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Towards Production of Abiotic Stress Tolerant Transgenic Rice Plants: Issues, Progress and Future Research Needs

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Rice is a vital crop for the entire world population. This crop constitutes the staple food for the inhabitants of the Asian countries. It is estimated that grain production in rice must be increased by 60% of the present value, to meet the demand which will be there at 2020 A.D. The increased rice production can play a significant role in upgrading the economic status of countries like India and China. In this context, it is notable that the grain yield of rice is not harvested in commensuration to its existing genetic potential in almost all rice- growing ecosystems. One of the major reasons behind this failure is the sensitivity of this crop to different stress factors in field-conditions. This is especially true for the abiotic stresses (particularly drought, submergence and salinity). The production of abiotic stress tolerant rice cultivars thus emerges as a priority issue. The areas of rice molecular biology and biotechnology have progressed appreciably in the past two decades and a number of different transgenic rice plants have already been developed. In this article, we assess the current status of raising rice tolerant to abiotic stresses and identify the research gaps which need to be filled up in this direction.

Key Words: Abiotic stress tolerance, Agrobacterium-mediated rice transformation, Genetic engineering, Transgenic technology

Introduction

The importance of rice as a food crop is second to none particularly in the context of Asian countries. A great deal of research has been carried out in the recent past on the molecular biology, genomics and biotechnology of rice crop. Employing recombinant DNA technology, remarkable progress has been made towards production of rice plants for increased yield (such as through production of hybrid rice varieties), improved nutritional quality (such as through introduction of provitamin A synthesizing genes, increased iron content etc.) and improved resistance to insects, viral, bacterial and fungal pathogens (see figure 1). Close to the dawn of 21st century, the understanding of rice molecular biology stands at a threshold point as this crop is now considered a model cereal crop for molecular studies (Khush & Toenniesen 1991, Sasaki & Moore 1997, Khush & Baenziger 1998).

Abiotic stresses (such as sub- and supra-optimal temperatures, excess salt levels, reduced water availability leading to drought stress, excess water resulting in flooding stress and oxidative stress caused by the combination of high light intensity with other stresses etc.) adversely affect almost all major field-grown crops. Rice crop is particularly sensitive to excess salts, reduced or excess water supply and sub-optimal temperature regimes (Widawsky & O'Toole 1990, Khush & Tonniessen 1991, Khush & Baenziger 1998). According to Hossain (1996), drought and submergence stresses affect rice cultivation more than the biotic stresses. On the issue of genetically transforming rice with genes

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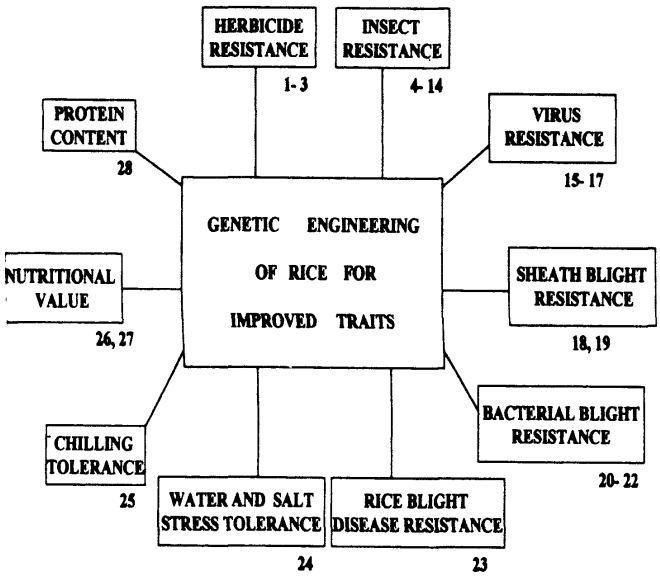

Figure 1 Various agronomic traits which have been engineered in rice using transgenic technology. Numbers under each block represent the corresponding reference of the work as detailed here (see 'References' in the text for complete details). **1**, Christou et al. (1991); **2**, Datta et al. (1992); **3**, Li et al. (1992), **4**, Fujimoto et al. (1993); **5**, Wunn et al. (1996); **6**, Ghareyazie et al. (1997); **7**, Datta et al. (1997); **8**, Nayak et al. (1997); **9**, Wu et al. (1997), **10**, Duan et al. (1996); **11**, Xu et al. (1996); **12**, Irie et al. (1996); **13**, Hosoyama et al. (1995); **14**, Xu et al. (1996 a); **15**, Hayakawa et al. (1992); **16**, Fang et al. (1996); **17**, Huntley & Hall (1996); **18**, Uchimiya et al. (1993); **19**, Lin et al. (1995); **20**, Song et al. (1996); **21**, Wang et al. (1996); **22**, Tu et al. (1998); **23**, Tada et al. (1996); **24**, Xu et al. (1996 b); **25**, Yokoi et al. (1998); **26**, Burkhardt et al. (1997); **27**, Goto et al. (1999); **28**, Zheng et al. (1995)

which can lead to superior tolerance against different abiotic stresses, previous studies have shown that

(i) the response of plants to abiotic stresses is multigenic in nature,

(ii) while some advances in understanding the nature of stresses and their interactions is made, the biochemical/ physiological reactions/ processes associated with tolerance to abiotic stresses are yet to be precisely identified (Khanna Chopra & Sinha 1998), and

(iii) there is a dearth of information on genes which would have a positive effect in imparting tolerance to abiotic stresses (Grover et al. 1993, 1998a, 1999).

