Prospects of improving flooding tolerance in lowland rice varieties by conventional breeding and genetic engineering

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Flooding is a recurrent phenomenon in several lowland rice-growing areas in India and elsewhere. Even though rice is a reasonably flooding-tolerant crop, the annual loss incurred by farmers due to floods is large. There are excellent traditional rice types with high level flooding tolerance. Combining high level flooding tolerance to high grain yield through conventional breeding has been successful to a limited extent so far but there are enormous opportunities for the same. There are also hopes that flooding tolerance can be genetically engineered in rice using a transgenic approach. We take a look on the prospects for improvement of rice to flooding stress through conventional breeding and through plant genetic engineering.

RICE is the most important food crop in the world consumed by nearly 3 billion people almost daily. Nearly 25% of the world's rice (i.e. 38 million hectares) is cultivated in the rainfed lowland ecosystem. However, the produce from rainfed lowland ecosystem accounts for only 17% of the global rice supply. Figure 1 shows distribution pattern of lowland rice areas in South and South-east Asia. India has the largest area (i.e. 17.2 million hectares) under rainfed lowland amongst the South-east Asian countries[1]. About 75% of the world lowland rice is in the belt across Eastern India, Bangladesh, Myanmar and Thailand.

Submergence due to flash-floods is the key factor limiting yield of lowland rices. Widawsky and O'Toole[2] showed that out of 42 biotic and abiotic stresses which prevail in rainfed lowland rice areas of Eastern India, submergence stress is the third most important limitation to rice production (surpassed by drought and weeds). Flash-floods are highly unpredictable and may occur at any growth stage of the rice crop and the yield loss may be anywhere between less than 10 and 100% depending on factors such as water depth, duration of submergence, temperature, turbidity of water, rate of nitrogen fertilization, light intensity and age of the crop[3]. Gases diffuse 10,000 times slower in water than in air[4]. Hence growth and survival during submergence of rice is affected by partial (hypoxia) or complete loss (anoxia) of O_2. Reduced supply of O_2 and CO_2 as well as reduced C_2H_4 dif-

fusion limit respiratory activities, photosynthesis and have a negative impact on elongation and growth of rice plants.

Rice plants respond to flooding stress through (a) elongation ability by which varieties in situation of stagnant or deep water flooding (water depth 60–100 cm) avoid complete submergence through elongation of leaf sheath, leaf lamina and internode leading to emergence of the plant above the rising flood water levels, and (b) submergence tolerance by which certain rice varieties survive submergence of 10 days or more particularly in shallow water [water depth up to 40 cm (as per the classification followed in India) up to 50 cm (as per the classification followed at the International Rice Research Institute (IRRI)); water depths mentioned are approximations as it is still a debatable issue] through metabolic adjustments. The latter situation prevails particularly in lowland ecosystems. Is it possible to change the genetic make-up of rice plant by conventional breeding and by genetic engineering to make it more tolerant to flooding stress? We discuss this theme in the present review.

Improvement of submergence tolerance through conventional breeding approach

Rice crop in lowland areas is invariably subjected to flooding stress continuously for varied periods. The traditional rice varieties have evolved suitable mechanism(s) to survive under this stress. Systematic screening of rice germplasm has shown that there are excellent flood-tolerant rice types locally available. Among these are especially the 'FR13A' and 'FR43B' of India, 'Kurkaruppan' of Sri Lanka and 'Goda Heenati' of Indonesia. The comparative performance of 'FR13A',

