GENETICALLY ENGINEERED BASMATI RICE FOR RESISTANCE AGAINST BACTERIAL BLIGHT

A THESIS SUBMITTED TO THE UNIVERSITY OF THE PUNJAB IN FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

By

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Zahid Mukhtar
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<th>Definition</th>
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<tr>
<td>°C</td>
<td>degree centigrade</td>
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<tr>
<td>µg</td>
<td>microgram</td>
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<tr>
<td>µm</td>
<td>micrometer</td>
</tr>
<tr>
<td>Ω</td>
<td>ohm</td>
</tr>
<tr>
<td>2,4-D</td>
<td>2,4-dichloro-phenoxy acetic acid</td>
</tr>
<tr>
<td>atm</td>
<td>atmosphere</td>
</tr>
<tr>
<td>BAP</td>
<td>benzyl amino purine</td>
</tr>
<tr>
<td>CIAP</td>
<td>calf intestinal alkaline phosphatase</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNTPs</td>
<td>2'-deoxynucleoside 5'-triphosphates</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>GUS</td>
<td>β-glucuronidase</td>
</tr>
<tr>
<td>gusA</td>
<td>β-glucuronidase gene</td>
</tr>
<tr>
<td>ha</td>
<td>hectare</td>
</tr>
<tr>
<td>Hg</td>
<td><em>Hydrargyrum</em> (mercury)</td>
</tr>
<tr>
<td>hpt</td>
<td>hygromycin phosphotransferase gene</td>
</tr>
<tr>
<td>IAA</td>
<td>indole acetic acid</td>
</tr>
<tr>
<td>kPa</td>
<td>kilo Pascal</td>
</tr>
<tr>
<td>KV</td>
<td>kilo volts</td>
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<tr>
<td>m</td>
<td>meter</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>mg L⁻¹</td>
<td>milli-gram per liter</td>
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<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>milli molar</td>
</tr>
<tr>
<td>NAA</td>
<td>napthalene acetic acid</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>psi</td>
<td>pounds per square inch</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>sssDNA</td>
<td>sheared salmon sperm DNA</td>
</tr>
<tr>
<td>TAE</td>
<td>tris-acetate EDTA</td>
</tr>
<tr>
<td>TE</td>
<td>tris-EDTA</td>
</tr>
<tr>
<td>X-Gluc</td>
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SUMMARY

Bacterial leaf blight caused by Xanthomonas oryzae pv. oryzae (Xoo) is a devastating disease of rice resulting in significant yield losses in different parts of the world as well in Pakistan. Since all the Basmati rice varieties are susceptible to this disease, therefore, development of bacterial blight (BB) resistant varieties is highly desirable. The present study was therefore undertaken to develop transgenic Basmati rice resistant to bacterial leaf blight. This study involved tissue culture and particle-bombardment methods for the recovery of transgenic plants. Therefore, optimization of conditions for embryogenic callus induction, their subsequent regeneration into complete plants and various biological and physical factors affecting gene transfer efficiency through particle-bombardment were essential before embarking on genetic transformation studies for BB resistance.

As a first step, an efficient system for embryogenic callus induction and subsequent plant regeneration was established in five local Basmati rice (Oryza sativa L.) varieties. Modified Murashige and Skoog (MS) medium with different concentrations and combinations of auxin (2,4-D) and cytokinin (BAP) were used to define suitable medium for high frequency embryogenic callus induction. Callus cultures were obtained at high frequencies from mature rice embryos. In general, all of the tested genotypes performed well in their response to callus induction. Treatments T4 and T5 (having 2.0 and 4.0 mg L⁻¹ 2,4-D, respectively) proved to be the best for embryogenic callus induction in B-Pak, B-385 and S-Basmati but non-significant differences were observed between these two treatments, therefore, T4 (having 2 mg L⁻¹ 2,4-D) was preferred in all the subsequent studies. Similarly T4 proved to be the most suitable medium for B-370 while T5 exhibited highest embryogenic callus induction frequency (ECIF). 2,4-D alone was sufficient to induce embryogenic callus in all of the varieties and there was generally a negative effect of BAP supplementation in the callus induction medium.
For plant regeneration, six different MS based media combinations either without growth regulators or supplemented with different combinations of BAP, NAA and kinetin either having sucrose or maltose were used. Highest plant regeneration frequency of 74.8% was achieved in S-Basmati followed by 70.4% in B-Pak, 66.8% in B-370, 58.0% in B-2000 and 44.2% in B-385. Plant regeneration efficiency remained low in media types R1 and R4 (without growth regulators) in all the varieties tested indicating that auxin and cytokinin combination was essentially required for induction of somatic embryos and subsequent plant regeneration. R6 (MS basal medium having kinetin 3 mg L⁻¹, NAA 1 mg L⁻¹, maltose 30 g L⁻¹, sorbitol 30 g L⁻¹ and phytagel 0.25%) generally proved to be the best medium for high frequency plant regeneration in almost all the varieties studied.

In order to develop a simple and reproducible system for transformation of Basmati rice varieties through particle-bombardment, a number of different factors which affect bombardment efficiency were studied and optimized for two types of explants (28-day old primary embryogenic calli and excised mature embryos) based on transient expression of the *gusA* reporter gene. Conditions were optimized by varying particle size, helium pressure, target distance and DNA/particle loads. Effect of pre- and post-bombardment osmotic treatment and effective concentration of hygromycin B for selection of transformed cells were also investigated. Both the embryogenic calli as well as mature embryos gave an equally good response in transient GUS expression, however, the later appeared to be more suitable giving higher selection efficiency. Gold particles of 1 µm size, helium pressure of 7,500 kPa, target distance of 9 cm and a particle to DNA ratio of 1:3 produced the highest transient GUS expression in embryos. Osmotic treatment resulted in a 2.7 to 2.9 fold increase in transient GUS expression. Hygromycin B at a final concentration of 50 mg L⁻¹ was optimum for effective selection of transformed cells. Transgenic plants were recovered at an average transformation frequency of 1.5%. Analysis of transgenic plants showed that 96% of the recovered plants were positive for both *gusA* and hpt genes. All the established plants were phenotypically normal and were fertile.