Moreover, it is often a difficult proposition to analyze the response of plants to different abiotic stresses in the experimental conditions, due to complex and variable nature of these stresses. However, in spite of all these complexities, remarkable success has been achieved in raising of transgenics for improved abiotic stress tolerance in the past six years of research work (1993-1999). Our group has discussed various issues related to the production of stress-tolerant transgenics in a number of recent publications (Grover et al. 1993, 1998a,b, 1999, Grover 1999, Katiyar-Agarwal et al. 1999, Minhas & Grover 1999, Mohanty et al. 2000). In these papers, details on genes encoding several structural proteins [i.e. glycerol 3-phosphate acyl transferase, mannitol 1-phosphate dehydrogenase, superoxide dismutase, betaine aldehyde dehydrogenase, choline dehydrogenase, o^1-pyrolline 5-carboxylate synthase (p-5-cs), levan sucrase, late embryogenesis abundant (LEA) protein, trehalose 6-phosphate synthase, choline oxidase, antifreeze protein and myo-inositol-o-methyl transferase] and regulatory proteins [i.e. C-repeat (CRT)/drought-responsive element (DRE) binding factor 1 or CRT/DRE binding factor1 (CBF1), DRE binding protein (DREB1A) and calcineurin] which have been shown to bring about superior abiotic stress tolerance in transgenic hosts as well as the associated limitations are given (also see Khanna-Chopra & Sinha 1998, Dhaliwal et al. 1998, Kasuga et al. 1999).

So far, most of the research work on production of abiotic stress tolerant crops has been carried out employing tobacco and *Arabidopsis thaliana*. It is, therefore, in place to ask how far are we from genetically producing crops with superior abiotic stress tolerance. However, it has been a general experience of plant biotechnologists that different plant species have varied requirements for the genetic transformation work. The regulatory sequences needed to bring about optimal expression of the given transgene varies between dicotylednous and monocotylednous species. The tissue culture responsiveness of the trans-host is another important parameter which governs the success in this respect (Bhaskaran & Smith 1990, Birch

1997, Hansen & Wright 1999). A large number of studies have shown that different rice cultivars exhibit different responses to tissue culture, regeneration and genetic transformation (Kyozuka et al. 1988, Peterson & Smith 1991, Rance et al. 1994, Datta et al. 1996). In this article, we review the existing information on genetic transformation of rice, discuss in detail the utility of *Agrobacterium tumefaciens* mediated genetic transformation approach for rice transformation and finally take a stock of efforts currently being made in the production of abiotic stress tolerant rice crop (see the recent paper by Tyagi et al. 1999, Hiei et al. 1999, Kloti & Potrykus 1999 for further details on crop improvement and genetic research on transgenic rice). The main objective in writing this article is to highlight the points of strength of rice transgenic technology and project the research gaps which need to be plugged in future years for raising transgenic rice tolerant to abiotic stresses.

Genetic Transformation of Rice

Following the successful demonstration in the early to mid seventies that chimeric DNA molecules can be constructed *in vitro* and in late seventies that T-DNA of *A. tumefaciens* can be manipulated to carry "foreign" genes into the plant cells, a great deal of momentum was generated in the early eighties to genetically transform different plants, mostly with antibiotic resistance or with reporter genes (Galun & Brieman 1997). Since the monocotyledonous plants were proven largely unresponsive to *Agrobacterium* in the early studies (Raineri et al. 1990, Chan et al. 1993, Vijaychandra et al. 1995, Park et al. 1996), a range of different methods of transforming rice cells with foreign DNA have been attempted. The procedural details on the different methods of plant transformation are summarized in a recent paper by Hansen & Wright (1999). The techniques of polyethylene glycol (PEG)- and electroporation-mediated DNA uptake into the protoplasts for rice transformation proved successful to a reasonable degree (table 1). However, the problems associated with handling of isolated protoplasts as well as lack of regenerability from isolated protoplasts in several cases deterred the large-scale exploitation of these methods (Lee et al. 1989, Lee et al. 1991, Park et al. 1996). These problems were circumvented to an extent with the introduction of microprojectile bombardment or biolistic method that was employed to deliver DNA directly into plant cells by shooting through the plant cell walls and cell membranes (Sanford et al. 1987). Several different versions of the microprojectile gun apparatus have been developed in the subsequent years, to make this technique less expensive and more effective (Christou 1997, Arencibia et al. 1998). Rice transformation work received a big impetus with the optimization of microprojectile gun mediated method (table 1). In fact, a large number of rice workers shifted their transformation approach from electroporation/ PEG to gun-based method in the early nineties. However, a major breakthrough was made at around the same time when it was shown that *A. tumefaciens* can be exploited for the genetic transformation of rice cells. Raineri et al. (1990) achieved *Agrobacterium*-mediated transformation of rice cultivar "Nipponbare" and showed the successful T-DNA transfer by DNA hybridization analysis. Hiei et al. (1994) showed that with suitable modifications in the procedure, it is possible to employ *Agrobacterium* for genetic transformation of rice at high frequency. Since then, *A. tumefaciens*- based method has become a method of choice with rice biotechnologists (table 1). Some other methods of genetic transformation of rice attempted so far include pollen tube-mediated method (Luo & Wu 1989), lipofectin-mediated method (Zhu et al. 1994) and laser-mediated method (Guo et al. 1995). However, the use of these methods has been to a limited significance only.

As of now, genetic transformation has been achieved in a score of different rice cultivars employing the above-mentioned methods (table 1). The following generalizations can be made from the work presented in table 1:

(i) Selection of transformed cells in rice can be exercised with several genes including

