

**SHORT COMMUNICATION****PRESENCE OF SIX DROUGHT RESPONSIVE CANDIDATE GENES IN TOLERANT AND SUSCEPTIBLE SUGARCANE VARIETIES****M. Roshni, T.S. Sarath Padmanabhan and G. Hemaprabha\*****Abstract**

Drought being a major stress factor affecting sugarcane productivity, research towards identifying drought responsive genes is important in the genetic improvement of sugarcane crop. In this study, six genes of known function in the drought metabolic pathway viz. ABA dependent Abscisic Acid Responsive Element Binding Factor 2 (ABF 2), Regulator of G- Protein signaling 1 (RGS 1), Trehalose-6-phosphate synthase 2 (TPS2), ABA independent Hardy transcription factor (HRD), NAC-like transcription factor 2 (NAC 2), and Gibberellins associated kgm gene were screened on three known drought tolerant and two susceptible cultivars to know their presence and variability. All the genes were amplified and were observed as multiple bands showing their multiallelic nature. Whereas five genes viz. ABF2, RGS 1, HRD, NAC 2 and kgm did not show specificity for drought tolerance, TPS 2 showed a fragment of 195 bp specifically present in tolerant clones, which needs to be validated in larger populations. The study, in continuation with the previous reports from this research laboratory, indicated that though these genes are present in sugarcane genotypes, the presence of specific alleles and gene expression are the two factors that decide the gross performance of a clone under drought stress.

**Key words:** Sugarcane, cultivars, drought tolerance, candidate genes

Sugarcane cultivation for sugar remains the most important goal, though by-products mainly electricity through co-generation and ethanol as biofuel are significant for its economic production. Drought is the most important limiting factor for crop production and is becoming an increasingly severe problem in many regions of the world. The percentage of drought affected land areas more than doubled from the 1970s to the early 2000s in the world. Apart from damaging the growth and development of sugarcane crop drought limits the areas suitable to agriculture.

To understand the molecular basis of drought tolerance, it is important to identify plant genes that respond to drought tolerance. Identifying suitable candidate genes would facilitate the crop

improvement process for drought tolerance. It is important to analyse the functions of stress-inducible genes, not only to understand the molecular mechanisms of stress tolerance and the responses of higher plants, but also to improve the stress tolerance of crops by gene manipulation (Seki et al. 2002). The ability of plants to counteract stress conditions depends on the efficiency and speed at which they recognize the stress, generate signal molecules and activate stress-protective mechanisms (Pasternak et al. 2005). Candidate genes, the DNA sequences with a predicted function, are used as a molecular marker tool to associate with the expressed phenotypes. Identification of the novel genes and their expression patterns are determined in response to the stresses.

---

Roshni M, Sarath Padmanabhan TS and G. Hemaprabha\*

Division of Crop Improvement, Sugarcane Breeding Institute, Coimbatore -641 007

\*email : ghemaprabha1@gmail.com

An improved knowledge of their functions in stress adaptation will provide effective engineering strategies to improve the stress tolerance (Swapna and Hemaprabha, 2012). Stress responsive genes can be expressed either through an ABA-dependent or ABA-independent pathway. ABA-dependent and ABA-independent pathways lead to rapid responses to drought or cold and function through members of the AP2/ERF family of transcription factors (Yamaguchi-Shinozaki & Shinozaki 1994; Kizis et al. 2006). Indeed, there are evidences demonstrating the presence of both ABA-independent and ABA-dependent regulatory systems governing drought-inducible gene expression. Both Cis-acting and Trans-acting regulatory elements functioning in ABA-independent and/or ABA-responsive gene expression induced by drought stress have been precisely analysed at the molecular level (Yamaguchi-Shinozaki & Shinozaki 2005).