Following optimization of particle-bombardment conditions, Basmati rice varieties were transformed with a cloned gene, *Xa21*, through particle bombardment method. Fifty plants
were recovered from the selected calli. Initial screening through PCR confirmed that 49 plants out of fifty were true transgenics. Southern analysis of transgenic plants confirmed the integration of \textit{Xa21} gene into the genome of these transgenic plants. Analysis of the T\textsubscript{1} generation of these transgenic lines confirmed that in most of the transgenic lines both \textit{hpt} and \textit{Xa21} genes were functional. Bioassays with two local isolates of bacterial blight pathogen (\textit{X. oryzae pv. oryzae}) showed that most of the transgenic lines were resistant to both the \textit{Xoo} isolates. Grain yield of the transgenic lines did not show any significant difference from the control; however the transgenic lines took 8-12 days longer to flower and showed reduced plant height compared to the control plants.
CHAPTER 1

INTRODUCTION

Today’s world population of six billion is expected to reach eight billion by 2020 (Datta, 2004). The biggest challenge facing the world today is to feed this burgeoning population. Even today there are 800 million people who do not have access to sufficient food to meet their needs primarily because of poverty and unemployment (Toenniessen et al., 2003). In the current scenario of food shortages and growing world population, breeders face major challenges to develop new varieties with a higher yield potential, with improved resistances to biotic and abiotic stresses, and adaptation to environmental changes (Negrao et al., 2008). It is therefore imperative to improve our food production in order to feed the growing world population. This can only be achieved through supplementation of modern biotechnology tools as an aid to conventional breeding to develop improved crop varieties with enhanced yields.

Importance of Rice (Oryza sativa L.)

Rice is one of the world’s most important food crops and a primary food source for half the people of the world (Zhu and Wu, 2008). It is a staple food for more than 80% of the people in Asia alone. More than 90 percent of the world’s rice is grown and consumed in Asia (Khush, 2005). Rice accounts for 35-75% of the calories consumed by more than 3 billion Asians. It is planted on about 154 million hectares annually or on about 11% of the world’s cultivated land.

Rice is a high valued cash crop of Pakistan and unlike other South and Southeast Asian countries, it is not considered a subsistence crop as it provides only 8% of the daily calorie intake, with only 10 kg/year annual per capita consumption compared to 124 kg/year that of wheat. Rice is the third major crop, after wheat and cotton, in contributing to the country’s GNP. It contributes 13%, with wheat and cotton each contributing around 30%. Rice is the second major export crop after cotton, contributing about 9% to the export earnings.

In Pakistan, rice (aromatic and non-aromatic) is grown on approx. 2.62 million ha which is about 12% of the total cultivable area (Pakistan Statistical Year Book, 2007). In year 2005-
06, rice production was 5.6 million tons with an average yield of 2.12 tons/ha. The contribution of Basmati rice in the total production was 2.9 million tons from an area of 1.67 million ha with average yield of 1.76 tons/ha. Pakistan earned US $ 1143 million in terms of foreign exchange through rice exports out of which Basmati contributed 0.84 million tons worth of US $ 474 million.

Rice is grown from latitude 24 °N to 36 °N, from sea level in the south to 2,500 m high altitude in north mountain valleys and terraces, in the arid hot zones to tropical humid areas. Soils in the rice growing areas are clay loam to heavy dispersing clays with minimum percolation losses. These types of agro-climatic parameters are ideal for bumper rice harvest. However, our national rice yields are low (3.0 tons/ha) compared to some of the other rice producing countries like USA (7.0 tones/ha), Japan (6.7 tones/ha), China (6.2 tons/ha), Indonesia (4.4 tons/ha) (Maclean et al., 2002). The most important reason for low national yields is the preferential cultivation of traditional Basmati rice varieties. Basmati grain yields are far less than the modern high yielding rice varieties. There has been concerted research efforts to increase Basmati yields; however, these have been largely unsuccessful, as attested by the stagnant national yields of Basmati varieties at around 1.8 tons/ha. Breeders have not been able to increase Basmati yields without sacrificing quality (especially aroma).

Basmati rice is premium long grain rice which is known for its characteristic aroma and excellent cooking qualities. Its high value stems from its unique cooking qualities and a characteristic aroma in both the raw and cooked state. On cooking, the grain elongates to almost twice the original grain length, and the cooked grain has a characteristic shape and integrity. Basmati is a traditional name for certain varieties of rice with these unique properties that are grown exclusively in specific areas of the Indo-Pak sub-continent, which currently includes the northern part of the Western Punjab (on both sides of the Indian and Pakistani border). Due to its premium quality, Basmati attracts a high market price as much as three times compared to coarse varieties. The Middle East is one of the major markets for Pakistani Basmati rice, which imports over 50 % of total Basmati rice exports. The Basmati varieties of rice are also exported to USA and European countries.
Basmati, besides being a natural low yielder, has a number of other constraints like abiotic and biotic factors which limit crop productivity. A number of bacterial, fungal and viral diseases in addition to losses incurred by insects take serious toll of crop production. Diseases, insects and weeds cause as much as 25% yield losses annually in cereal crops (Khush, 2005). Similarly crop yields are affected and the production fluctuates greatly as a result of abiotic stresses such as salt, drought, excess water (submergence), mineral deficiencies and toxicities and abnormal temperatures.

Rice production needs to increase by 755 million tones by 2012 to meet the predicted demands of increasing population (Brookes and Barfoot, 2003). One alternative is to increase the area under rice cultivation, which is getting harder as more farm areas are being converted to residential development due to increasing population in the developing world (Fischer et al., 2000). Therefore, the increased demand of rice will have to be met with less land, less water and less labour (Negrao et al., 2008).

