana.* Plant Mol. Biol., 63: 465-477.

Kermode, A. R. (2005). Role of abscisic acid in seed dormancy. J. Plant Growth Regulation, 24: 319- 344.

Kim, J. C. S. H. Lee, Y. H. Cheong, C. M. Yoo, S. I. Lee, H. J. Chun, D. J. Yun, et al. (2001). A novel cold-inducible zinc finger protein from soybean, SCOF-1, enhances cold tolerance in transgenic plants. Plant J., 25: 247-259.

Kim, S. H., J. K. Hong, S. C. Lee, K. H. Sohn, H. W. Jung and B. K. Hwang (2004). CAZFP1, Cys2/His2-type zinc-finger transcription factor gene functions as a pathogen-induced early-defense gene in *Capsicum annuum.* Plant Mol. Biol., 55: 883-904.

Knight, H. and M. R. Knight (2001). Abiotic stress signaling pathway: specificity and cross-talk. Trends Plant Sci., 6: 262-267.

Krishna, S. S., I. Majumdar and N. V. Grishin (2003). Structural classification of zinc fingers. Nucleic Acids Res., 31: 532-550.

Leonhardt, N., J. M. Kwak, N. Robert, D. Waner, G. Leonhardt and J. I. Schroeder (2004). Microarray expression analyses of *Arabidopsis* guard cells and isolation of a recessive abscisic acid hypersensitive protein phosphatase 2C mutant. Plant Cell, 16: 596-615.

Liang, P. and A. B. Pardee (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science, 257: 967-971.

Macherel, D., A. Benamar, M. H. l. Avelange Macherel and D. Tolleter (2007). Function and stress tolerance of seed Mitochondria. Physiologia Plantarum, 129: 233-241.

Marc, F., P. Weigel, C. Legrain, Y. Almeras, M. Santrot, N. Glansdorff and V. Sakanyan (2000). Characterization and kinetic mechanism of mono- and bifunctional ornithine acetyltransferases from thermophilic microorganisms. Eur. J. Biochem., 267: 5217-5226.

Mauch-Mani, B. and F. Mauch (2005). The role of abscisic acid in plant–

pathogen interactions. Current Opinion Plant Biology, 8: 409-414.

Meng, X. B., W. S. Zhao, R. M. Lin, M. Wang and Y. L. Peng (2006). Molecular cloning and characterization of a rice blast-inducible RING-H2 type Zinc finger gene. The Journal of Sequencing and Mapping, 17: 41-48.

Mochida, K., K. Kawaura, E. Shimosaka, N. Kawakami, T. Shin, Y. Kohara, Y. Yamazaki and Y. Ogihara (2006). Tissue expression map of a large number of expressed sequence tags and its application to in silico screening of stress response genes in common wheat. Molecular Genetics and Genomics, 276: 304-312.

Moffatt, B. A., P. S. Summers, J. D. Snider, M. l. Drebenstedt, K. J. Alexander and E. A. Weretilnvk (2001). Maintaining methylation activities during salt stress. The involvement of adenosine kinase. Plant Physiol., 125: 856-865.

Mohr, P. G. and D. M. Cahill (2003). Abscisic acid influences the susceptibility of *Arabidopsis thaliana* to *Pseudomonas syringae* pv. Tomato and *Peronospora parasitica*. Funct. Plant Biol., 30: 461-469.

Moons, A., R. Valcke and M. Van Montagu (1998). Low-oxygen stress and water deficit induce cytosolic pyruvate orthophosphate dikinase (PPDK) expression in roots of rice, a C3 plant. The Plant Journal, 15: 89-98.

Rabbani, M. A., K. Maruyama, H. Abe, M. A. Khan, K. Katsura, Y. Ito, K. Yoshiwara, M. Seki, et al. (2003). Monitoring expression profiles of rice genes under cold, drought and high–salinity stresses and abscisic acid application using cDNA microarray and RNA gel-blot analysis. Plant Physiol., 133: 1755-1767.

Roosens, N., E. Hal Bitar, K. Loenders, G. Angenon, and M. Jacobs (2002). Overexpression of ornithine-δ-aminotransferase increases proline biosynthesis and confers osmotolerance in transgenic plants. Mol. Breed., 9: 73-80.