Table 1 *Selective reports on genetic transformation studies in rice*

	Variety/ cultivar	Explant	Configuration of the plasmid construct	Reference
Using electroporation-method				
Transient	Tainung 67 (japonica)	protoplasts	p35S: *cat*, pCopia*: *cat*	Ou-Lee et al. 1986
	————	protoplasts	p2'*: *nptII*, p1'*: *dhfr**, p35S: *bar*, pNos: *nptII*	Dekeyser et al. 1989
	Taipei 309 (japonica)	leaf bases	p2'*: *nptII*	Dekeyser et al. 1990
	Nipponbare (japonica)	suspension cells	pLhcp*: *gus*, p35S: *hpt*	Tada et al. 1991
	Basmati 370, improved Sabarmati (indicas)	cell suspensions	p35S: *gus*	Chaudhry et al. 1994
	Basmati 370 (indica)	mature embryo	p35S: *gus*	Chaudhry et al. 1995
	Radon (japonica), IR54 (indica)	immature embryos, suspension cells	p35S:*bar*::pAct:*gus*	Rao 1995
Stable	Yamahoushi (japonica)	protoplasts	p35S: a*phT**	Toriyama et al. 1988
	Taipei 309 (japonica)	protoplasts	p35S: *npt II*	Yang et al. 1988
	Taipei 309 (japonica)	protoplasts	p35S: *npt II*	Zhang et al. 1988
	Yamahoushi (japonica)	suspension cells	p35S: *hpt*, pO12*: *gus*	Matsuki et al. 1989
	Nipponbare (japonica)	protoplasts	p35S: *hpt*	Shimamoto et al. 1989
	Taipei 309 (japonica)	protoplasts	p35S: *nptII* :: p35S: *gus*	Battraw & Hall 1990
	Norin-8 (japonica)	protoplasts	p35S: *gus*, p35S: *hpt*	Tada et al. 1990
	Nipponbare (japonica)	protoplasts	p35S: *gus*	Terada & Shimamoto 1990
	Taipei 309 (japonica)	protoplasts	p35S: *npt II*	Davey et al. 1991
	Nipponbare (japonica)	protoplasts	pAdh1*: *adh*1: *gus*	Kyozuka et al. 1991
	Taipei 309 (japonica)	protoplasts	p35S: *npt II*:: p35S: *uidA*	Battraw & Hall 1992
	Taipei 309 (japonica)	protoplasts	p35S: *npt II*	Lynch et al. 1992
	Yamahoushi (japonica)	protoplasts	pUbi: *exon1*: *intron1*: *bar*	Toki et al. 1992
	Nipponbare (japonica)	protoplasts	pRbcS*: *gus*, pRbcS*: *gus*	Kyozuka et al. 1993
	IR36 (indica)	mature embryo	pNos: *npt II*	Xu & Li 1994

(Contd.)

Table 1 *(contd.)*

	Variety/ cultivar	Explant	Configuration of the plasmid construct	Reference
Using PEG-mediated DNA uptake method				
Transient	Taipei 309 (japonica)	protoplasts	pUbi: *luc*	Cornejo et al. 1993
	M202	protoplasts	p35S: *adh*1*: *luc,* pAdh1:*adh*1: *luc*	Sadasivam & Gallie 1994
Stable	L.c 5924	protoplasts	pNos: *aphII**	Uchimiya et al. 1986
	Taipei 309 (japonica)	protoplasts	p35S: *nptII*	Yang et al. 1988
	Pi-4, Taipei309 (japonicas)	protoplasts	pAdh: a*dh gus*	Zhang & Wu 1988
	Chinsurah Boro II (indica)	protoplasts	p35S: *hpt*	Datta et al. 1990
	Nipponbare, Taipei 309 (japonicas)	protoplasts	pNos: *hpt,* p35S: *hpt*	Hayashimoto et al. 1990
	IR54 (indica)	protoplasts	p35S: *nptII,* p35S: *gus*	Peng et al. 1990
	Fang7, H124 (japonicas)	suspension cultures	p35S: *gus*:: p35S: *npt II*	Lee et al. 1991
	Taipei309 (japonica)	protoplasts	p35S: *dhfr**:: pNos: *nos,* p35S: hpt:: p2'*: *gus*	Meijer et al. 1991
	Nipponbare (japonica)	protoplasts	p35S: *hpt*	Zheng et al. 1991
	IR72 (indica)	protoplasts	p35S: *hpt,* p35S: *bar*	Datta et al. 1992
	Nipponbare (japonica)	protoplasts	p35S: *csr1-1**	Li et al. 1992
	IR54 (indica)	protoplasts	p35S: *nptII,* p35S: *gus*	Peng et al. 1992
	Taipei 309 (japonica)	protoplasts	pUbi: *gus,* pUbi: *luc,* pUbi: *bar*	Cornejo et al. 1993
	Radon, Nortai (japonicas)	protoplasts	p35S: *bar,* pAct1: *gus*	Rathore et al. 1993
	IR 54 (indica)	protoplasts	p35S: *nptII* p35S: *gus*	Peng et al. 1995
	Miara (japonica)	protoplasts	pUbi: *exon1*: *intron1*: *uid*A, p35S: *hpt,* p35S: *nptII,* p35S: *bar,* pLtp*: *uidA*	Chair et al. 1996
	Chinsurah Boro II, IR 72, IR 51500 (indicas)	protoplasts	p35S: *tlp**	Datta et al. 1999

(Contd.)