**Challenges and Opportunities for Breeding Improved Crops**

Conventional plant breeding methods have contributed greatly to the improvement of rice varieties. However, there are limitations to conventional plant breeding because it relies on the exchange of genetic information based on sexual compatibility of two diverse parental genotypes of same or closely related species. Recent advancements in deciphering the genetic code of rice have brought this cereal crop at the focal point of functional genomics. The International Rice Genome Sequencing project (IRGSP) sequence published in 2002 will serve as a gold standard for all future investigations regarding the identification of the function of each of the 60,000 rice genes. Since cereals have a high degree of homology in their genome structure (Sandhu and Gill, 2002), therefore, a comparative genomic analysis will help assign a tentative gene function to a gene in other species. The recent developments in cell and tissue culture, plant molecular biology and gene delivery techniques have led to cellular and molecular approaches to crop improvement. These approaches show promise to increase the efficiency of traditional breeding methods and also provide new unconventional tools for tailoring crops with improved characteristics. The most practicable option, therefore, is to increase productivity by breeding efforts supplemented with advanced
biotechnological tools. Biotechnology can play a vital role in reducing the crop losses caused by various biotic and abiotic factors. Transgenic crops have the potential to instigate revolutionary changes in agriculture and the realization of such benefits can already be seen with the rapid adoption of transgenic crops worldwide (James, 2007). In addition to this, rice being the staple food for most of the developing world, the nutritional improvement of rice can also help reduce malnutrition (Sakamoto and Matsuoka, 2004). It has been suggested that these advances in rice and other crops will realize a second green revolution through genetic engineering of food crops.

**Tissue Culture of Rice**

Cell and tissue culture is a prerequisite for any genetic manipulation work. The regeneration of plants from cell cultures still has a delightful element of empiricism as many plant species are still difficult to regenerate from *in-vitro* cultures. The recovery of fertile plants from a transgenic callus is a critical component of many cereal transformation systems. However, efficient regeneration of plants from transformed embryogenic callus is often limited to specific genotypes that exhibit vigorous plant regeneration.

Regeneration frequency depends on genotype and its interaction with culture conditions (Ozawa *et al*., 2003). The strong influence of genotype on plant regeneration was observed in several rice cultivars belonging to japonica, indica and japonica-indica hybrids (Abe and Futsuhara, 1984). Some of them showed high capacity for plant regeneration while others did not. Abe and Futsuhara (1986) studied callus formation and subsequent regeneration of mature seeds of 60 different japonica, indica and javanica cultivars which indicated both intra and inter-varietal differences. Generally, the japonica rice cultivars are considered easier to regenerate *in-vitro* compared to indica cultivars (Ge *et al*., 2006) indicating that callus induction and plant regeneration in rice are genetically controlled. It has been reported that both nuclear and cytoplasmic genes control regeneration ability in rice cultures (Peng *et al*., 1990). Genetic markers have been used to find genes involved in the induction of embryogenic calli (Armstrong *et al*., 1992; Taguchi-Shiobara *et al*., 1997; Takeuchi *et al*., 2000), but these genes have not yet been isolated (Ozawa *et al*., 2003).
Various factors in addition to genotype, including physiological and developmental status of the explant, the composition and concentration of the basal salts, and the organic components and plant growth regulators in the culture medium are known to influence callus induction and plant regeneration ability in rice (Ge et al., 2006). The first success in regeneration of plants from callus derived from mature seeds of rice was obtained by Nishi et al. (1968). Numerous explants were then used by different workers for callus induction and plant regeneration like mature embryos (Ozawa et al., 2003), immature embryos (Lai and Liu, 1982), immature panicles (Ling et al., 1983; Li et al., 1992), roots (Abe and Futsuhara, 1985), young inflorescences (Chen et al., 1985), anthers or pollen (Chen et al., 1980; Toriyama et al., 1986), leaf bases (Abdullah et al., 1986) and leaf sheath (Bhattacharya and Sen, 1980). Among these explants, immature embryos and mature seeds are most commonly used for callus induction and subsequent studies.

Frequency of plant regeneration depends upon the type of callus. Various workers have described the nature and properties of highly regenerable calli also termed as embryogenic calli. According to Vasil and Vasil (1982), embryogenic calli are white to pale yellow in colour, globular, compact but fragile and usually dry in appearance. Non-embryogenic calli on the other hand are usually yellow to brown in colour, watery, crystalline in appearance and not easily separable.

Some of the factors that affect plant regeneration efficiency from callus are the concentration of gelling agents, osmoticum and combination of plant growth regulators (Tsukahara and Hirosawa, 1992). Kavi-Kishor (1987) observed that osmolarity of both growth and regeneration media was important for obtaining highly regenerable rice callus and retaining its regeneration potential. It has been observed that lower water content of callus cultured on a medium containing mannitol and a high concentration of agar was one of the key factors for efficient regeneration of plants from callus (Lai and Liu, 1988). An increase of regeneration frequency upto 47% with a simple dehydration treatment was observed compared to less than 5% in the un-treated controls (Tsukahara and Hirosawa, 1992). Higher
shoot regeneration frequency (1.2 to 5.6 fold) in indica rice cvs. HKR-46 and HKR-126 were also observed following partial desiccation of calli (Saharan et al., 2004).

Peterson and Smith (1991) studied the effect of various growth regulators and callus size on plant regeneration of a number of rice cultivars. They observed that smaller callus pieces (10 mg) produced two to three times as many plants as were regenerated from those of larger (100 mg) pieces. Several reports indicate that regeneration can be enhanced by manipulating media constituents and inclusion of different organic supplements like L-proline, L-tryptophan etc. Sucrose is the most commonly used carbon source in rice tissue culture, however, other carbon sources like maltose, lactose, sorbitol and mannitol have also been used. Ghosh-Biswas and Zapata (1993) observed enhanced regeneration from indica rice cell cultures by using maltose instead of sucrose in the regeneration medium. Kavi-Kishor and Reddy (1986) obtained high regeneration frequency in root and embryo derived callus using a medium containing sorbitol or mannitol. Asano et al. (1994) improved the regeneration response of creeping bentgrass and japonica rice by including maltose and lactose in the regeneration medium.