Sakamoto, H., T. Araki, T. Meshi and M. Iwabuchi (2000). Expression of a subset of the *Arabidopsis* Cys(2)/His(2) type zinc finger protein gene family under water stress. Gene, 248: 23-32.

Sambrook, J., E. F. Fritsch and T. Maniatis (1989). Molecular cloning: A laboratory manual, 2 nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sanger, F., S. Nicklen and A. R. Coulson (1977). Biochemistry DNA sequencing with chain-terminating

inhibitors (DNA polymerase/ nucleotide sequences/bacteriophage 4X174). Proc. Nati. Acad. Sci. USA, 74: 5463-5467.

Seo, H. S., S. K. Kim, S. W. Jang, Y. S. Choo, E. Y. Sohn and I. J. Lee (2005). Effect of jasmonic acid on endogenous gibberellins and abscisic acid in rice under NaCl stress. Biologia Plantarum, 49: 447-450.

Slocum, R. D. (2005). Genes, enzymes and regulation of arginine biosynthesis in plants. Plant Physiol. Biochem., 43: 729-45.

Takahara, K., K. Akashi, and A. Yokota (2005). Purification and characterization of glutamate N-acetyltransferase involved in citrulline accumulation in wild watermelon. FEBS Journal, 272: 5353-5359.

Thaler, J. S. and R. Bostock (2004). Interactions between abscisic acid mediated responses and plant resistance to pathogens and insects. Ecology, 85: 48-58.

Verbruggen, N., X. J. Hua, M. May and M. Van Montagu (1996). Environmental and developmental signals modulate proline homeostasis: evidence for negative transcriptional regulator. Proc. Natl. Acad. Sci. USA, 93: 8787-8789.

Xiong, L., H. Lee, M. Ishitani and J. K. Zhu (2002). Regulation of osmotic stress-responsive gene expression by the LOS6/ABA1 locus in *Arabidopsis*. J. Biol. Chem., 277: 8588-8596.

Table (1): Expression patterns of the cDNA fragments obtained from $T_{11}G$/AP3, $T_{11}G$/AP4, $T_{11}G$/AP5 and $T_{11}A$/AP1, $T_{11}A$/AP2, $T_{11}A$/AP4 combinations.

Cluster	Pattern	Control	3 hrs	24 hrs	DD fragments
I	1	-	-	++	RAP4-16, RGP3-16, RAP4-2
	2	-	-	+++	RGP3-1, RGP3-2, RGP3-3, RGP3-4, RGP3-13, RGP4-3, RGP4-6, RGP4-7, RGP4-8, RGP4-9, RGP5-1, RGP5-5, RGP5-6, RGP5-10, RAP1-1
	3	-	++	++	RAP1-3, RAP4-6
	4	+	+++	+++	RGP3-6RGP3-6
	5	-	++	+++	RGP4-4, RGP4-5, RGP5-4
II	1	-	++	-	RAP4-1, RAP4-7, RAP4-12
	2	-	++	+	RPA2-3
	3	-	+++	-	RAP4-5, RAP4-3, RGP3-5, RGP3-10, RGP3-11, RGP3-14, RGP3-15, RGP4-1, RGP4-2, RGP5-3, RGP5-7, RGP5-8, RGP5-9, RGP5-11
	4	-	+++	++	RGP3-12, RGP5-2, RAP4-4
	5	-	+++	+	RGP3-7, RGP3-8
	6	++	+++	++	RAP2-1, RAP2-2
III	1	++	-	++	RAP4-13, RAP4-14, RAP4-15
IV	1	++	-	-	RAP4-9, RGP5-12, RGP5-13, RGP3-9
	2	++	++	-	RAP1-2, RAP4-8, RAP4-10, RAP4-11

(-) very weak or no expression, (+) weak expression, (++) normal expression and (+++) high expression.

Table (2): Hits results from computer analysis of the selected DD fragments.

