Comparative expression of two abscisic acid-inducible genes and proteins in seeds of aromatic indica rice cultivar with that of non-aromatic indica rice cultivars

Aryadeep Roychoudhury*, Supratim Basu & Dibyendu N Sengupta
Department of Botany, Bose Institute, 93/1, Acharya Prafulla Chandra Road, Kolkata 700 009, India

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As an integral part of stress signal transduction, the phytohormone abscisic acid (ABA) regulates important cellular reactions, including up-regulation of stress-associated genes, the products of which are involved directly or indirectly in plant protection. Being accompanied by an increased endogenous ABA level, the matured seeds, embryo and aleurone tissues of cereals accumulate several genes and proteins, associated with desiccation. The present study was aimed at investigating how the contrasting rice genotypes, varying in their salt-stress sensitivity, differ with respect to the expression pattern of two abiotic stress-inducible genes, \textit{Rab16A} and \textit{SamDC}, and corresponding proteins, in the seeds, at the background level (dry or water-imbibed state) and ABA-imbibed conditions, which could be related to the varietal differences in tolerance. The rice genotypes selected were M-1-48 (salt-sensitive), Nonabokra (salt-tolerant) and Gobindobhog (aromatic). An extremely low abundance of \textit{Rab16A} or practically undetectable \textit{SamDC} transcripts were observed in M-1-48 and Gobindobhog seeds under control conditions, induced only after exogenous ABA treatment, whereas they were expressed at a much higher level even in dry and water-imbibed seeds of Nonabokra, and lesser induced by ABA. The \textit{RAB16A} (= dehydrin) and \textit{SAMDC} protein expression in the three varieties were also identical to the gene expression patterns. Thus, the expression was stress-inducible in M-1-48 and Gobindobhog, while constitutive in Nonabokra. Our study reflected the similarity of the molecular responses to exogenous ABA of the seeds of the aromatic rice Gobindobhog to that of the salt-sensitive M-1-48, in exhibiting lower expression of stress-tolerant proteins only after stress. This work also proved that variation in gene/protein expression in seeds could be highly correlated with the variation in the tolerance mechanism of rice varieties.

**Keywords**: Abscisic acid, Aromatic indica rice, Gene expression, \textit{Oryza sativa}, \textit{Rab16A}, Rice seeds, \textit{SamDC}, Varietal difference

Abscisic acid (ABA) mediates various developmental and physiological processes, including responses of plants to environmental stress, such as high salinity, drought and low temperature as well as seed development and is pivotal for stress resistance. ABA appears to be perceived at several sites both extracellularly and intracellularly. During seed development, ABA aids the induction of storage protein and lipid synthesis, desiccation tolerance, prevention of germination and loading and unloading of assimilates under stress conditions\textsuperscript{1-3}. The concentration of ABA increases during late embryo development shortly before the onset of desiccation of seed tissues or seed dormancy. Desiccation stimulates ABA biosynthesis, which is, in turn, associated with \textit{de novo} expression of specific genes, affecting intracellular osmolarity or other protective functions\textsuperscript{5}. The gene expression in late seed development is tightly associated with drought tolerance and plays a cardinal role in adaptation process by encoding proteins involved in protective functions like ion sequestration, ion compartmentalization, water binding, osmolyte biosynthesis, free radical scavenging, polyamine biosynthesis, membrane stabilization etc\textsuperscript{5}. One such group of genes, like wheat \textit{Em}, rice \textit{Osem} and \textit{Rab16A-D}, barley \textit{HVA1} and \textit{HVA22} and so on, commonly induced by ABA, is called late embryogenesis abundant (\textit{lea}) genes because they are usually expressed during the late phase of seed development in resting grains, correlating with the seed desiccation stage\textsuperscript{6}. Their expression can also be triggered in immature embryos during exogenous ABA treatment. The study on transgenic plants has shown that LEA proteins can increase plant tolerance to water stress, has ion-binding activity and acts as
antioxidants under abiotic stress\(^7\). Apart from *lea*, the other groups of genes, induced by ABA, include genes involved in the production of enzymes for osmolytes and polyamines, detoxification or antioxidative enzymes, ion channels and membrane transporters on one hand, whereas genes encoding protein kinases and phosphatases or transcription factors on the other hand\(^8\).