Nutrient medium is another important parameter for successful callus induction and plant regeneration. Different types of media have been used for callus induction and plant regeneration including MS (Murashige and Skoog, 1962), N6 (Chu et al., 1975), B5 (Gamborg et al., 1968) or LS (Linsmair and Skoog, 1965). 2,4-D is the most commonly used growth regulator for callus induction in rice tissue culture (Yang et al., 1999; Ge et al., 2006). Various rice explants express embryogenic competence in the presence of 2,4-D (Maggioni et al., 1989). Heyser et al. (1983) observed varying responses among rice varieties in producing embryogenic competent cultures with use of 2,4-D and 2,4,5-trichlorophenoxy acetic acid (2,4,5-T). A combination of auxins and cytokinins was found to be suitable for embryogenic callus initiation in several cultivars of rice (Fatokun and Yamada, 1984; Grewal et al., 2005; Zaidi et al., 2006). Callus initiated in 2,4-D regenerated plants when transferred to hormone free medium in some cultivars (Heyser et al., 1983), while others required a cytokinin in the regeneration media for shoot formation (Abe and Futsuhara, 1985; 1986; Sun
et al., 1983). Endogenous levels of abscisic acid (ABA) also appear to play significant role in rice for initiation of embryogenic cultures. Torrizo and Zapata (1986) observed that exposure of the calli to ABA prior to plant regeneration increased the frequency of plant formation in all the varieties tested. Inoue and Maeda (1981) achieved enhanced regeneration of rice using ABA in the initiation medium.

**Genetic Transformation of Rice**

Genetic transformation of plants has emerged as one of the most important tools in plant biology and during the past few years, advances in cell culture and recombinant DNA technology have generated the potential to transfer genes from diverse organisms into plants. Transgenic plants are not only used for basic studies like gene regulation, identification of gene function, examination of steps in primary and secondary metabolism in plant sciences, but are also becoming a primary tool for the development of new varieties of crops with different quality traits.

The introduction of an enzymatic procedure for the isolation of plant protoplasts (Cocking, 1960) and regeneration of plants from isolated protoplasts (Takebe et al. 1971) provided the basis and a new and promising facet to the plant cell and tissue culture and opened up a whole new area of studies in plant cell biology. Protoplast culture provided excellent opportunities for research on plant improvement, first by exploring genetic variations among the existing crops and then attempting transfer of genetic information from one species to another either through fusion of protoplasts or transfer of foreign genes through direct DNA transformation of protoplasts. Protoplasts have been the most common recipients of foreign DNA in rice transformation. Transgenic cereal plants were first obtained from protoplast transformation system with DNA uptake mediated by electroporation and/or polyethylene glycol (PEG) (Toriyama et al., 1988; Zhang and Wu, 1988; Zhang et al., 1988; Shimamoto et al., 1989). These studies on the production of transgenic rice plants exemplify the key role that protoplasts have played in cereal cell transformation. However, regeneration of fertile plants from protoplasts is highly genotype-dependent, time consuming and labour intensive.
Therefore, despite of their use in engineering of some of the economically important genes, these two methods have fallen out of favour and scientists prefer other methods of gene delivery (Tyagi and Mohanty, 2000).

Currently, the two most widely used methods for genetic transformation of plants are *Agrobacterium* and particle bombardment. *Agrobacterium* has many advantages as a system for introducing genes into plant cells. *Agrobacterium* mediated transformation does not require the use of protoplasts thus by-passing one of the major bottlenecks for transformation of many important rice cultivars. Also in contrast to direct DNA uptake, integration of *Agrobacterium* T-DNA into the recipient genome is relatively precise and usually results in the insertion of a single un-arranged copy of the gene of interest (Klee *et al*., 1987). In most of the dicots, a strong wound response is part of the process of callus formation and plays a key role in *Agrobacterium* mediated transformation. However, in cereals the wound response is absent and wounding leads to cell death (Hooykaas-Van Slogteren *et al*., 1984). The *Agrobacterium* mediated transformation system has proved to be efficient in many dicots and some monocots plants including major food crops as wheat, rice and corn. Remarkable progress has been made during the recent couple of years in the development of efficient systems for *Agrobacterium tumefaciens*-mediated transformation in rice. About 40 different genotypes of *indica*, *japonica* and *javanica* rice have already been transformed using this approach (Shrawat and Lorz, 2006) which has prompted a renewed interest for transformation of cereals using this method of gene delivery.

A method of gene delivery which can circumvent the limitations of regeneration of protoplasts into plants and *Agrobacterium* host specificity is to use micro-projectile mediated transformation, also called the particle bombardment or biolistic method. The biolistic method has emerged as a method of choice especially for the transformation of recalcitrant crops like some graminaceous monocots. Biolistic method employs high velocity metal particles to deliver biologically active DNA into plant cells. First apparatus developed by Klein *et al*. (1987) was based on gun powder charge in a blank cartridge. Afterwards, acceleration of the particles was achieved by different devices using air (Oard *et al*., 1990), nitrogen gas (Morikawa *et al*., 1989; Iida *et al*., 1990), helium gas (Williams *et al*., 1991;
Russell et al., 1992) and electric discharge (Christou et al., 1989). The first report on the development of transgenic plant through particle bombardment appeared in 1988 when transgenic soybean (Glycine max L.) plants were produced (McCabe et al., 1988). Recovery of transformed maize (Zea mays L.) callus (Klein et al., 1988a and b) validated the general applicability of this technology for plant transformation. By 1990, transgenic cotton (Gossypium hirsutum L.; Finer and McMullen, 1990), papaya (Carica papaya L.; Fitch et al., 1990), maize (Fromm et al., 1990; Gordon-Kamm et al., 1990) and tobacco (Nicotiana tabacum L.; Tomes et al., 1990) were reported. In the two years following the engineering of aforementioned plants, almost all major crops like rice (Christou et al., 1991; 1992), wheat (Triticum aestivum L.; Vasil et al., 1992; Weeks et al., 1993), sugarcane (Saccharum officinarum L.; Bower and Birch, 1992) were transformed using particle bombardment. One of the most important features of the biolistic process is that it is of broad utility, and may prove to be something like a universal gene delivery system (Sanford, 1990). Different explants can be used as target cells including embryogenic cell suspensions (Wang et al., 1988; Finer et al., 1992; Schopke et al., 1996), meristems (McCabe et al., 1988), embryos (Vain et al., 1995), immature embryos (Christou et al., 1988; McCabe et al., 1988), embryogenic calli (Vasil et al., 1992), and pollens (McCabe et al., 1988; Twell et al., 1989). In addition to this, micro-projectile bombardment is the only method with which mitochondria and chloroplasts were transformed (Klein et al., 1992). The ability to deliver foreign DNA into regenerable cells, tissues or organs appears to provide the best method for achieving truly genotype-independent transformation in agronomic crops, thus by-passing Agrobacterium host specificity and tissue culture related regeneration constraints (Christou, 1994). The greatest advantage of this procedure is that almost any kind of tissue having the capacity to regenerate plants can be used. Moreover, gene(s) may be delivered directly to the explant which can be used to initiate embryogenic cultures, thus offering several advantages. First, compact cultures can be readily obtained from immature embryos, seeds, and anthers of most rice cultivars. Second, gene delivery directly to the explant and then culturing of callus under selective conditions removes the labour intensive aspects of maintaining a culture collection for the use of transformation targets. Third, the time it takes from gene delivery to obtaining transgenic plants can be reduced by almost half compared to the time it takes with
relatively un-differentiated cells or suspension cultures. Finally, this reduced tissue culture time results in fewer problems about the fertility of the regenerated plants.