Already this research laboratory has identified over 30 genes present in drought tolerant hybrids clones, from which 14 new sugarcane specific genes could be identified which were deposited in Genbank database (Swapna and Hemaprabha 2012; Priji and Hemaprabha 2014, 2015). In this study, six candidate genes were taken up as a step towards validating them in five sugarcane cultivars/ elite genotypes with known levels of tolerance and susceptibility to drought response. These are ABA dependent

Abscisic Acid Responsive Element Binding Factor 2 (ABF 2), Regulator of G- Protein signaling1 (RGS 1), Trehalose-6-phosphate synthase 2 (TPS2), ABA independent Hardy transcription factor (HRD), NAC-like transcription factor 2 (NAC 2), and Gibberellins associated kgm genes.

A set of known tolerant and susceptible commercial varieties was subjected to candidate gene analysis. The commercial sugarcane hybrid clones used in candidate gene analysis were Co 740, Co 0212, and Co 0216 (tolerant) and Co 775 and Co 419 (susceptible). DNA from these genotypes was isolated using standard protocols (Doyle and Doyle 1987). Six genes were selected to test the drought response in the selected varieties. The details of primers designed using PRIMER 3 software for the study are shown in Table1.

DNA was isolated following CTAB method and DNA quantified using Nanodrop DNA-RNA quantifier. PCR reaction was carried out using specific primers. PCR was carried out with 20ng of template DNA for amplification using 0.3 unit of Taq DNA polymerase in a 10µl reaction mixture containing 20pg each of forward and reverse primers and 2mM of each dNTPs. The amplification conditions consisted of initial denaturation - 94°C for 2 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 54 - 58°C for 50

**Table 1. Six drought responsive candidate genes and their forward and reverse primers**

| <b>Primer</b> | <b>Forward primer 5'-3'</b> | <b>Reverse primer 3'-5'</b> |
|---------------|-----------------------------|-----------------------------|
| ABF 2         | ggctcaggctcagagtcaga        | gctcctgcgacgatgaactt        |
| RGS 1         | ctgttgacccaaacgagcca        | aaacccaaatccgaaccggaca      |
| TPS 2         | tcgcatctagactattgceg        | tgccacccttcctctctctgt       |
| HRD           | tggcggcaatagcctatgac        | ctattcatgcaagccacaccac      |
| NAC 2         | ggttgctggccaccatttct        | gccgtttgttaccttctctgt       |
| KGM           | tgggaggccggcagttaata        | ggtggtgctgatgatgcgaa        |

seconds (for different primers calculated and standardized according to the melting temperature of each primer), and extension at 72°C for 50 seconds, followed by a final extension at 72°C for 7 minutes. Upon completion of the PCR cycles, the amplified products were mixed with two micro litres of loading dye (6X) and separated on 1.2% agarose gels through electrophoresis. The gels were stained using Ethidium bromide (0.2mg/ml) and gel photographs documented for amplification of the specific fragments.

Candidate gene analysis was used to identify sugarcane genes/ alleles which are differentially present in drought tolerant and susceptible varieties. Stress induced genes protect cells from unfavourable conditions by producing important protective metabolites and proteins, and also regulating the stress response signal transduction pathways. As the introduction of many stress-inducible genes via gene transfer resulted in improved plant stress tolerance (Zhang et al. 2004), this study is carried out to find the presence of six major genes in drought metabolism pathway in three tolerant and two susceptible varieties for drought.

### **ABA dependent ABF 2**

ABF 2, an ABF subfamily member, is a basic leucine zipper (bZIP) protein that regulates ABA dependent stress-responsive gene expression by interacting with the ABA-responsive elements. Its' over expression altered ABA sensitivity, dehydration tolerance and the expression levels of ABA / stress-regulated genes and promoted glucose-induced inhibition of seedling development, whereas its mutation impaired glucose response (Kim et al. 2004). Additionally, ABF may function in different stress response pathways. ABFs can transactivate ABRE-containing reporter genes. It has been demonstrated that ABA-dependent post translational modification, probably phosphorylation,