Rice is mostly categorized among the salt-sensitive crops. However, genotypic variation of indica rice cultivars to sensitivity of abiotic stress is enormous. Among the lowland rice genotypes, the low yielding varieties like Pokkali, Nonabokra, Oormundakon etc are classified as highly tolerant ecotypes on the basis of various physiological parameters. On the other hand, the high yielding rice varieties like M-1-48, IR-29 and IR-72 etc are sensitive to NaCl toxicity, dehydration or ABA-mediated stress injuries. The endogenous ABA accumulation upon stress imposition is found to be larger, more rapid and less transient in the tolerant than in sensitive cultivars\(^9\). In addition, the aromatic indica rice varieties like Pusa Basmati, Radhunipagal, Badshabhog, Kataribhog, Kalonunia, Gobindobhog etc not only hold a major place in the domestic market, but are also of commercially high demand globally because of their superior qualities including pronounced odor and perfumed aroma. However, their poor productivity, low yield potential and restricted distribution within a particular eco-geographical area can be accounted for by their susceptibility to biotic and abiotic stress conditions. Hardly there are reports of high quality aromatic rice linked with salt or drought tolerance\(^10\). Hence, proper observation and documentation of the response of aromatic rice with respect to their overall physiology and regulation of gene expression in presence of stressors (high salinity, water deficit or ABA) might provide useful information regarding the understanding or manipulation of their general tolerance mechanism so as to increase their agricultural output and global demand.

In the present communication, we showed the comparative expression profiles of two ABA-inducible genes *Rab16A* and *SamDC* and their encoded proteins RAB16A and SAMDC in the dry, water-imbibed and ABA-imbibed seeds of three indica rice cultivars namely M-1-48 (salt-sensitive), Nonabokra (salt-tolerant) and Gobindobhog, the most popular indigenous aromatic rice confined within several districts of West Bengal, India. Rice gene, *Rab21*; (=*Rab16A*; Responsive to Abscisic acid) belongs to group 2 *lea* or the dehydrin (D11) group of protein of molecular weight 21 kDa. The transcript was earlier reported to accumulate at high levels and survive as long-lived mRNAs in the resting grains and also in young seedlings or vegetative tissues in response to any abiotic stress like high salt, water deficit or exogenous ABA treatment\(^11\). The other rice gene *SamDC* encodes the S-adenosylmethionine decarboxylase (SAMDC, EC 4.1.1.50) enzyme that converts the polyamine putrescine (Put\(^2+\), a diamine) to the higher polyamine spermidine (Spd\(^3+\), a triamine) in conjunction with another enzyme spermidine synthase (SPDS, EC 2.5.1.16). The higher polyamines, spermidine and spermine, are known to play protective functions against a large variety of environmental stresses, while excess putrescine level is toxic, inhibitory to plant growth and marks sensitivity to abiotic stress\(^12\). The *SamDC* gene expression has been previously observed in rice shoots during salinity, polyethylene glycol and ABA treatment\(^13\). For the study of the changes in gene expression and protein accumulation, we selected matured seeds, tissue responsible for maximum accumulation of dehydrin proteins, and also preferred organs of polyamine metabolism, accumulation and storage rather than the vegetative tissues\(^14\). Furthur, it was thought that the current study conducted with seed tissues and with ABA treatment, would further substantiate or corroborate our earlier observations showing the susceptibility or tolerance of these three cultivars to salinity stress\(^15\).

**Materials and Methods**

*Plant materials and treatments*—Seeds of *Oryza sativa* L. cv. M-I-48 were obtained from International Rice Research Institute (IRRI, Manila, Philippines), Nonabokra seeds from Central Soil Salinity Research Institute (CSSRI, Canning, West Bengal, India) and Gobindobhog seeds from State Agricultural Research station (Bethuadahari, Nadia district, West Bengal). All the varieties were multiplied in Madhyamgram Experimental Farm of Bose Institute. Dehusked rice seeds, either dry, water-imbibed (6 h) or soaked in water, supplemented with 50 \(\mu M\) ABA for 6 h, were used for our experiments. The samples of equal weight (5 g each) were homogenized for the preparation of total RNA or total protein.