Genetic transformation performed either by *Agrobacterium* or particle bombardment has its own merits and limitations (Dai *et al.*, 2001). It has been observed that T-DNA generally integrates into transcriptionally active regions of genome (Eamens *et al.*, 2004; Sha *et al.*, 2004) which can also result in integration of multiple elements either at different loci or in the form of inverted or tandem repeats (De Buck *et al.*, 2000; Eamens *et al.*, 2004). Generally, *Agrobacterium*-mediated transformation results in lower copy insertions of transgene (Dai *et al.*, 2001). Moreover it is a simple and inexpensive method. Where available, it is unlikely to be replaced by particle bombardment (Bajaj and Mohanty, 2005).

Efforts have, however, been made to develop transgenic rice free of elements, which otherwise are essential for transformation such as selectable markers or vector backbone, but do not add to the value of the desired trait. Presence of vector backbone sequences have been reported in transgenic plants developed either by *Agrobacterium* (Yin and Wang, 2000; Kim *et al.*, 2003) or through particle bombardment (Kohli *et al.*, 1999). Integration of these sequences may have undesirable effects on the transgene stability and/or expression (Breitler *et al.*, 2002). Therefore, transformation methods using particle bombardment with minimal linear gene cassette have been developed (Fu *et al.*, 2000; Loc *et al.*, 2002). Transgenic plants harbouring minimal transgene cassettes exhibited low-copy number, simple integration events and fewer rearrangements compared to transgenic plants produced with intact plasmids (Fu *et al.*, 2000). This study showed that that particle bombardment with minimal expression cassette offers a superior alternative to *Agrobacterium* (Fu *et al.*, 2000) and it is very likely that future genetic transformation studies will rely on minimal expression cassettes rather than whole plasmids (Bajaj and Mohanty, 2005).

**Genes for Plant Transformation**

A number of different genes isolated from diverse organisms are employed in transformation experiments in addition to the agronomically important genes. These include selectable markers and reporter genes. In the transformation experiments, very few cells in the targeted
population become transformed, therefore the selection of transformants demands the use of selectable markers. The commonly used selectable marker genes are those which encode enzymes which detoxify either antibiotics like kanamycin or hygromycin or herbicides such as phosphinothricin or glyphosate (Miki and McHugh, 2004). Reporter genes are useful tools in the analysis of gene expression and have been used to identify regulatory sequences in genes, to characterize the sequences involved in gene responses to environmental and physiological stimuli, to determine the activity of heterologous promoters from different origins and most importantly to evaluate parameters that influence the transfer and expression of DNA. A number of reporter genes have been used in studies of gene expression in higher plants which include *E. coli* β-galactosidase (*lacZ*), *E. coli* β–glucuronidase (*gusA*), chloramphenicol acetyl transferase (*CAT*), nopaline synthase (*nos*), octopine synthase (*ocs*), firefly luciferase (*luc*), bacterial luciferase (*lux* A and *lux* B), and a green fluorescent protein (*GFP*) from jelly fish (*Aequorea victoria*) etc. The *E. coli* β-glucuronidase (*gusA*) gene has been developed for use as a reporter gene in transgenic plants (Jefferson, 1987; Jefferson et al., 1987a), animals (Jefferson et al., 1987b), bacteria (Sharma and Singer, 1990), fungi (Schmitz et al., 1990). GUS is encoded by the *gusA* (formerly *uidA*) of *E. coli* which is now widely used as a reporter gene in plants and other organisms (Jefferson et al., 1987a and b). This gene has a number of properties that makes it excellent for gene fusion experiments. The enzyme is quite stable to high temperature denaturation, tolerate detergents and varying ionic conditions, can be assayed over a broad range of pH values (4.0 - 8.0; Gallagher, 1992). One of the most important features that have led to the extensive use of *gusA* as a reporter gene in plant molecular biology is the lack of appreciable GUS activity in plant tissues except few exceptions.

Transgenic crops mostly rely on the use of either an antibiotic resistance or an herbicide tolerance gene for the selection of transformed cells over the non-transformed cells. Public concerns over the safety of such genes have led to the development of either new selectable markers that are more acceptable or to produce marker-free transgenic plants (Joersbo, 2001; Datta et al., 2003; Rommens et al., 2004). The most simple and straightforward approach to
remove selectable marker gene is by segregation, provided that the gene of interest and marker gene are integrated at independent loci (Tu et al., 2003). Some other techniques like cre/loxP (Gleave et al., 1999), IPT-MAT vector system (Endo et al., 2002), maize Ac/Ds transposon system (Cotsaftis et al., 2002), and dual T-DNA binary vector system (Komari et al., 1996; Breitler et al., 2004) for the recovery of marker-free transgenic plants have been used.