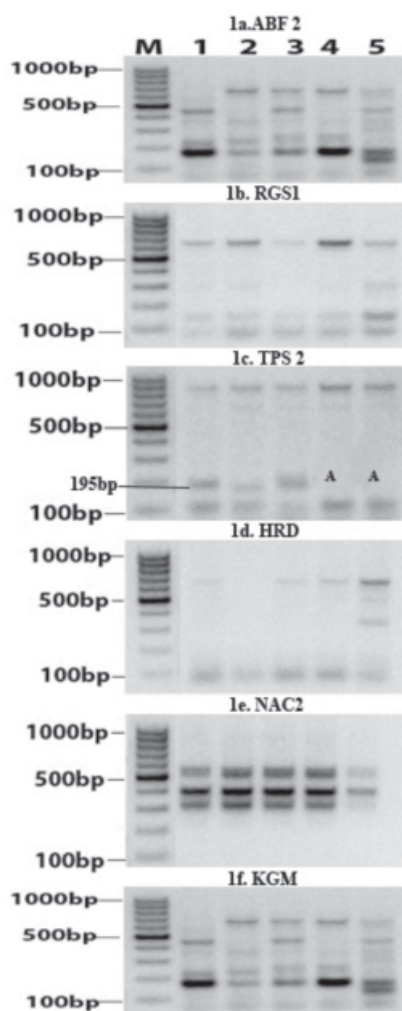
is required for the maximal transcriptional activity of ABF 2. In the present study, ABF2 amplified in all the genotypes and showed the multi allelic form of the gene (Fig. 1a). There were no specific alleles, visualized as bands to differentiate between susceptible and tolerant clones.

### **ABA dependent RGS 1**

Heterotrimeric G-proteins transduce extracellular signals from G-protein-coupled receptors and mediate intracellular processes critical for many cellular processes, such as plant growth responses to hormones, drought, light, and pathogens, and many developmental events. The G-protein-mediated signal transduction chain consists of many components, including,  $\alpha$ ,  $\beta$ , and  $\gamma$  subunits of the G-protein heterotrimer, G-protein-coupled receptors (GPCRs), regulator of G-protein signalling (RGS) protein, and many other downstream effectors. RGS protein (RGS 1) was recently identified in the *Arabidopsis* genome (AtRGS 1). AtRGS 1 protein was implicated in the responses of seed germination to sugars and abscisic acid (Chen et al. 2006). In this study, RGS1 amplified an 800bp band in all the five clones (Fig. 1b).

### **ABA dependent TPS2 (Trehalose-6-phosphate synthase 2)**

Trehalose is a non-reducing disaccharide that functions as a stress protection metabolite and carbohydrate reserve in many organisms (Goddijn and van Dun 1999). It is also known to have high water retention activity, which maintains the fluidity of membranes under dry conditions (Muller et al. 1995). Trehalose may also function as a regulator of plant metabolism and development (Goddijn et al. 1999) and has been found to be more effective than other sugars in increasing lipid bilayer fluidity (Crowe et al. 1984) and in preserving enzyme stability during drying (Colaco et al. 1992).



**Fig.1.** Amplification of six drought responsive genes in drought tolerant and susceptible genotypes of sugarcane. M - 100bp Ladder; lanes 1- 3, drought tolerant clones: 1 - Co 740, 2 - Co 0212, 3 - Co 0216; lanes 4- 5, drought susceptible clones: 4 - Co 775, 5 - Co 419; A- Absent

Transgenic plants that expressed the trehalose – 6 phosphate (T-6-P) synthase (TPS) and/or T-6-P phosphatase (TPP) genes from microorganisms not only exhibited increased drought tolerance but also showed strong developmental alterations (Romero and Ruiz 2010). TPS 2 gene implied in high tolerance levels to different abiotic stresses showed differentiated profile with 195bp fragment confined to tolerant clones and absent in susceptible

genotypes viz., Co 775 and Co 419 (Fig. 1c). Hence TPS2<sub>195</sub> as a candidate gene marker to be further validated for its use in marker assisted selection of drought tolerant clones.

### ABA independent HRD

The *HRD* gene belongs to a class of AP2/ERF-like transcription factors, classified as group III b in a recent comprehensive classification of the AP2 / ERF family. There are 17 members reported in this subfamily. Ectopic overexpression of HRD increases the density of the root network and improves water and salt stress tolerance in *Arabidopsis*, while in rice it caused an increase in plant biomass and drought resistance (Karaba et al. 2007). This study showed amplification of the gene in all the clones and did not show any specificity between susceptible and tolerant clones (Fig. 1d).