Table 1 *(contd.)*

	Variety/ cultivar	Explant	Configuration of the plasmid construct	Reference
Using particle gun-mediated transformation method				
Transient	Taipei 309	suspensions cells	pAdh1: *adh*1*: *gus*, p35S: *adh*1: *cat*	Wang et al. 1988
	cv japonica	scutellar callus	pAdh: *adh*1: *gus*, p35S: *adh*6: *gus*	Oard et al. 1990
Stable	M101	seedlings	pPhy*: *cat*, pUbi: *luc*	Bruce et al. 1989
	Gulfmont (javanica), IR 54, IR 72, IR 26, IR 36 (indicas)	immature embryos	p35S: *gus*:: p35S: *hpt*	Christou et al. 1991
	Taipei 309 (japonica)	suspension cells	pAct1: *exon*1: *intron*1: *bar*, p35S: *bar*	Cao et al. 1992
	Taipei 309, 77125 (japonicas) Tetep, TN1 (indica) 8706 (ind x jap)	immature embryos, embryogenic calli	pAct1: *gus*, pNos: *hph*, p35S: *hph*	Li et al. 1993
	Taipei 309 (japonica)	protoplasts	pPin2: *gus*, pPin2: *act*1: *gus*, p35S: *bar*	Xu et al. 1993
	IR 72, IR 54 (indica) Koshihikari, Gulfmont (javanica)	immature embryos	p35S: *hpt*:: p35S: *gus*, p35S: *hpt*:: pGt3*: *gus* p35S: *hpt*:: pSSU*: *gus*, p35S: *hpt*:: pSSU*: *gus*, p35S: *hpt*:: pSSU*: *gus*, p35S: *bar*:: p35S: *hpt*:: p35S: *gus*	Cooley et al. 1995
	Gulfmont (javanica)	immature embryos	p35S: *phy*A	Clough et al. 1995
	Pusa	suspension	pAct1: *gus*,	Jain et al. 1996
	Basmati 1 (indica)	cells	pAct 1: *hva**:: p35S: *bar*, pPin2*: *act. pin*2:: p35S: *bar*	
	Gulfmont. (javanica) IR 72 (indica) Koshihikari	immature embryos	p35S: *adh*1: *bar*:: p35S: *aph*IV*:: p35S: amv*: *uid*	Oard et al. 1996
	TN1 (indica)	calli	pUbi: *ubi: hpt*, pUbi: *gus*	Sivamani et al. 1996
	IR24 IR64, IR72, IR57311-95-2-3	suspension cells	p35S: *uid*A:: p35S:*hpt*	Zhang et al. 1996

(Contd.)

Table 1 *(contd.)*

Variety/ cultivar	Explant	Configuration of the plasmid construct	Reference
Taipei 309 (japonica)	seed embryos	AMV*enh**: p35S: *btcry*IIIA, pUbi: *bar*	Kumpatla et al. 1997
Zhonghua8 Zhonghua10 (japonicas)	callus	p35S: *nptII* :: p35S: *rdvs8**	Zheng et al. 1997
Vaidehi (indica)	scutellar callus	p35S: *cry*1A(b): *pepc**, p35S: *hpt*	Alam et al. 1998
———	immature embryos	p35S: *gus* :: p35S: *hpt*, p35S: *adc**	Capell et al. 1998
Taipei 309	embryogenic callus, suspension cells	14 different constructs carrying *hpt, uid*A, *bar, luc, gfp* and coding sequences of RTSV*, RYMY*, RTBV*	Chen et al. .1998
IR 72 IR 64 CBII IR 51500 IR 68899B Vaidehi (indicas) IRRI hybrid Taipei 309 (japonica)	immature embryos, embryogenic callus	p35S: *cry*1A(b):*pepc**, pPepc: *cry*1A(b): *pepc**, pPith: *cry*1A(b): *pepc**, pAct1: i*ntron. cry*1A(b) p35S: *hpt*	Datta et al. 1998
Basmati 370 M7 (indicas)	scutellar callus	p35S: *gus*: p35S: *hpt*, *cry2A* in pROB	Maqbool et al. 1998
ASD 16 M5 M12 (indicas)	immature embryos	pRss1: *gna**, pUbi: *gna**, p35S: *gus* :: p35S: *hpt*	Rao et al. 1998
Kenfong	suspensions cells	(ABRC-ACT1-100IP-HVA22)*: *uid*A:: p35S: *bar*, (4ABRC1-ACT1-100P-HVA22)*: uidA:: p35S: bar	Su et al. 1998
ASD 16 M5 M12 (indicas) FX92 (japonica)	immature embryos	pRss1*: *gna**, pUbi: *ubi. gna*	Sudhakar et al. 1998
IR 72 (indica)	immature embryos	p35S: *hpt*, *xa21* in pC822	Tu et al. 1998
ITA212 IDSA6 LAC23 WAB56-104	immature embryos	p35S: AMV*: *ocs*δ*dd*86*:: p35S: *aphIV*, pUbi: *gus*	Vain et al. 1998
Notohikari (japonica)	scutellar tissue	pAct1: *bar*, p35S: ntfad3*	Wakita et al. 1998

(Contd.)

Table 1 *(contd.)*

	Variety / cultivar	Explant	Configuration of the plasmid construct	Reference
	IR 64 IR 72 Minghui 63 BG90-2 (indicas)	suspensions cells	p35S: *hpt*, *xa21*in pC 822	Zhang et al. 1998
	Chinsurah Boro II, IR72, IR 51500 (indicas)	immature embryos	p35S: *tlp**	Datta et al. 1999
Using Agrobacterium-mediated transformation method				
Transient	8 indicas, 7 japonicas, 6 African rices	leaf, root seed	p35S: *gus/intron**, pMas/35S*: *gus*, pMas/35S*: *gus/intron*	Li et al. 1992
	IR 64 (indica), Lemont	shoot segments	p35S: *gus*: nos* p35S: *gus*: ocs* p35S: *gus/intron*: ocs*	Liu et al. 1992
Stable	Nipponbare, Fujisaka 5 (japonicas)	mature embryos	pMas/35S: *gus*	Raineri et al. 1990
	Tainung 62 (japonica)	immature embryos	pNos: *nptII*: paaAmy8*: *uid*A	Chan et al. 1993
	Tsukinohikari, Asanohikari, Koshihikari (japonicas)	shoot segments, root segments, root derived calli, scutella derived calli, cell suspensions, immature embryos	pNos: *nptII*: p35S: *intron: gus*: p35S: *hpt*	Hiei et al. 1994
	Co43 (indica)	coleoptile, scutellum, scutellar callus leaf blade, leaf base, root	virE-lacZ* virB-lacZ* extra copies of virG,C,D,E*	Vijayachandra et al. 1995
	Nortai, Radon (japonicas)	immature embryos	pMas/35S: *gus intron*, pNos: *nptII*: p35S: *intron: gus*: p35S: *hpt*	Aldemita & Hodges 1996
	Taipei309 (japonica), Gulfmont, Jefferson, (javanica)	scutellar calli	pNos: *nptII*: p35S: *intron: gus*: p35S: *hpt*	Dong et al. 1996
	Maybelle (japonica)	shoot meristems	p35S: *bar* pAct1: *act1*: bar:: pNos: *nptII*	Park et al. 1996

(Contd.)