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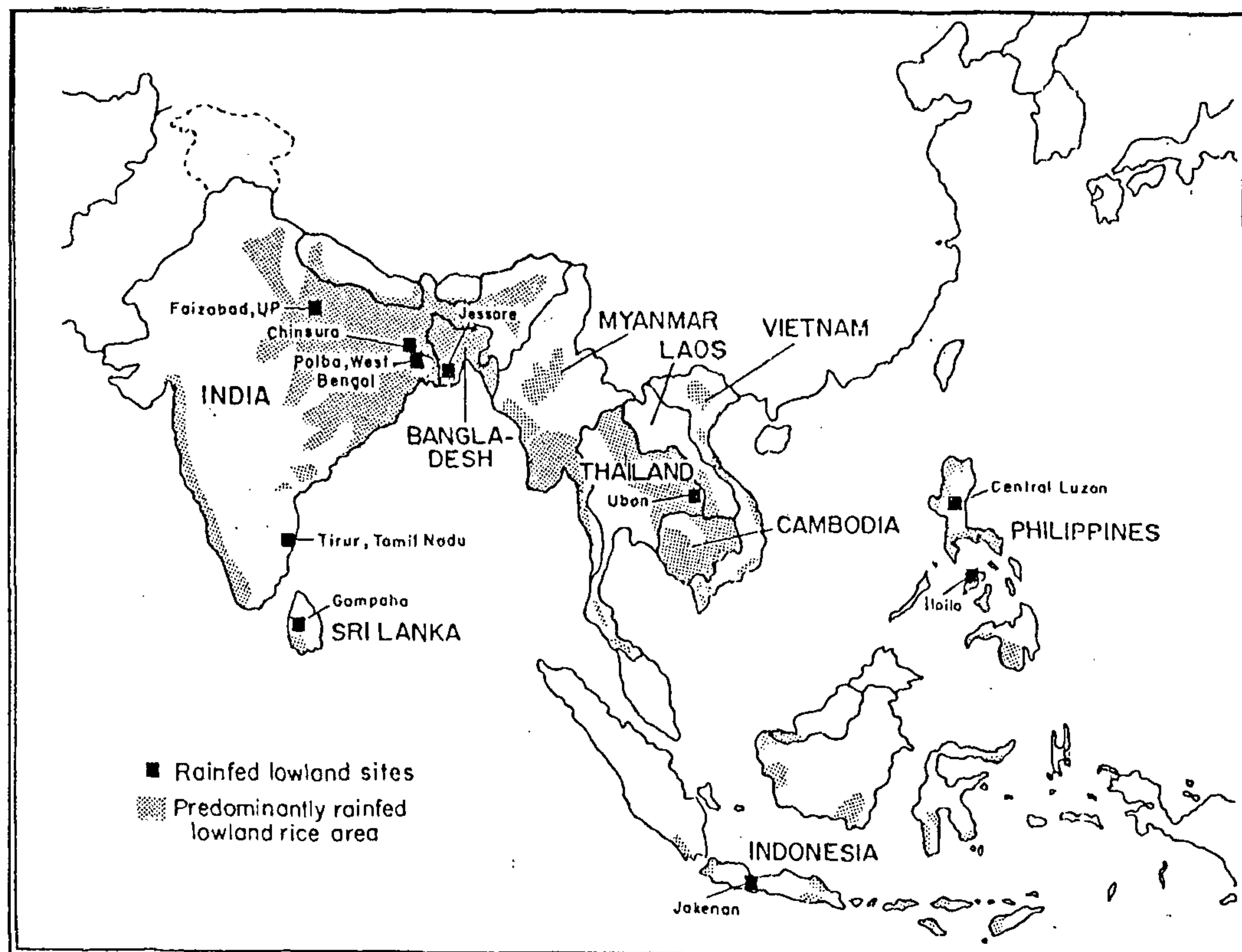*For correspondence. (e-mail: grover_anil@hotmail.com)

Figure 1. Predominant rainfed lowland rice areas and proposed rainfed lowland key sites in South and South-east Asia (from IRRI 1992 – rainfed lowland rice research consortium).

'FR43B' and several other rice types is shown in a field experiment[5] in Figure 2. Detailed study on such contrasting rice types has indicated that flooding tolerance is controlled by one or a few genes with major effects and additional genes with smaller, modifying effects[6]. However, the traditional lowland rice varieties are lodging-prone because of their tall stature. Further, these varieties are susceptible to diseases and insect pests. Such varieties are unsuitable for large-scale cultivation. It is therefore an attractive research proposition to transfer submergence tolerance from traditional cultivars into more productive modern varieties.