Fragment ID	Molecular size (bp)	Homology results GenBank accession number, E-value	Reference
Up-regulated			
RAP1-1	824	X: NS, N: NS	
RAP1-3	879	X: NP_347437.1,1e-18 Phosphoenolpyruvate synthase [*Clostridium acetobutylicum*] N: NS	Nolling *et al.* (2001)
RGP3-16	428	X: ABD32606.1 3e-47 FAD-dependent pyridine nucleotide-disulphide oxidoreductase (*Medicago truncatula*) X: NP 567283.1 NAD(P)H dehydrogenase/ disulfide oxidoreductase (*Arabidopsis thaliana*) X: NP 193880.2 3e-31 NADH dehydrogenase (*Arabidopsis thaliana*) N: AC149577.3, 6e-74, chromosome 2 BAC clone mth2-13b10, (*Medicago truncatula*) N: NM118269.2, 3e-08, NADH dehydrogenase (*Arabidopsis thaliana*)	Town *et al*. (2006) Town *et al.* (2006)
RGP4-3	200	X: NS, N: NS	
RGP4-4	273	X: NS, N: NS	
RGP4-5	202	X: NS, N: NS	
RGP4-6	198	X: NS, N: NS	
RGP4-8	206	X: NS, N: NS	
RGP4-9	190	X: NS, N: NS	
Up- and down-regulated			
RGP3-7	374	X: *ABE85361.1, 2e-42,* Zinc finger, CCHC-type (*Medicago truncatula*) X: *ABE93466.1, 5e-33* IMP dehydrogenase/GMP reductase (*M. Truncatula*) N: NS	Shaull *et al*. (2006) Shaull *et al*. (2006)
RAP4-3	403	X: NS, N: NS	
RAP4-4	350	X: The same RGP3-7 blast N: NS	
RAP4-5	347	X: ABE83101, 2e-58 Phosphoribosyltransferase/ uridine kinase (*M. Truncatula*) X: NP_175977, 2e-56 ATP binding / kinase/ uracil phosphoribosyl transferase/ uridine kinase *(Arabidopsis thaliana)* N: NM_179117.1, 3e-4*1* ATP binding / kinase/	Shaull *et al.* (2006)

Table (2): Cont

Fragment ID	Molecular size (bp)	Homology results GenBank accession number, E-value	Reference
		uracil phosphoribosyl transferase/ uridine kinase *(Arabidopsis thaliana)*	
RAP2-1	273	N: NM 106085.1, 3e-68) DNAJ heat shock N-terminal domain-containing protein (*Arabidopsis thaliana* X: NP_177565, 4e-24 DNAJ heat shock N-terminal containing protein *(Arabidopsis thaliana)* X: NP_001066831, ABA98642, 5e-16 DnaJ domain containing protein, expressed *[Oryza sativa*]	Matsumoto *et al*. (2006) Buell *et al.* (2005)
RGP3-12	312	X: NS, N: NS	
RGP5-11	363	N: NS X: AAK68750, 3e-23	Nguyen *et al*. (2001)
RGP4-1	189	X: NS, N: NS	
RGP4-2	190	X: NS, N: NS	
RGP5-3	258	X: NS, N: NS	
RAP2-2	453	X:NS, N: ACO22306.6, 0.0 Homo sapiens chromoosome 15 clone RP11-295H24 map 15sq21.1	Rowen *et al*. (2002)
RAP2-3	663	X:NS N: AE017283.1, 0.0 Hypothetical protein (Propionibacterium acnes KPA171202)	Bruggemann *et al*. (2004)
Down-regulated			
RGP5-12	420	N: AC153125.101, 2e-14, AY452686.1,1e-09 X: ABE87632.1, 3e-37, AF098971, *2e-35*, AAS49213, *4e-33*	Lin *et al*. (2006) Ashfield *et al.* (2004) Lin *et al.* (2006) Creusot *et al*. (1999) Hayes *et al*. (2004)
RGP5-13	424	X: ABE87632.1, 3e-37, AC119414.51, 1e-04	Lin *et al*. (2006) Shaull *et al*. (2006)
RAP1-2	887	X: ZP_01271247.1, 6e-61 Glutamate N-acetyltransferase N:CP000323, 9e-04 Arginine biosynthesis bifunctional protein ArgJ (*Psychrobacter cryohalolentis*)	Copeland *et al*. (2006) Copeland *et al*. (2006)

*: See text for details. NS is no significant similarities.