Table 1 *(contd.)*

Variety/ cultivar	Explant	Configuration of the plasmid construct	Reference
B370 (indica)	scutellar calli	pNos: *nptII*:: p35S: *intron: gus*:: p35S: *hpt*	Rashid et al. 1996
Binnatoa (indica)	scutellar calli	pNos: *nptII*:: p35S: *intron: gus*:: p35S: *hpt*	Rasul et al. 1997
Nipponbare, Kitaake (japonicas)	seed callus	pNos: *nptII*:: p35S: *intron: gus*:: p35S: *hpt* pNos: h*pt*:: pUbi: *bar*	Toki 1997
Taipei 309 (japonica)	scutellar callus	p35S: *hpt*: nos*, p35S: *hpt*: tm1'*, p35S: *hpt-cat*-hpt*, p35S: *hpt-haem*-hpt* pUbi: *ubi*: *hpt*	Wang et al. 1997
Nipponbare (japonica)	scutellar callus	pNos: *nptII*:: p35S: s*odCc2**, *cod*A:: p35S: *hpt* pNos: *npt*II:: p35S: s*odCc2**: TP: *cod*A:: p35S: *hpt*	Sakamoto et al. 1998
Yamahoushi (japonica)		Km^r*: pUbi: gpat*: Hyg^r	Yokoi et al. 1998
Kitaake, Nipponbare (japonicas)	seed callus	pNos: *npt*II: *pepc*:: p35S: *hpt*	Ku et al. 1999

* pCopia encodes promoter of *copia* long terminal repeat from *Drosophila*; p1' and p2' encode promoters of transcripts of octopine T-DNA; *dhfr* codes for methotrexate resistance; pLhcp encodes promoter of rice light harvesting chlorophyll *a/b* binding protein of photosystem II; *aph* codes for geneticin resistance; pO12 encodes promoter of ORF 12 gene (*rolC*) of Ri plasmid; pAdh1 encodes promoter of maize alcohol dehydrogenase; pRbcS codes for small subunit of ribulose bisphosphate carboxylase, two constructs were employed — one had a promoter from rice and the other from tomato; *adh*1 gene encodes alcohol dehydrogenase protein; *aph*II gene encodes for kanamycin resistance; p2' encodes 1',2' mannopine synthase locus of *Agrobacterium*; *csr* 1-1 gene represents a mutant acetolactate synthase gene for herbicide resistance; pLtp represents rice lipid transfer protein gene promoter; *tlp* gene for thaumatin-like protein is responsible for enhanced resistance to *Rhizoctonia solani* (sheath blight pathogen); *adh* gene encodes alcohol dehydrogenase protein; *phy* gene encodes for oat phytochrome protein; pGt3 denotes rice glutenin promoter; SSU denotes small sub-unit of RUBISCO protein; *pin*2 gene codes for potato protease inhibitor 2 protein; *aph*IV gene codes for hygromycin resistance; amv stands for alfalfa mosaic virus leader sequence; enh denotes enhancer element; *rdv*s8 is the eighth largest segment of the rice dwarf virus; *ocs*δ*dd*86 stands for mutated *Oryza cystatin* gene; pRss1 represents promoter from rice sucrose synthase gene; *gna* codes for snowdrop lectin from *Galanthus nivalis* agglutenin against rice brown plant hopper; *adc* encodes arginine decarboxylase protein; (ABRC-Act1-100IP/100P-HVA22) represents ABA-responsive promoter complex consisting of G-box from barley (*Hordeum vulgare*) HVA22 gene; *pepc* represents the PEP Carboxylase intron; *xa21* codes for light-resistance; pPith represents tissue specific promoter from green tissue; *gfp* stands for green fluorescent protein; RTSV, RYMY, RTBV stand for rice tungro spherical, rice yellow mottle and rice tungro bacilliform viruses respectively; *ntfad*3 codes for ω-3 fatty acid desaturase gene from tobacco; *tlp* encodes thaumatin-like protein responsible for enhanced resistance to *Rhizoctonia solani* (sheath blight pathogen); Mas/35S represents chimeric promoter of mannopine and a truncated CaMV 35S; ocs encodes octopine synthase terminator; *gus/intron* gus gene stands for *gus* gene containing plant intron; αAmy8 represents 5' upstream fragment of α *amylase* gene;virE-lacZ, virb-lacZ and Extra copies of vir G,C,D,E were present in constructs to study induction of vir genes; nos' is the terminator sequence of *Agrobacterium tumefaciens* nopaline synthase; tm1'is the transcription termination sequence of an *Agrobacterium tumefaciens* T-DNA borne tumour morphology large gene; *cat* represents castor bean catalase-1 gene intron; *haem* represents a *Parasponia andersonii* haemoglobin gene intron; *sodCc2* represents the first intron of rice superoxide dismutase gene; *cod*A encodes choline oxidase gene that converts choline to glycine betaine for osmoprotection; TP stands for the transit peptide sequence of small subunit of ribulose bisphosphate carboxylase; Km^r and Hyg^r represent genes for kanamycin and hygromycin resistance, respectively; *gpat* codes for glycerol-3-phosphate acyltransferase gene from *Arabidopsis* that confers unsaturation of fatty acids and chilling tolerance; *pepc* stands for the maize C_4-phosphoenol pyruvate carboxylase gene with all its exons, introns promoter and terminator.

those providing resistance against kanamycin, hygromycin, bialophos, glufosinate, phosphinothricin etc.

(ii) Reporter genes tested and proved useful for rice transformation work include β-glucuronidase, chloramphenicol acetyl transferase, luciferase etc.