### ABA independent NAC 2

NAC is a plant specific transcription factor family with diverse roles in development and stress regulation. NAC was derived from the names of the first three described proteins containing the DNA-binding domain, namely NAM (no apical meristem), ATAF1-2 and CUC2 (cup-shaped cotyledon) (Aida et al., 1997). NAC proteins appear to be widespread in plants. Although more than 100 members of this family have been suggested in both rice and *Arabidopsis* genomes (Xiong et al., 2005), only a few of them have been characterized and the reported NAC transcription factors have diverse functions. AtNAC1 mediates auxin signaling to promote lateral root development (Xie et al., 2000). The specific primers amplified multiple copies and did not show specificity between susceptible and tolerant clones (Fig. 1e).

### Gibberellins associated kgm

Gibberellins (GAs) are diterpenoid hormones that play crucial roles in plant growth and development,

including seed germination, leaf expansion, stem elongation, and flower and fruit development (Hooley, 1994). GAMYB transcription factor operates within the GA-response pathway and also functions as a target of antagonistic effects by abscisic acid (Zentella et al. 2002). KGM, a GAMYB-binding protein is involved in response to abiotic stress in barley (Tondelli et al. 2006). The specific primers amplified multiple copies and did not show specificity between susceptible and tolerant clones (Fig. 1f).

The presence of drought responsive genes in the genotypes with tolerance and susceptible nature examined in the present study would indicate that drought tolerance is determined by many genes and all genes would not be absent in the susceptible clones. Sugarcane is a 12 month crop experiencing drought stress at any point of time in the field and these genes provide adaptive mechanism to tide over adversity. These results also substantiate the finding that sugarcane is basically drought tolerant. Drought tolerance mechanisms depend on the presence as well as the expression of the genes in the drought metabolic pathway. Though the gene may be present, the occurrence of the different allelic forms will be crucial that decide tolerance /susceptibility. Multiple banding with the gene specific primers (Fig 1) indicated the presence of more alleles of the same gene. These alleles might be contributed by the genomes of both wild as well as cultivated species clones and present in more copies due to the polyploidy and heterozygous nature of the crop. Another major factor to be considered is gene expression, where a battery of regulatory genes governs the time specific, tissue specific and stress specific responses. Swapna and Hemaprabha (2012) have shown the differential expression of such genes in drought tolerant and susceptible parents and mapping populations. Hence a detailed study on the presence of such alleles in the wild species clones

as well as their expression in the commercial clones on imparting stress is expected to give insight into the complex physiological process leading to drought stress tolerance and the results of the earlier work are encouraging in identifying the best species clones harbouring more number of drought responsive genes (Priji and Hemaprabha 2014, 2015). The genes identified could be used for their overexpression in susceptible clones and also can serve as candidate gene markers in marker assisted selection to develop future sugarcane cultivars with climate resilience and suitability for cultivation under suboptimal conditions of water availability.

## References

- Aida M, Ishida T, Fukaki H, Tasaka M (1997) Genes involved in organ separation in *Arabidopsis*: an analysis of the cup-shaped cotyledon mutant. *Pl Cell* 9: 841-857.
- Chen Y, Ji F, Xie H, Liang J, Zhang J (2006) The regulator of G – protein signaling (RGS) proteins involved in sugar and abscisic acid signaling in *Arabidopsis* seed germination. *Pl Physiol* 140: 302–310.
- Colaco C, Sen S, Thangavelu M, Pinder S, Roser B (1992) Extraordinary stability of enzymes dried in trehalose simplified molecular biology. *Biotechnology* 10: 1007–1011.
- Crowe JH, Crowe LM, Chapman D (1984) Preservation of membranes in anhydrobiotic organism the role of trehalose. *Science* 223: 701–703.
- Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem Bull.* 9: 11-15.
- Goddijn, OJM, Van Dun K (1999) Trehalose metabolism in plants. *Trends Pl Sci* 4: 315–319.
- Goddijn OJM, Verwoerd TC, Voogd E, Krutwagen RWHH, de Graaf PTHM, Poels J, Van Dun K, Ponstein AS, Damm B, Pen J (1997) Inhibition of trehalase activity enhances trehalose accumulation in transgenic plants *Pl Physiol.* 113: 181–190.