One of us (H.K.M.) took up this challenge in the early eighties and isolated a number of promising breeding lines with good plant type and submergence tolerance such as 'IR 26702-25-3' ('FR13A' source) and 'IR 31406-333-1' ('Kurkaruppan' source)[7]. Subsequent breeding efforts at IRRI led to evolution of tolerant lines with good agronomic traits. The line 'IR 49830-7-2-2' combines high tolerance levels with higher yield potential and resistance to diseases and insect pests and it has been extensively used as a donor parent in the breeding

programme. 'Sudhir' is another variety which has been developed from the 'FR13A' x 'Biraj' crosses[8]. This variety has been released by the 'Central Variety Release Committee, Indian Council of Agricultural Research, Government of India' in 1999.

In spite of the above developments, till date no variety has been developed which combines desirable levels of flooding tolerance with grain yield. It is attributed to the fact that 'FR 13A' is a poor combiner for yield and other agronomic traits. Apart from 'FR 13A', 'BKNFR 76106-16-0-1-0' and 'Kurkaruppan' have major dominant gene for submergence tolerance. Flooding tolerant rice 'Goda Heenati' apparently does not have the same submergence tolerance gene. This fact offers hope for pooling diverse genes for submergence tolerance to increase tolerance even above what we have at present. It has been suggested that there are at least three submergence tolerance genes in 'FR13A', 'Kurkaruppan' and 'Goda Heenati'. Further, though 'FR13A' is considered to be an excellent source of submergence tolerance specially at young seedling stage (10 days), 'CN 540 (Suresh)' is found to be more resistant than 'FR13A' at

Major attempts have also been made in breeding for improved submergence tolerance through the use of double haploid lines (DHLs), developed using crosses between submergence tolerant and sensitive rice cultivars[10]. The role of DHLs is to screen up several hundred lines within each population for submergence tolerance in representative areas of flash-flooding. Two DHL populations for submergence tolerance have been developed at IRRI using the cross combination of (1) 'IR 49830 × CT 6241' and (2) 'FR13A × IR 42'. These populations were evaluated simultaneously in Thailand and Philippines. In Thailand, a distinct bimodal distribution of lines for survival after submergence was observed supporting the concept of a single gene for submergence tolerance[11]. However, the result from Philippines did not support this theory. Differential responses of DHL populations for submergence tolerance to varying environments signifying G × E interaction, suggest the need for location-specific breeding efforts. The inability of reproducing the result at different locations is a major concern in current research with DHL populations.

Use of DHLs has recently been reported for identification of two RFLP (restriction fragment length polymorphism) markers for submergence tolerance of rice which are mapped to a segment of chromosome 9. This chromosome segment [Sub 1(t)] accounted for 70% of the phenotypic variance in submergence tolerance of this population[12]. It would be rewarding if the Sub 1(t) locus is cloned through map-based cloning technique. Recently, a high-resolution map has been constructed around the Sub 1(t) locus through the use of RFLP and AFLP (amplified fragment length polymorphism) markers. Present attempts are focussed on cloning of tightly-linked AFLP markers for eventual screening of gene libraries[13].

The 'International Conference on Lowland Rice' in 1984 showed that none of the countries in South and South-east Asia had an effective breeding programme for submergence tolerance with the exception of Thailand where two promising tolerant lines 'BKNFR 76106-16-0-1' ('FR13A' source) and 'BKNFR 76109-7' ('FR43B') were developed[14]. Release of short/ intermediate height (90–120 cm) high-yielding varieties like 'Jagannath' (OUAT) and 'Pankaj' (IRRI) in India in 1969 and 'Mahasuri' in Malaysia in 1971 was a landmark in lowland rice breeding[15]. The two releases 'Savitri' (Central Rice Research Institute; CRRI) and 'IR42' (IRRI) in the early eighties possessed high yield potential (up to 9 t/ha in 'Savitri') and resistance to diseases and insect pests (in 'IR42') and also adaptability to adverse soils in the latter. However, these varieties lack submergence tolerance to the tune of 'FR13A'. Presently, these are being used as the base material in breeding for submergence tolerance. A significant change in the breeding approach in the nineties is the