*Cloning of rice Rab16A full-length gene and rice SamDC cDNA*—Genomic DNA was isolated from the
leaves of 5 day-old seedlings of salt-tolerant rice cultivar Pokkali using a modified CTAB method\(^\text{19}\). Total RNA was isolated from the roots of salt-treated (200 mM NaCl, 16 h) 10 day-old Pokkali seedlings using GITC method\(^\text{17}\). The full length Rab16A gene (promoter + the ORF, interrupted by a short intron) was amplified from the genomic DNA using Rab21-5 forward and Rab21-3B reverse primers as described earlier\(^\text{18}\). The SamDC cDNA (complementary DNA) was amplified from the total RNA, using RSam5 forward and RSam3 reverse primers as described earlier\(^\text{15}\). Both the genes were subcloned separately in pBSKS (Stratagene). The sequences of all the primers were shown in Table 1. DNA manipulations and cloning were carried out according to the standard protocol\(^\text{19}\).

**RT-PCR (Reverse transcriptase-polymerase chain reaction) analysis**—Total RNA was isolated from dry, water-imbibed (6 h) and ABA-imbibed (50 μM, 6 h) seeds of M-1-48, Nonabokra and Gobindobhog, by SDS-phenol method\(^\text{20}\). The RNA samples were treated with RNase-free DNase I (Boehringer Mannheim). The RT reactions were carried out with 5 μg of total RNA using Thermoscript\(^\text{TM}\) RT-PCR system kit (Life Technologies, USA). For Rab16A, the primer pairs used were Rab16-5A and Rab21-3B, and for SamDC, the primer pairs were RSam5 and RSam3, whose sequences are given in Table 1. RT-PCR was also done with the primers specific to rice actin cDNA, designed according to the published sequence of this gene (Accession No. X16280); this was used as an internal control to ensure equivalent loading of total RNA from all the samples.

**Protein immunoblot analysis**—The protein extracts were prepared from dry, water-imbibed (6 h) and ABA-imbibed (50 μM, 6 h) seeds of M-1-48, Nonabokra and Gobindobhog, following the method described earlier\(^\text{21}\) with slight modifications. After transferring 50 μg of total protein from each sample electrophoretically to a PVDF membrane, it was blocked with 1% (w/v) non fat dried milk in 1X Tris-buffered saline (TBS) containing 0.05% (v/v) Tween 20 for 2 h at room temperature (25°C), washed repeatedly with the same buffer and incubated either with anti-dehydrin (RAB16 like) antibody (a generous gift from Prof. Timothy J. Close) or with SAMDC antiserum (generated in our laboratory against the full length SAMDC protein overexpressed in E. coli by sub-cloning 1.2 Kbp SamDC cDNA, encoding the entire open reading frame; following the standard protocol\(^\text{22}\)). Each antibody was used at 1:1000 dilutions and incubated overnight at 4°C. The membrane was next incubated with goat anti-rabbit IgG alkaline phosphatase conjugate at 1:1000 dilutions. The cross-reacted bands of RAB16A or SAMDC protein were detected using 4-nitroblue-tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) substrates.

### Results and Discussion

India is rich in diverse scented rice varieties, having much stronger aroma and hence wider acceptance globally than ordinary non-aromatic rice. Unfortunately, there is dearth of consolidated experimental studies on aromatic rice varieties, especially with respect to their molecular responses during several abiotic stress conditions, at the genomic or proteomic level. In our earlier communication, we compared the physiological and molecular responses of Gobindobhog, to high salinity\(^\text{15}\), with non-aromatic M-1-48 (salt-sensitive) and Nonabokra (salt-tolerant) rice, and found Gobindobhog to resemble more like M-1-48 in its salt susceptibility. In this short communication, we have chosen to study the comparative effect of ABA on abiotic stress-inducible gene expression in the seeds of the above three rice varieties, because, this hormone plays a central role in seed development as well as in the response of rice plants to water stress, the two important agronomic traits. The two genes (and the corresponding proteins) selected for our studies were Rab16A and SamDC, since we have been working on the regulation of these two ABA-inducible genes for over a decade. We thought that the knowledge of the function of such ABA-responsive proteins will aid in understanding of

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### Table 1—Different oligonucleotide primers used for PCR and RT-PCR