Transgenic plants with improved characteristics, such as pest and herbicide resistance, are most essential as no inherent resistance has been demonstrated within the crop species or related species (Byrne et al., 2001). Currently intensive research on transgenic plants is being focused to decrease the dependency on chemical pesticides, enhance yields, facilitate earlier and easier harvesting, tailor crop varieties with improved nutritional qualities, tolerance to abiotic stresses and increase the flexibility in crop management. For the end users, this should lead to reduced food costs, higher food quality, and increase availability of food. So, these new techniques are playing an increasingly important role in crop improvement and in our daily lives.

Bacterial diseases are among the major factors which limit crop productivity and are of great economic significance for many crops, with the highest losses occurring in cereals, vegetables and fruits (Morgues et al., 1998). In most cases, application of protective agrochemicals is not adequate to control bacterial diseases. Moreover, the use of chemicals is subject to increasing restrictions because of their potentially harmful impact on the human health and the environment. The occurrence of natural sources of resistance to bacterial diseases has helped in the development of a number of resistant varieties through conventional breeding. However, classical breeding is obviously restricted to within species (or between closely related species), while potentially effective resistance mechanisms may also exist elsewhere. In addition, it may be rather difficult and time consuming to introduce resistance from wild species into commercial cultivars (Morgues et al., 1998). During the recent years, improvement in plant transformation techniques and progress in understanding the plant pathogen interactions has enabled us to use genetic engineering for the rational
creation of disease resistant plants. Unlike conventional breeding, genetic engineering permits the modification or introduction of one or more resistant traits into susceptible varieties.

**Bacterial Leaf Blight of Rice**

Bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a devastating disease of rice in Asia and Africa resulting in an estimated loss of US$ 250 million annually (Zhang et al., 1998). The yield losses due to bacterial blight can be as high as 50% when plants are infected at the maximum tillering stage (Wei et al., 2007). This disease is not only widespread throughout Asia but also occurs in Australia, the United States and in several rice growing countries of Latin America (Chu et al., 2006). In addition to several epidemic outbreaks in Asia during the past two decades, it has resulted in extensive damage to rice crop in West Africa particularly in Niger where irrigated rice suffered heavy losses due bacterial blight in 1982 (Noor et al., 2006). Bacterial blight was identified for the first time in Japan in 1884 (Ou, 1985) and from Punjab Pakistan in 1976 (Mew and Majid, 1977). Its occurrence has been reported from almost all provinces of Pakistan (Akhtar and Akram, 1987). Its incidence on farmer’s field was 10-15, 15-20 and 20-25 % in Sindh, Punjab and NWFP respectively (Akhtar and Sarwar, 1986). Bacterial blight only became significant in Pakistan in 1985 due to the introduction of a susceptible rice variety – Basmati 385. Since then, the disease has become one of the most serious problems on rice in the Punjab province (Gill et al., 1999). The incidence of this disease is increasing in Pakistan especially in the kallar belt which is famous for producing premium quality rice (Khan et al. 2000a).

Bacterial blight is a water-borne disease (Ronald, 1997). *Xoo* enters into the plant tissues through the hydathodes, multiplies in the epithelium, and ultimately moves to the xylem vessels where the infection becomes systemic. The first symptoms of the disease appear as water-soaked lesions on the edges of the leaf blades near the leaf tip. The lesions expand and turn yellowish and eventually grayish white, thus causing severe leaf damage and reducing yield.
Numerous donors for resistance to *Xoo* have been identified (Kinoshita, 1995; Lin *et al*., 1996). Some of the resistance genes, such as *Xa4*, have been incorporated into improved rice varieties using the traditional breeding approaches, and these varieties are widely grown (Khush *et al*., 1989). However, the large-scale and long-term cultivation of these varieties with *Xa4* has led to the significant shifts in the *Xoo* race (Mew *et al*., 1992). In many areas in Indonesia, India, China, and the Philippines where rice varieties with *Xa4* have been widely grown, new races of the BB pathogen have emerged (Tu *et al*., 1998). Therefore new genes for BB resistance are being incorporated into elite rice varieties. A wild rice species, *O. longistaminata*, was found to be resistant to *Xoo* (Khush *et al*., 1989; Ikeda *et al*., 1990; Khush *et al*., 1990). In 1997, researchers in India evaluated this species for resistance to various strains of *Xoo* and found that it could resist all the strains they tested. After twelve years of intensive breeding, International Rice Research Institute (IRRI) developed a bacterial blight resistant variety IRB-20 from IR-72 and *O. longistaminata* using conventional techniques. It was also shown that the resistance was conferred by a small region located on chromosome 11, which was perhaps a single gene, which they named as *Xa21* (Ronald *et al*., 1992). Researchers believe that the protein produced by *Xa21* gene is able to detect diseases, such as bacterial blight. Once it detects the disease, it sends an alert signal, causing the cell to activate its defence mechanism against the disease. Song *et al*. (1995) cloned the *Xa21* gene using a map-based cloning strategy and discovered that it belongs to a small closely linked multigene family containing eight members. Wang *et al*. (1996) showed that the transgenic plants expressing the cloned *Xa21* gene conferred resistance to 29 out of 32 diverse *Xoo* isolates collected from eight different countries. *Xa21* encodes a receptor kinase-like protein with leucine-rich repeats (LRRs) in the extra-cellular domain, a single pass trans-membrane domain, and a serine/threonine kinase intracellular domain. Compared with other cloned plant R genes, the structure of *Xa21* is unique in having both the receptor domain (LRR), presumably for recognition, and the kinase domain for subsequent signal transduction (Wang *et al*., 1996).

Due to its ability to confer broad-spectrum resistance to bacterial blight, this gene has been introduced to several rice varieties either through transgenic or conventional breeding approaches (Tu *et al*., 1998; Zhang *et al*., 1998; Wang *et al*. 2005; Maruthasalam *et al*.,
This gene seems to be the best candidate so far to produce bacterial blight-resistant transgenic plants (Bajaj and Mohanty, 2005). It can also be used along with other genes to provide multiple stress tolerance (Datta et al., 2002; Narayanan et al., 2002).

Most commonly $R$ genes are arranged in clusters (Afzal et al., 2007), and genes within one cluster are mostly derived from a common ancestral gene (Richly et al., 2004). This clustering feature is considered to facilitate the expansion of $R$ gene numbers and race-specificities through recombination and positive selection (Michelmore and Meyers, 1998).