Figure 2. Comparative performance of FR13A and several other rice types. (Top panel), Submergence treatment was given for 14 days at water depth of 75 cm by placing the pots in submergence tanks; (Middle panel), Comparison of IR 42 and FR13A varieties following desubmergence at young seedling stage. Pots placed behind the Labels represent control plants; (Lower panel), Comparative performance of submerged and control plants of different rice varieties. This experiment was performed employing 50-days-old plants. The submergence treatment lasted for 12 days at 100 cm of water (see Mallik[5] for more details).

an older stage (40 days) and 'FR43B' remains resistant at all growth stages[9]. Therefore, it is proposed that the development of a bridge parent combining both types of resistance (such as 'FR43B') might result in genotypes with better submergence tolerance.

operation of the 'Shuttle Breeding Programme' in different tracts in South and South-east Asia. The 'Eastern India Programme' started operating in Kharif 1992 and is jointly coordinated by CRRI and IRRI. IRRI supplied seeds of F_2 generation of target crosses for selection under local conditions. Locally-developed elite lines and breeding lines from IRRI including DHLs and somaclonal variants were evaluated in shuttle breeding at a number of cooperating centres in Eastern India. A number of tolerant lines with good agronomic features have been identified such as 'IR 67626-2-2-1' at Chinsurah and 'TCA 95-6', 'IR 67637-14-3-3' and 'IR 67637-11-2-6' at Masodha[16]. The variety 'Sabita' released by one of us (S.M.) is a national check for the semi-deep water ecosystem since 1987 and also a check in 'Shuttle Breeding Programme' since its inception. This variety has been utilized by IRRI in more than 30 crosses and the progenitors from these crosses have been shown to possess a good grain type, wide adaptability and submergence tolerance. As the Cooperator of this programme, S.M. is involved in exchange of F_1 and F_2 seeds amongst different institutions, to make sure that it is a multi-way trafficking of research findings.

The promising tolerant lines of 'Shuttle Breeding Programme' were not included in the shallow water lowland trials for Eastern India of 'All India Co-ordinated Rice Improvement Programme (AICRIP)' for a while which was a matter of concern. However, beginning with Kharif 1999, the materials developed from 'Shuttle Breeding Programme' (such as 'NDR9730004, NDR96007, NDR96006') have been included in the 'National Programme (AVT- shallow water)'. Further, the 1999 workshop of AICRIP (Directorate of Rice Research, Hyderabad) recommended release of the lowland lines 'OR877-ST-4-2' (developed at OUAT from 'IR42/Savitri//IR42') and 'CN1035-61' (developed at Chinsurah from 'Pankaj/IR38699-43-1-2//IR41389-20-1-5') for semi-deep situations for their moderate level of tolerance to submergence and high and stable yields. The line 'CN1035-61' has also been tested through the 'Shuttle Breeding Programme' and considering its good performance during the last two years, it has been reconsidered for inclusion in 'AVT- shallow water'.

Improvement of flooding tolerance in rice through plant genetic engineering approach

Research on rice molecular biology and biotechnology has made great strides during the past 15 years. Rice has emerged as a model cereal crop for research on molecular and genetic studies. Research on this crop has received tremendous support through different funding bodies including the Rockefeller Foundation, USA through its 'Rice Improvement Programme' and from the Japanese Government through its 'Rice Genome Programme'. The salient features of rice genome, rice molecular biology and biotechnology can be seen elsewhere[17]. Tools and techniques for improvement of rice through genetic engineering approach have been perfected to a great extent in several International and National laboratories. Genetic transformation of rice in the early studies was achieved through protoplast-based methods. However, this task has become more routine with the availability of biolistic approach (through the use of microprojectile gun) and more recently, through the *Agrobacterium tumefaciens*-based approach. A number of different regulatory sequences are now available for controlling expression of transgenes in rice and as a result, the transgene can be either constitutively expressed or expressed in response to a specific stimulus (including anaerobic stress; through use of promoters from genes which strongly respond to flooding stress). The bottom line in this discussion is that transgenic rice with specified genes can be produced[18]. This contention is further supported by the fact that transgenic rice plants tolerant/resistant to viruses, insects, fungal pathogens, herbicides, salts and low temperature stresses are a reality now[18].