<table>
<thead>
<tr>
<th>DNA</th>
<th>Complementary oligos</th>
<th>Nucleotide sequences</th>
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<tbody>
<tr>
<td>Rab16A full length gene</td>
<td>Rab21-5 (32-mer)</td>
<td>5′-CGACAAAGTTCTCACGCGCACTACACACACAT-3′</td>
</tr>
<tr>
<td>Rab16A cDNA</td>
<td>Rab21-3B (37-mer)</td>
<td>5′-ATAGAGCTCTGGATCTCCTAGTCTGCGCGGCGAGCTT-3′</td>
</tr>
<tr>
<td>SamDC cDNA</td>
<td>RSam5 (39-mer)</td>
<td>5′-CATGGTGTAACAATGGGAGACTTGCTGCCTGACCG-3′</td>
</tr>
<tr>
<td></td>
<td>RSam3 (38-mer)</td>
<td>5′-CTCGGATCTATTACCTCATAATCACAACCCTATCTGCCTC-3′</td>
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physiology of seed maturation and of salt/drought tolerance, particularly in aromatic rice.

Comparative gene expression analysis of Rab16A and SamDC in the seeds—Genes encoding LEA proteins were consistently represented in differential screens for transcripts with increased levels of drought and associated with the maturation process in embryo. Previously, high levels of Rab16A mRNA were found in embryos between 20 DAF and maturity (30 DAF), i.e., the level of expression was increased as maturation proceeded. The long lived transcript was found in mature dry grains, but was rapidly degraded or turned over at the onset of seed germination, especially after imbibition of isolated dry embryos in water. However, exogenously added ABA causes the precocious accumulation or re-induction of mRNA in immature embryos and in germinating seeds\(^{1,23}\). Similarly, it has been observed with another lea, i.e., Osem mRNA\(^{23,24}\). The highest level of Rab16A mRNA has been obtained on treatment with 50 µM ABA\(^{23}\). In fact, we have earlier characterized the abscisic acid responsive elements (ABREs) or ABRE-like sequences of Rab16A and their interacting trans-acting factor(s) in rice\(^{23,26}\), which justifies that ABA acts as a signal in affecting the steady state level of Rab16A mRNA. Rice seeds, being the least metabolically active tissue, have earlier been shown to accumulate polyamines at higher levels than vegetative tissues\(^{27}\). ABRE and/or ABRE-related motifs have been reported in the promoters of SamDC\(^{28}\) as well, and the gene has been found to be induced through ABA-dependent pathway after exogenous application of ABA to rice shoots for 6 h and 24-48 h\(^{13}\).

In the present study, the variation in expression pattern of Rab16A and SamDC was monitored in dry, water-imbibed (6 h) and ABA-imbibed (50 µM, 6 h) seeds of the three varieties M-1-48, Nonabokra and Gobindobhog. RT-PCR with equal amount of total RNA from dry, water-imbibed and ABA-imbibed seeds showed a very low level of Rab16A transcript (0.5 Kbp), both in dry and water-imbibed M-1-48 seeds, the expression was prominent only after ABA treatment of seeds. Similar trend was also observed for the aromatic cultivar Gobindobhog, where the transcript level was still lesser than M-1-48 under control conditions and triggered to a high level only by ABA. In case of Nonabokra, the tolerant cultivar, conspicuously higher transcript level could be detected all throughout, i.e., in dry; ABA-imbibed and even in water-imbibed seeds (Fig. 1A). Likewise for SamDC, the maximum transcript (1.2 Kbp) level was observed constitutively in Nonabokra, while in M-1-48 and Gobindobhog, it was inducible only upon ABA imbibition, but undetectable in dry or water-imbibed seeds (Fig. 1B). PCR with no-RT reaction using each RNA sample did not show any product (data not shown). The rice actin cDNA, consisting of 1.1 Kbp fragment, was detected constitutively when a control RT-PCR was done with each of the above RNA samples, and the transcript level appeared to be equal in all the lanes (Fig. 1C), suggesting equal amount of total RNA used.

Comparative expression of RAB16A and SAMDC proteins in the seeds—Hydrophilic proteins such as dehydrins (to which RAB16A protein belongs to) are

Fig. 1—RT-PCR analysis of Rab16A and SamDC gene expression in dry, water-imbibed (6 h) and ABA-imbibed (50 µM, 6 h) seeds of M-1-48, Nonabokra and Gobindobhog. The levels of ~0.5 Kbp Rab16A transcript (A); and ~1.2 Kbp SamDC transcript (B) are represented. Equal amount of each RT mix was taken and No-RT reactions were also done as negative control (data not shown). A control RT-PCR, monitoring the constitutive expression of ~1.1 Kbp actin transcript with all the RNA samples (C), is also represented to show the equal loading of the total RNA.
the common products protecting the bio-membranes in matured seeds, whose accumulation is induced especially by drought, salinity and ABA treatment\textsuperscript{29,31}. The SAMDC enzyme protein is also very vital, since it is the rate limiting enzyme in polyamine biosynthetic pathway with a rapid turnover and hence, having a very short half-life\textsuperscript{32}. This enzyme plays a key role in the biosynthesis of the triamine Spd\textsuperscript{3+}, which confers stress tolerance.