(iii) Transgene expression in rice transformation work has been tested using a range of different promoters including cauliflower mosaic virus 35S promoter (CaMV 35S), alcohol dehydrogenase1 promoter, actin1 promoter, *copia* long terminal repeat promoter of *Drosophila*, rice light harvesting chlorophyll a/b binding protein of photosystem II (LHCP II) promoter, nopaline synthase promoter and ubiquitin1 promoter. While the CaMV 35S promoter has been extensively employed in rice transformation work (table 1), recent studies have shown that this promoter has a recombination hotspot which can lead to illegitimate recombination (Kohli et al. 1999). The actin1 and ubiquitin1 promoters have in particularly proven useful for the high-level constitutive expression of the transgene in rice.

As mentioned above, the optimization of *A. tumefaciens* based approach for genetic transformation of rice in early to mid nineties is regarded as a turning point in rice biotechnology research. This technique is now greatly favoured over the other methods described in table 1 since it gives a high frequency of transformation, number of copies of trans-gene integrated into the genome are fewer with this method and this procedure is most cost-effective (Sonti & Sarma 1995, Rashid et al. 1996, Wang et al. 1997, Datta et al. 1999). A general protocol to achieve rice transformation through this approach involves the following steps:

(i) raising of embryogenic calli from mature seed embryos,

(ii) co-cultivation of embryogenic calli with the competent *A. tumefaciens* cells harbouring the gene of interest,

(iii) selection of the transformed calli from the non-transformed calli by repeated subcultures on hygromycin (which is often used as a selection agent) containing media,

(iv) regeneration of shoots from the putatively-transformed calli and induction of rooting,

(v) transfer of tissue culture-grown seedlings from test tubes to pots through proper hardening treatments,

(vi) optimization of conditions for raising the transformed plants to maturity up to seed setting and

(vii) analysis of putatively transformed plants by polymerase chain reaction (for checking integration of the "foreign" gene) and by Southern blotting (for checking the integration as well as for finding copy number of the "foreign" gene in transformed tissues).

Selected steps involved in this procedure are shown in figure 2 (legend for more details on protocols). The seed to seed cycle in this exercise takes 6-7 months in usual practice (figure.3 for details on the durations of the different steps involved in this procedure in one of the experiments conducted in the laboratory of the authors on Taipei 309 rice cultivar transformation) which indicates that it is possible to obtain T_2 seedlings for genetic analysis within a period of nearly one year. The above arguments hold true for both japonica and indica rice cultivars and the time frame is comparable in both the cases.

Transgenic Rice for Abiotic Stresses

The noteworthy progress made in rice molecular biology and genetic engineering on production of several different kinds of transgenic rice plants as evidenced from figure 1 and table 1, points out that the stage is all set for the production of transgenic rice plants. The present scenario of research on production of abiotic stress tolerant rice can be summed up in the following two directions:

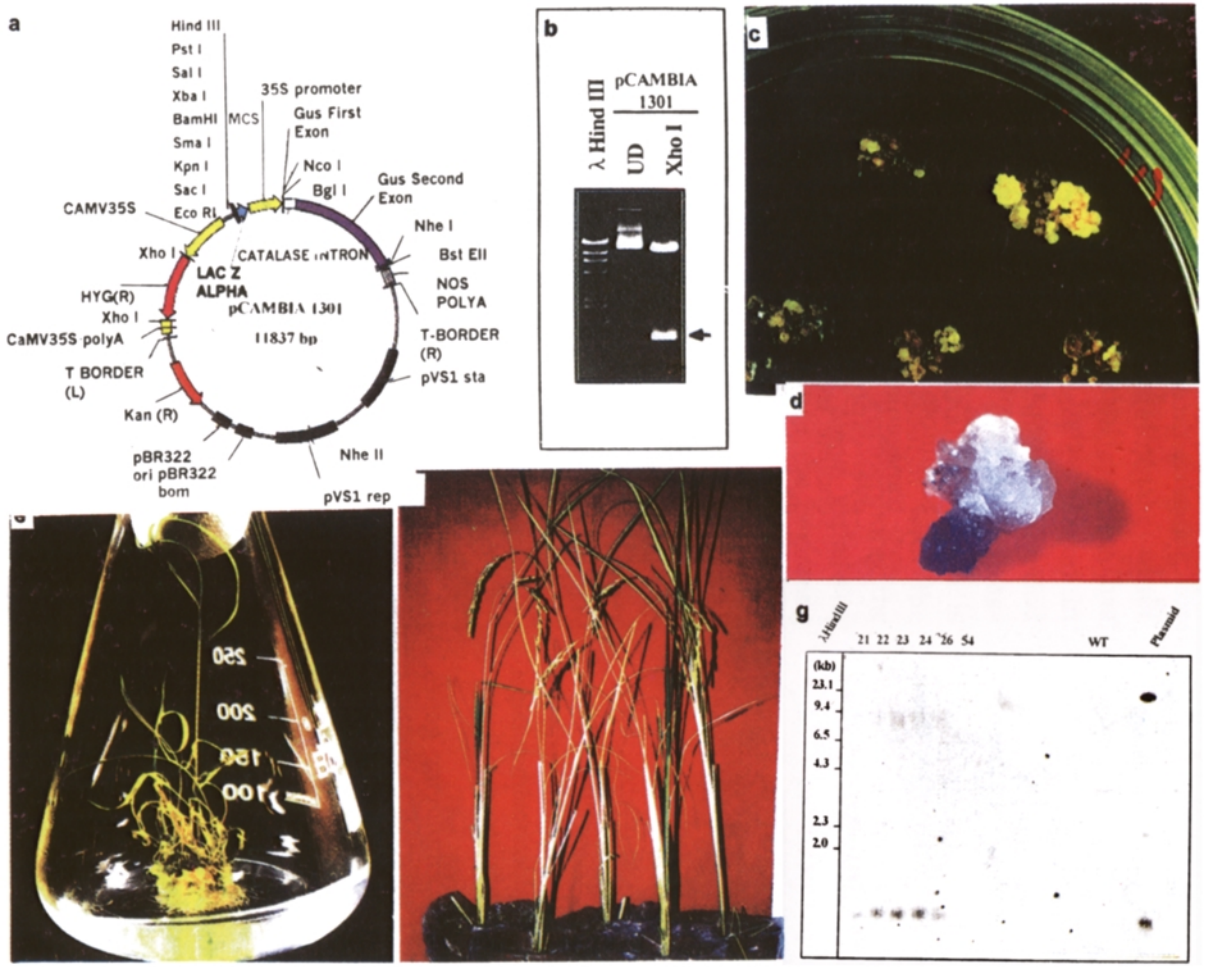

Figure 2: *Agrobacterium*- mediated genetic transformation of Taipei 309 rice cultivar with *gus*/ *hyg* genes. a. Restriction map of the binary vector pCAMBIA 1301 which was used for *Agrobacterium*- mediated transformation of scutellar callus of Taipei 309 cultivar of rice. It has *gus* as the reporter gene and resistance to hygromycin as the selectable marker. b. Restriction analysis of the plasmid pCAMBIA 1301 with Xho I. This construct has two Xho I sites flanking the hygromycin resistance gene. pCAMBIA 1301 was cut with Xho I to obtain the requisite-sized insert (shown by arrow). UD: undigested plasmid. c. Taipei 309 calli on hygromycin selection (30 mg L^{-1}) following co-cultivation. Note proliferation of hygromycin resistant calli. d. Histochemical staining of GUS in hygromycin- resistant calli of Taipei 309. e. Hygromycin-resistant shootlets of Taipei 309 rice cultivar on rooting medium. f. Primary transformants (T_0) at the time of harvesting. g. Southern analysis of 6 primary (T_0) putative transformants (lines 21-24, 26, 54) plants and an untransformed plant (WT) of Taipei 309. Transformation was carried out with the binary vector pCAMBIA 1301 which has the *gus* and hygromycin resistance genes. 5 mmg of genomic DNA was digested with Xho I, separated by electrophoresis, blotted onto nitrocellulose membrane and hybridized with radioactively- labelled *hyg* coding region (Xho I fragment of pCAMBIA 1301). The plasmid pCAMBIA 1301 digested with Xho I was loaded as a positive control (extreme right). The cross-reacting band in the transgenics is shown by arrow. Molecular markers are indicated on the left.

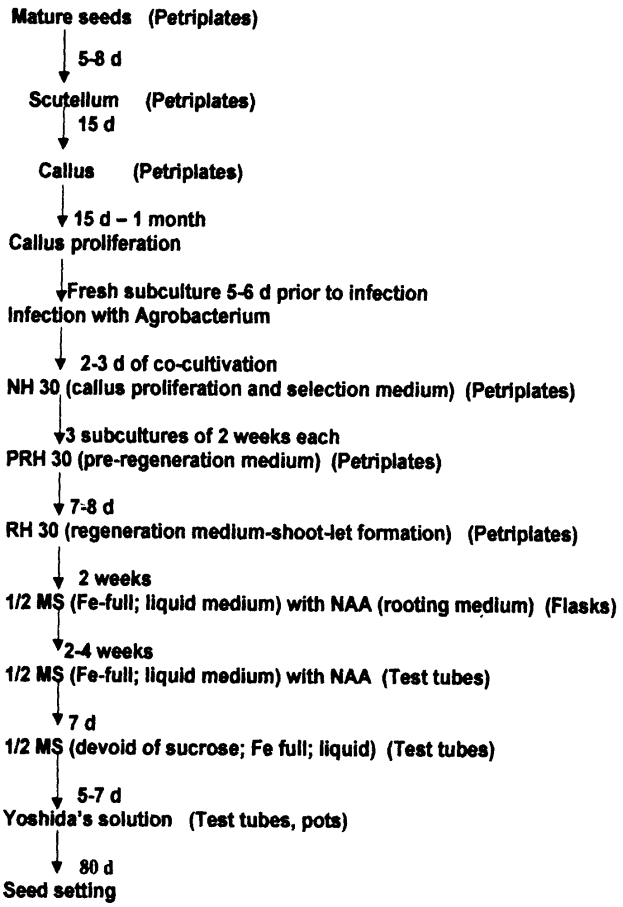

Figure 3 Summary sheet showing different stages of raising genetically transformed rice plants (cultivar Taipei 309). The approximate duration of each stage is indicated.

(a) raising rice transgenics employing genes which have already been characterized and shown to work in other systems (such as tobacco and *Arabidopsis*) and

(b) isolation and characterization of novel genes from rice for augmenting the present level of abiotic stress tolerance (in rice but this may hold true for other crops also once it is proven for rice).

The critical inputs for pursuing the first direction as mentioned above involve subcloning the requisite gene(s) in vectors which work optimally with rice transformation, analysis of the progenies for appropriate expression levels and finally, testing the transformed plants for stress tolerance at biochemical/ physiological levels. This work appears straight-forward horizontal expansion of scientific pursuits with clear technological gains. The following results have been obtained using this approach so far:

(i) Xu et al. (1996b) have raised transgenic rice plants (cultivar "Nipponbare") which over-express *hva1* gene (encoding LEA protein). R_1 generation transgenic rice plants showed significantly increased tolerance to water deficit and salinity. Transgenic rice plants maintained higher growth rates than non-transformed control plants under stress conditions. The increased tolerance was also reflected by delayed development of damage symptoms caused by stress and by improved recovery upon the removal of stress conditions.

(ii) Sakamoto et al. (1998) have produced transgenic rice (cultivar "Nipponbare") over-expressing *codA* gene (encoding choline oxidase), in chloroplasts as well as in cytoplasm. Treatment with 0.15 M NaCl inhibited the growth of both wild type and transgenic plants in this work. However, the transgenic plants began to grow again at the normal rate after a significantly less time than the wild type plants after cessation of the salt treatment. Inactivation of photosynthesis, used as a measure of cellular damage, indicated that chloroplast-COD plants were more tolerant than cytoplasm-COD plants to photoinhibition under salt stress and low temperature stress.