The question here is how developments in rice molecular biology research can help in raising flooding tolerant rice. Studies have shown that identification/isolation/cloning of genes which are associated with improved flooding stress tolerance appear to be the limiting factors[18]. A great deal of research in this context is focused on carbohydrate metabolism for the obvious reason that a reduced O_2 supply directly hampers normal respiration resulting in decreased levels of ATP synthesis. It has come to light that the pathway of respiration switches over from oxidation to fermentation mode during anaerobiosis. The induction of ethanolic fermentation pathway is considered to be an important component of responses which are elicited in rice (and other plants) against flooding stress[18,19]. A good correlation between anoxia tolerance and rate of ethanolic fermentation is further evidenced through analysis of null mutants of *Zea mays* and *Arabidopsis thaliana* which are unable to produce ethanol and die more rapidly under anaerobic stress than the wild type plants[18,20,21]. The operation of ethanolic fermentation (pyruvate to ethanol) is a relatively simpler trait involving only two enzymes (i.e. pyruvate decarboxylase or PDC and alcohol dehydrogenase or ADH). The availability of cloned *pdc* and *adh* genes (from microbial as well as higher plants) has naturally prompted interest of molecular biologists to employ these for transgenic experiments. Cris Kuhlemeier's groups (University of Berne, Switzerland) has produced transgenic tobacco plants which have a constitutive capacity of ethanolic fermentation by expressing *pdc* gene obtained from obligate anaerobe *Zymomonas mobilis* that had been subcloned to CaMV 35S promoter. This group noted that

while over-expression of the bacterial *pdc* caused only a moderate increase in acetaldehyde and ethanol production in the transgenic roots under anoxia compared to wild type roots under the same conditions, the increased ethanolic flux did not enhance anoxia tolerance[22,23]. However, it is too early to make conclusions that increased *pdc* (and also *adh*) has little or no role in submergence tolerance because the following pertinent possibilities are yet to be explored: (a) What happens if different promoters (with varying strengths and not only constitutive but also anoxia-induced) are employed to bring differential levels of *pdc* expression? (b) What happens if concomitant high level *pdc* and *adh* expression is brought in the same cell? (c) What happens if rice rather than tobacco is employed as the host system for such work because tobacco and rice may differ in their response to flooding? A group at CSIRO, Australia is currently working on genetically altering levels of *pdc* and *adh* in rice. One of us (A.G.) is associated with this group for the past 6 years in the programme in which 3 different rice *pdc* genes (*pdc1*, *pdc2* and *pdc3*) have been cloned and sequenced; *pdc1* cDNA has been subcloned at the 3' end of three different promoters (CaMV 35S, actin 1 and anoxia-induced 6X ARE promoter which is a synthetic promoter) in both sense and antisense orientations and these plasmid constructs have been introduced into rice to yield a large number of transgenic lines[21,24,25]. One of the above gene constructs (actin 1-*pdc1*, sense) has been employed at IRRI for the production of transgenic rice with enhanced metabolic capacity under anaerobiosis conferring submergence tolerance[26]. In this study, tillers of confirmed T_0 plants showed higher PDC activities and ethanol production compared to untransformed control and consequently ethanol production of tillers of T_0 transgenic plants was positively correlated with survival after submergence. The group at CSIRO has also made considerable progress in altering levels of *adh* gene in transgenic rice plants[27]. However, the assessment of the lines with altered ADH levels with respect to submergence tolerance has not yet been completed[25].