In the present study, equal amounts of total protein (50 µg) from each sample were loaded and resolved through 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for immunoblot analysis. Detection of the polypeptide band of 21 kDa, that cross-reacted with the anti-dehydrin antiserum (a generous gift from Prof. Timothy J. Close) was confounded at an abundant level in Nonabokra, particularly in dry and ABA-imbibed seeds with a slight decrease on water imbibitions. Actually, water imbibition, programming the seeds towards germination, might have reduced the protein level, supporting the earlier observations\textsuperscript{11,23,24}. The seeds of M-1-48 and Gobindobhog could accumulate the protein in considerable amounts only after ABA imbibitions, but not in dry or water-imbibed conditions (Fig. 2A). Earlier, the dehydrin protein accumulation to strikingly high levels was clearly induced in response to ABA in both Pokkali and Nonabokra roots, but not detectable in Taichung Native\textsuperscript{9}. A similar varietal difference was noted here in case of rice seeds as well. The polypeptide band of 35 kDa (probably the larger α subunit fragment of rice SAMDC protein) that cross-reacted with the anti-SAMDC antiserum (generated in our laboratory) was detected abundantly and uniformly in dry, water-imbibed and ABA-imbibed Nonabokra seeds. However, only the ABA-imbibed seeds of M-1-48 and Gobindobhog accumulated SAMDC protein, which was untraceable in dry and water-imbibed seeds (Fig. 2B). These observations indicated that both RAB16A (dehydrin) and SAMDC proteins were expressed at a much higher level in Nonabokra seeds compared to M-1-48 and Gobindobhog, which exhibited only stress-inducible expression of the two proteins.

**Conclusion**

Linking the expression of a gene and its encoded protein to a higher degree of tolerance within a genotype provides an important argument for its role in adaptation. The susceptibility or tolerance to abiotic stress in plants is a co-ordinated action of multiple stress responsive genes. The endogenous ABA concentration increases in different plant tissues during drought, salinity or cold induced reduction in water availability\textsuperscript{33}. Exposure to exogenous ABA mimics the induction of genes similar to stress exposure. However, only a few studies have compared the gene expression in response to exogenous ABA in rice genotypes differing in tolerance. Particularly, the comparison of the level of Rab16A and SamDC in seeds in response to ABA in rice varieties differing in their level of tolerance has not been done before. The present study suggested that the aromatic rice cultivar Gobindobhog exhibited closeness to the salt-sensitive rice cultivar M-1-48 in expressing Rab16A and SamDC genes or corresponding proteins in seeds especially during ABA treatment, but low or almost nil at the background level, i.e., in dry or water-imbibed seeds. On the other hand, the salt tolerant cultivar Nonabokra showed higher expression level of the two stress-inducible genes or proteins and that also at the constitutive level, i.e., even in dry or water-imbibed seeds, which was slightly triggered by ABA. The induction by ABA of both the genes in seeds thus appeared to be controlled by similar mechanism.
This work threw light on correlation of regulation of stress-inducible gene expression with ABA in seeds of aromatic indica rice variety. A comparison of the level of stress-inducible genes or transcription factors have been done earlier in response to salt stress in the vegetative tissues (leaves) of salt-sensitive and salt-tolerant cultivars with that of a popular aromatic rice, which reveals higher and constitutive expression pattern of stress-inducible genes in the tolerant cultivar. Another group has also reported higher level of SamDC transcript with more rapid accumulation in the salt-tolerant rice varieties. In conclusion, the differences in the levels of the ABA-responsive proteins RAB16A (dehydrin) and SAMDC are definitely associated with the differences in varietal stress tolerance of rice, and the gene induction in the sensitive and aromatic cultivars following ABA treatment can be correlated with the acquisition of desiccation tolerance.

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