(iii) Cheng & Wu (1998) have produced transgenic rice plants (cultivar "Kenfeng") over-expressing *cor47* (encoding cold-regulated protein of 47 kDa) gene. To analyze the response of *cor47*- transgenic plants to drought stress, water was withheld from the trays for 5 days. The stressed plants were then supplied with water for 2 days to allow the plants to recover. The preliminary data from this experiment showed that R_1 generation *cor47*- transgenic plants are more tolerant to water deficiency than control plants.

(iv) Zhu et al. (1998) have raised transgenic rice plants over-expressing o^1-pyrolline

5-carboxylate synthase (p-5-cs) gene. In this case also, the assessment of the stress response of the transgenic and control plants was made on growth basis The 2nd generation R1 transgenic plants showed increased biomass than the control plants in this experiment.

It is to be noted here that transgenic plants resulting from the above experiments have not so far been subjected to vigorous testing for parameters which actually define the water relations of the cells. Rather, the approach has been simply to test for stress protection in terms of growth etc. during the stress period or during recovery phase following stress treatment. It is important that this issue is taken in view in future research with the available transgenic plants. Furthermore, the production of transgenic rice plants expressing other genes (those mentioned in the beginning of this article) must hopefully be now a matter of time.

The second direction involving isolation, cloning and characterization of novel abiotic stress tolerant genes represents a virgin area of research. The opportunities in this venture are enormous as only a handful of genes with a clear role in abiotic stress tolerance have been analyzed so far (Grover et al. 1999). In recent years, it has been shown that regulatory genes governing synthesis of trans-acting factor proteins and stress- signalling molecules may prove extremely useful in bringing about tolerance to abiotic stresses (Grover et al. 1999). The methods for isolation and cloning of such genes (through differential screening of gene libraries, differential display of cDNAs, analysis of expressed sequence tags, genome sequencing project) and unveiling functionality of such clones through different methods (such as through transgenic approach) have been optimized for rice to a great deal. The detailed molecular analysis undertaken on rice plant may facilitate the work on isolation of novel genes from this plant species more than from other crops.

Directions for Future Research

In order to achieve the goal of producing high-level abiotic stress tolerant rice plants, production of abiotic stress tolerant rice must be taken as a programmed activity in our view. This venture must involve efforts from breeding, molecular biology, tissue culture and physiology and biochemistry disciplines. The action plan of this programme needs to address to the following points:

(i) The molecular biology activity in such a programme should provide inputs in making suitable plasmid constructs with the known genes. The exercise of vector designing must be based on the information available on regulatory sequences available through different sources (McElroy et al. 1995, Su et al. 1998, Vain et al. 1999). The selection of promoter sequence for driving expression of the transgene is a critical input (Chen et al. 1998). It has recently been shown that the most suitable strategy to express stress- related transgenes is through driving its expression by a promoter which is stress-responsive, rather than depending on constitutively-expressed promoters (Grover et al. 1999, Kasuga et al. 1999).

The pyramiding of selective genes by devising suitable cloning strategies and vector systems is another important area of research in this context (Grover et al. 1999). The selection of single genes to be employed for pyramiding must be based on inputs from physiological/ biochemical components of the programme.

Intensive basic research aiming at identification, isolation and cloning of newer stress-responsive genes is the need of the hour in work on stress molecular biology. This task involves establishment of routine protocols for construction of gene libraries, screening methods, differential display of mRNAs, and analyses of stress proteins. The newly-identified stress- induced gene clones

would need to be characterized with respect to both their structural and functional properties. The functional properties are best characterized by banking on reverse genetics approach or through complementation of mutants, if available. The specialized groups with experience in microbiological techniques are needed for the complementation work.

(ii) The component of tissue culture laboratory in this programme should involve in optimizing rice transformation procedures on turn-key basis. The choice of the rice cultivar to be transformed so far has been driven by the availability of the method for transformation. This approach relies on the fact that once the transgene has been put in rice, it should be possible to horizontally transfer it to the destined rice cultivar by backcrossing. The approach has another angle also. It is possible that the gene introduced in "transformation-friendly" rice cultivar may not show expression and physiological effects comparable to the one which is the destined cultivar grown locally in a particular stress- prone ecosystem but is relatively recalcitrant to transformation procedures. It is therefore important that the gene to be introduced must get the proper host. The cultivars which need to be the recipients of the trans-genes in different ecosystems must be identified with appropriate help from physiologists and biochemists.

(iii) The physiology/ biochemistry component in this activity should involve in sharing the responsibility of providing/ and testing various reactions/ processes for their role in stress tolerance using suitable plant material provided by breeders. This group should also develop methodologies for checking stress tolerance with appropriate tissue amounts, in a large- scale field experiment. It is possible that this activity might need specific tanks/ chambers for subjecting plant to stress in a field experiment. This is perhaps the most basic requirement for the work in consideration.

Apart from these, development of proper infrastructure for work on rice transgenics is an important input. The rice plantlets regenerated in tissue culture must be taken to field-conditions as soon as they grow up to the requisite growth stage in the growth chamber/ room. Since rice is a seasonal crop with specific temperature, humidity and photoperiodic requirements, the chances of pre-mature death of the seedlings in culture conditions has proven a deterrant in many experiments on this theme. There is a need that proper infra-structure is developed for raising rice transgenics which may allow production of fertile transgenic rice plants round the year. The future attempts must also address to the inheritence of the transgenes in succeeding generations as well as well to the issues concerning stability or the silencing of the introduced transgenes (Grover et al. 1999).

Finally, it is important to appreciate that research on abiotic stresses has so far been supported through public sector governmental and other donor agencies. This was needed because this area of research has always looked like an open- ended endeavor. With the current developments in view, the private seed industries would find themselves in a good position to reap the golden harvests through production of stress tolerant crops. The additional support for R & D in this venture from private agencies may make goal of achieving higher grain yield in rice very much a reality in near future.

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