Future work has to be taken up on the following lines: (1) Apart from PDC and ADH, several other enzymes play a role in (a) mobilization of carbon from complex carbohydrate forms (i.e. starch, sucrose etc.) to simpler six-carbon forms which readily enter glycolysis (i.e. glucose); and (b) metabolizing the six-carbon simpler sugars to pyruvate[19,28]. These include glucose phosphate isomerase, phosphofructokinase, aldolase, triose phosphate isomerase, glyceraldehyde phosphate dehydrogenase, enolase, pyruvate kinase and phosphoglycerate kinase. Most of these enzymes show an up-regulation (in terms of enzyme activity as well as transcript levels) in response to anoxia stress[19]. However, till date no attempts directed at making transgenics over-expressing these genes have been reported. There is a need to follow reverse genetics approach to find utility of these genes/proteins. (2) Abiotic stresses (including flooding stress) elicit multiple responses. Therefore, expression of the entire battery of stress-responsive genes would obviously have a greater beneficial effect on stress tolerance than the individual genes. Recent experiments show that by changing the transcription factor genes, it is possible to alter levels of several target genes at the same time[29,30]. For this to be achieved in the context of anoxia stress, understanding of the transcriptional activation of anoxia-induced genes is a prerequisite. (3) Another level in the hierarchy of genetic controls which have a bearing in regulating stress responses is exercised at signal transduction pathways. This is evidenced by the findings of Pardo *et al.*[31] who recently raised salt-tolerant transgenic plants by altering a signal transduction component. The plant stress signalling involves participation of G proteins, cAMP, cGMP, calcium/calmodulin, inositol phospholipids and kinases/phosphatases. Ca^{++} has been found to play an important role in induction of several stress responsive genes (including flooding stress). The strategies for altering signalling pathway in flooding stress response are yet to be discussed and tested.

Future outlook

The conventional breeding approach has made reasonably good progress in producing flooding tolerant rice. However, there are several avenues for future research in this endeavour. The range of flooding tolerance donors from divergent sources must be extended in the future breeding programmes. Screening methods for flooding tolerance need re-addressal. The screening method presently followed using 21-days-old seedling submerged for 14 days appears to be drastic and thereby promising breeding lines with even adequate level of tolerance get eliminated. Screening for submergence tolerance at adult stage is equally important. Besides submergence tests, the breeding material must be subjected to other on-farm biotic and abiotic stress factors. The material thus developed might possess desirable traits needed for rainfed lowland areas including high degree of submergence tolerance. In on-farm evaluation test, breeding lines should be exposed to natural floods. Selection efficiency in breeding population could be enhanced by use of molecular markers. Identification of the RFLP/AFLP markers flanking Sub (1)t locus is an important step in this direction. Yield and submergence tolerance are generally negatively associated. If this association is linkage, attempts need to be made to break this linkage by repeated back-crossing to produce lines that combine high yields and submergence tolerance.

More basic research on regulation of flooding response in terms of transcriptional activation of stress-responsive genes and signal transduction mechanisms is

the need of the hour to make transgenic rice for improved flooding tolerance. There is also a need to identify, isolate and clone genes which are induced in response to flooding stress and to test the functional relevance of such genes in rice flooding tolerance response through the reverse genetics approach. The construction of suitable gene libraries, differential display, subtractive hybridization and other related techniques would be helpful to obtain this goal. The recently initiated 'Rice Genome Project (RGP)' aims to provide nucleotide sequence of the complete rice genome. The information from this project would hopefully provide function and location of additional genes important for flooding tolerance.

Finally, it is argued that IR 36, the most adapted stable variety across countries in irrigated ecosystem has diverse genes from 18 parents originating from 11 countries. Why can the same not be achieved for the lowland ecosystems? Effective integration of conventional breeding and biotechnological efforts might lead to a variety like IR 36 for the diverse, unpredictable and flood-prone environments of rainfed lowland ecosystems. Indian laboratories have the requisite support in terms of germplasm availability to carry out this programme. However, it is important to supplement this through additional support for exploiting this germplasm for desired goals. It must be borne in mind that such programmes have lucrative ends in sight and therefore, one has to move against time to make a dent in international markets for the superior products (i.e. seeds of the elite types). Adaptation to flooding in semi-deep water, deep-water and floating rice will be reinforced if they have seedling submergence tolerance in addition to elongation ability.

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