Study of multiple, genetically linked $R$ gene families forms the basis for understanding the molecular mechanisms of $R$ gene evolution and the generation of novel recognition specificity. Currently, more than 40 plant $R$ genes have been cloned and characterized, which include five $R$ genes (i.e. $Xa1$, $xa5$, $Xa21$, $Xa26$ and $Xa27$) for rice bacterial blight resistance, (Song et al., 1995; Yoshimura et al., 1998, Iyer and McCouch, 2004; Sun et al., 2004; Gu et al., 2005). Out of these 40 cloned $R$ genes, only three genes are recessive (Buschges et al. 1997; Martin et al. 2003; Iyer and McCouch 2004) while others are dominant. The products of most of the dominant $R$ genes seem to function as receptors interacting directly or indirectly with pathogen elicitors to initiate hyper-sensitive responses (Martin et al., 2003). However, the recessive $R$ genes that have been cloned each have very different structures, suggesting that they might function differently (Chu et al., 2006).

Consequent to the successful cloning and characterization of over 40 resistance ($R$) genes from different plant species over the past few years, significant insight into the molecular basis of disease resistance in plants has been acquired (Hulbert et al. 2001). Sequence analysis of the predicted proteins revealed that $R$ genes of diverse origin and pathogen specificity share similar structural motifs such as leucine-rich repeats (LRR), kinase domains and nucleotide binding sites (NBS). The structural similarity of different $R$ genes also suggests the existence of a common or limited number of resistance pathways in plants (Wang et al., 2004). Dissection of these pathways is expected to provide insight into the number of genes required to activate a defense response, and the possible roles these genes play.
The use of resistant cultivars is one of the most effective and economical methods to control this disease. Breeding for disease resistance is the most usual and obvious choice for crop improvement but it requires a lengthy back-crossing procedure which may span over 7-10 years. On the other hand, genetic engineering provides an opportunity to transfer resistance genes to the target plant and resistant cultivars can be released within a short period of 3-4 years.

Keeping in view the importance of rice in Pakistan’s economy and the emerging problem of bacterial leaf blight in the traditional rice growing areas of the Punjab province, the present study was aimed

i) to develop procedures for high efficiency regeneration system from *in-vitro* cultures of Basmati rice,

ii) to optimize conditions for foreign gene transfer to *in-vitro* cultures of Basmati rice, and

iii) to develop transgenic Basmati rice lines resistant to bacterial leaf blight.

This study will pave the way to improve Basmati rice against other factors (both biotic and/or abiotic) which limit crop productivity. Thus by adopting innovative technologies and introduction of transgenic varieties resistant to biotic and abiotic stresses, our national yields can be substantially increased.
CHAPTER 2

MATERIALS AND METHODS

IN-VITRO STUDIES ON RICE

Five locally cultivated Basmati rice (*Oryza sativa* L. sub sp. indica) cultivars namely ‘Basmati-370’ (B-370), ‘Basmati-2000’ (B-2000), ‘Basmati-Pak’ (B-Pak), ‘Basmati-385’ (B-385) and ‘Super Basmati’ (S-Basmati) were used for embryogenic callus induction and subsequent plant regeneration studies. Rice seeds were obtained from Mutation Breeding Division of the Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan.

**Callus induction**

Mature seeds were manually dehusked and surface sterilized with 70% ethanol for 1 minute followed by immersion in 50% (v/v) commercial bleach (containing 5.25% sodium hypochlorite) for 30-45 minutes with continuous stirring on a magnetic stirrer. After sterilization, the seeds were washed 3-4 times with sterile distilled water. Ten to twelve seeds were placed in baby-food jars (Sigma, St. Louis, Mo, USA) or 20-25 seeds in sterile disposable Petri plates (20x100 mm) containing 25-30 ml callus induction medium. The cultures were kept in a dark room maintained at 25 ±1 °C.

To define optimum medium combination for high frequency of embryogenic callus induction, 10 treatments (Table 2.2) involving different concentrations/combinations of 2,4-D (0.0, 0.5 1.0, 2.0 and 4.0 mg L\(^{-1}\)) and or BAP (0.0 and 0.5 mg L\(^{-1}\)) in modified MS (Murashige and Skoog, 1962) basal media (Table 2.1) were used. Sucrose (3%) was used as the carbon source. pH of the medium was adjusted to 5.8 and the medium was solidified with 1% bacto agar (Difco Laboratories, Detroit, MI, USA) before autoclaving at 100 kPa for 15-20 minutes. After 28 days of incubation in dark, the embryogenic callus induction frequency (ECIF; i.e. number of explants producing embryogenic callus divided by the total number of explants used multiplied by 100) was determined. The calli which were nodular, pale yellow to white in colour, friable and dry in appearance were considered as embryogenic.
Table 2.1. Murashige and Skoog (MS) Medium (Murashige and Skoog, 1962)