- Karaba A, Dixit S, Greco R, Aharoni A, Trijatmiko KR, Marsch-Martinez N, Arjun K, Karaba NN, Makarla U, Pereira A (2007) Improvement of water use efficiency in rice by expression of HARDY an *Arabidopsis* drought and salt tolerance gene. *Agrisci* 104 (39): 15270–15275.
- Kim S, Jung-youn, Kang Dong-Im, Cho Ji, Hye Park, Soo, Young Kim (2004) ABF2 an ABRE-binding bZIP factor, is an essential component of glucose signaling and its over expression affects multiple stress tolerance. *PI J* 40: 75–87.
- Kizis D, Pages M, Saleh A, Lumbreras V, Lopez C, Dominguez-Puigjaner E (2006) Maize DBF1-interactor protein 1 containing an R3H domain is a potential regulator of DBF1 activity in stress responses. *PI J* 46: 747–757.
- Pasternak T, Potters G, Caubergs R, Jansen MAK (2005) Complementary interactions between oxidative stress and auxins control plant growth responses at plant, organ, and level J. *Exp Bot* 56: 1991-2001.
- Priji PJ, Hemaprabha G (2014) Sugarcane specific drought responsive candidate genes belonging to ABA-independent pathway identified from tolerant and susceptible clones of *Saccharum* and *Erianthus* species. *Indian J Genet* 74(1): 64-72.
- Priji PJ, Hemaprabha G (2015) Sugarcane specific drought responsive candidate genes belonging to ABA dependent pathway identified from basic species clones of *Saccharum* sp. and *Erianthus* sp. *Sugar Tech*: 17(2):130–137
- Romero L, Ruiz JM (2010) Study of the ionome and uptake fluxes in cherry tomato plants under moderate water stress conditions. *PI Soil* 335:339–347.
- Seki M, Ishida J, Narusaka M, Fujita M, Nanjo T, Umezawa T, Kamiy A, Nakajima M, Enju A, Sakurai T, Satou M, Akiyama K, Yamaguchi-Shinozaki K, Carninci P, Kawai J, Hayashizaki Y, Shinozaki K (2002) Monitoring the expression pattern of around 7000 *Arabidopsis* genes under ABA treatments using a full-length cDNA microarray. *Funct Integr Genom* 2:282–291.
- Swapna S, Hemaprabha G (2012) Sugarcane specific drought responsive candidate genes identified through differential expression in resistant and susceptible genotypes of sugarcane (*Saccharum* sp.). *Int Sug J* 114 (1366): 731-737.
- Tondelli A, Francia E, Barabaschi D, Aprile A, Skinner JS, Stockinger EJ, Stanca AM, Pecchioni N (2006) Mapping regulatory genes as candidates for cold & drought stress tolerance in barley. *Theor Appl Genet* 112 (3): 445-454.
- Xie Q, Frugis G, Colgan D, Chua NH (2000) *Arabidopsis* NAC1 transduces auxin signal downstream of TIR1 to promote lateral root development. *Genes Dev* 14: 3024–3036.
- Xiong Y, Liu T, Tian C, Sun S, Li J, Chen M (2005) Transcription factors in rice genome-wide comparative analysis between monocots and eudicots. *PI Mol Biol* 59: 191-203.
- Yamaguchi-Shinozaki K, Shinozaki K (2005) Organization of *cis* acting regulatory elements in osmotic and cold-stress-responsive promoters. *Trends Pl Sci* 10: 88–94.
- Zentella R, Yamauchi D, Ho T-HD (2002) Molecular dissection of the gibberellin / abscisic acid signaling pathways by transiently expressed RNA interference in barley aleurone cells. *PI Cell* 14: 2289-2301.
- Zhang JZ, Creelman RA, Zhu JK (2004) Using information from *Arabidopsis* to engineer salt, cold & drought tolerance in crops. *PI Physiol* 135:615–621.