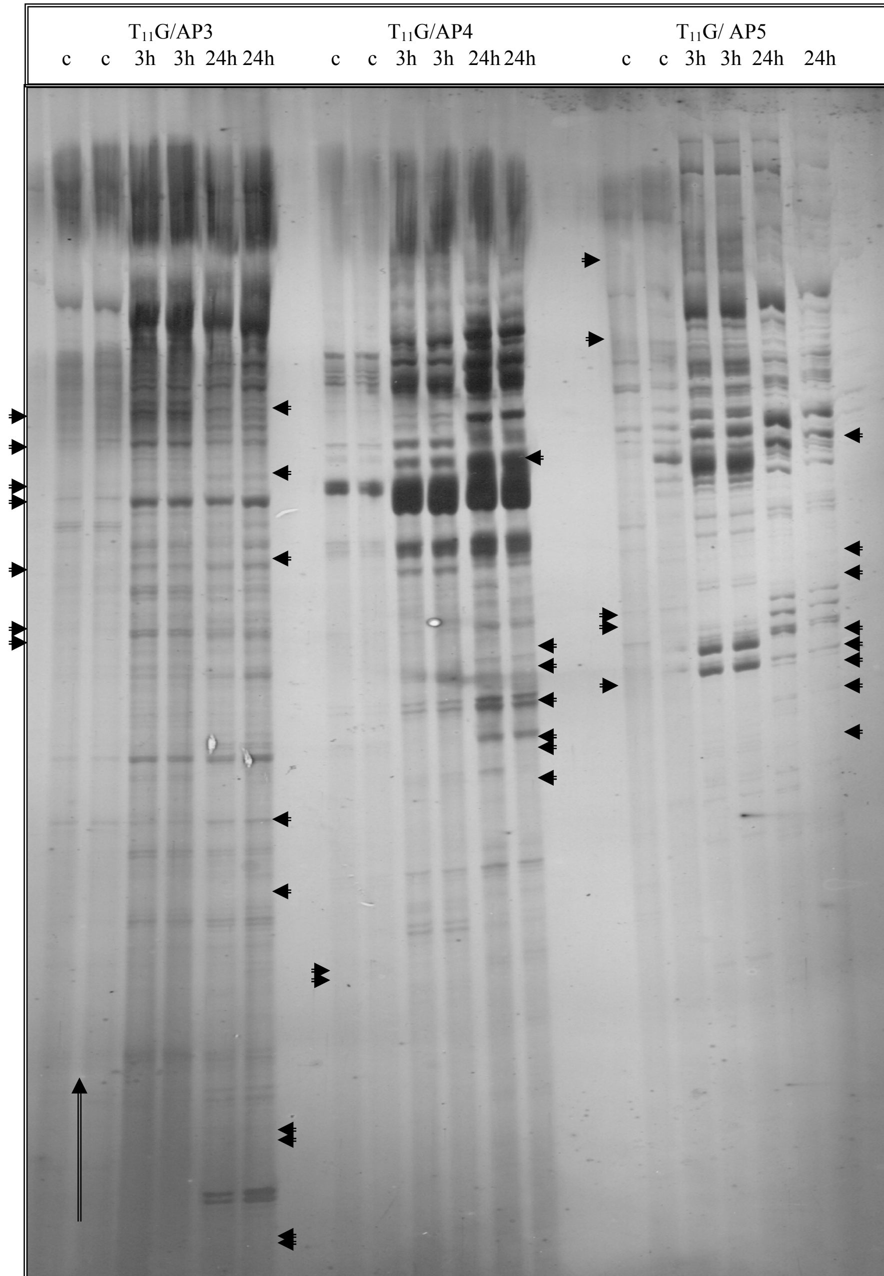

Fig. (1): Polyacrylamide gels of DD cDNA fragments from untreated (C) and ABA treated *Vicia villosa* shoots (3 and 24 hrs) using different primer combinations of anchored primer $T_{11}G$, with arbitrary primer 3, 4 and 5 (AP3, AP4 and AP5). Arrows indicate a number of differentially expressed bands on a duplicate basis. The long arrow shows the direction of bands selection (from the botton to the top of the gel).