<table>
<thead>
<tr>
<th>Components</th>
<th>Final Conc. (mg L(^{-1}))</th>
<th>Stock (g L(^{-1}))</th>
<th>Use per liter (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macronutrients I:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgSO(_4).7H(_2)O</td>
<td>370.0</td>
<td>3.7</td>
<td>100.0</td>
</tr>
<tr>
<td>KH(_2)PO(_4)</td>
<td>170.0</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>KNO(_3)</td>
<td>1900.0</td>
<td>19.0</td>
<td></td>
</tr>
<tr>
<td>NH(_4)NO(_3)</td>
<td>1650.0</td>
<td>16.5</td>
<td></td>
</tr>
<tr>
<td><strong>Macronutrients II:</strong></td>
<td></td>
<td></td>
<td>100.0</td>
</tr>
<tr>
<td>CaCl(_2).2H(_2)O</td>
<td>440.0</td>
<td>4.4</td>
<td></td>
</tr>
<tr>
<td><strong>Micronutrients:</strong></td>
<td></td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>H(_3)BO(_3)</td>
<td>6.2</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>MnSO(_4).4H(_2)O</td>
<td>22.3</td>
<td>22.3</td>
<td></td>
</tr>
<tr>
<td>ZnSO(_4).7H(_2)O</td>
<td>8.6</td>
<td>8.6</td>
<td></td>
</tr>
<tr>
<td>Na(_2)MoO(_4).2H(_2)O</td>
<td>0.25</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>CuSO(_4).5H(_2)O</td>
<td>0.025</td>
<td>0.025</td>
<td></td>
</tr>
<tr>
<td>CoCl(_2).6H(_2)O</td>
<td>0.025</td>
<td>0.025</td>
<td></td>
</tr>
<tr>
<td>KI</td>
<td>0.83</td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td>FeNaEDTA</td>
<td></td>
<td></td>
<td>10.0</td>
</tr>
<tr>
<td>FeSO(_4).7H(_2)O</td>
<td>27.8</td>
<td>2.78</td>
<td></td>
</tr>
<tr>
<td>Na(_2)EDTA.2H(_2)O</td>
<td>37.3</td>
<td>3.73</td>
<td></td>
</tr>
<tr>
<td><strong>Vitamins and Amino Acids:</strong></td>
<td></td>
<td></td>
<td>10.0</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>0.1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Myoinositol</td>
<td>100.0</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>2.0</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>5.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.2. Different combinations of callus induction media (CIM) formulated for embryogenic callus induction from different Basmati rice cultivars.

<table>
<thead>
<tr>
<th>Media type</th>
<th>Basal medium</th>
<th>2,4-D (mg L⁻¹)</th>
<th>BAP (mg L⁻¹)</th>
<th>Sucrose (g L⁻¹)</th>
<th>Agar (g L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>MS</td>
<td>0.0</td>
<td>0.0</td>
<td>30.0</td>
<td>10.0</td>
</tr>
<tr>
<td>T2</td>
<td>MS</td>
<td>0.5</td>
<td>0.0</td>
<td>30.0</td>
<td>10.0</td>
</tr>
<tr>
<td>T3</td>
<td>MS</td>
<td>1.0</td>
<td>0.0</td>
<td>30.0</td>
<td>10.0</td>
</tr>
<tr>
<td>T4</td>
<td>MS</td>
<td>2.0</td>
<td>0.0</td>
<td>30.0</td>
<td>10.0</td>
</tr>
<tr>
<td>T5</td>
<td>MS</td>
<td>4.0</td>
<td>0.0</td>
<td>30.0</td>
<td>10.0</td>
</tr>
<tr>
<td>T6</td>
<td>MS</td>
<td>0.0</td>
<td>0.5</td>
<td>30.0</td>
<td>10.0</td>
</tr>
<tr>
<td>T7</td>
<td>MS</td>
<td>0.5</td>
<td>0.5</td>
<td>30.0</td>
<td>10.0</td>
</tr>
<tr>
<td>T8</td>
<td>MS</td>
<td>1.0</td>
<td>0.5</td>
<td>30.0</td>
<td>10.0</td>
</tr>
<tr>
<td>T9</td>
<td>MS</td>
<td>2.0</td>
<td>0.5</td>
<td>30.0</td>
<td>10.0</td>
</tr>
<tr>
<td>T10</td>
<td>MS</td>
<td>4.0</td>
<td>0.5</td>
<td>30.0</td>
<td>10.0</td>
</tr>
</tbody>
</table>

pH of all the media was adjusted to 5.7-5.8 and autoclaved at 100 kPa for 15-20 minutes or longer depending on the quantity of the medium.
Embryogenic calli were carefully selected under the stereo-microscope and used for further studies.

Plant regeneration from calli

Plant regeneration from Basmati rice calli was achieved in two steps. Embryogenic calli were placed first on the pre-regeneration medium (PRM-1) and kept in dark for 10-12 days thereafter these were shifted to regeneration medium (PRM-2) and kept under 16/8 hours light/dark period in a growth room maintained at 25 ± 1 °C. To define a suitable medium for plant regeneration, six different media combinations (Table 2.3) with basic composition of MS (Murashige and Skoog, 1962; Table 2.1) were used. MS medium was either used without growth regulators or supplemented with different combinations of kinetin, IAA and NAA (Table 2.4). Sucrose or maltose (3%) was used as the carbon source. Both PRM-1 and PRM-2 media were supplemented with 3% sorbitol. The PRM-1 medium differed from the PRM-2 medium only in having additional 10 mg L⁻¹ abscisic acid (ABA). All the media were solidified with 0.3% phytagel. The pH of the medium was adjusted to 5.8 before autoclaving. Pre-regeneration or regeneration medium (25-30 ml) was poured into 100x20 mm petri plates (disposable, sterile). Calli (6-8 in number) were placed in each plate containing PRM-1 medium and the plates were sealed with parafilm. After two weeks the cultures were transferred to PRM-2 medium. After two weeks, the regenerating calli were transferred to fresh PRM-2 medium. Emerging plantlets were transferred to root proliferation medium (Table 2.5) in culture tubes containing 20 ml medium. Regeneration frequency was determined after 4-6 weeks. Regeneration frequency was calculated as the number of calli producing plantlets divided by the total number of calli multiplied by 100. The ratio of green to albino plants was also determined for each of the regeneration medium.

Establishment of Regenerated Plants in Hydroponics/Soil

The regenerated plantlets were transferred to root proliferation medium (Table 2.5). After 2-3 weeks, the plantlets (10-15 cm in size) were removed from the culture medium and the roots were washed in running tap water to remove any adhering culture medium. Two different
Table 2.3. Different combinations of plant regeneration media formulated for *in-vitro* plant regeneration from callus cultures of different Basmati rice cultivars.

<table>
<thead>
<tr>
<th>Media Type</th>
<th>Pre-regeneration (PRM-1) medium composition</th>
<th>Regeneration (PRM-2) medium composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>MS salts &amp; vitamins + sucrose 30 g L(^{-1}), sorbitol 30 g L(^{-1}), abscisic acid 10 mg L(^{-1}), phytagel 0.25%</td>
<td>MS salts &amp; vitamins + sucrose 30 g L(^{-1}), sorbitol 30 g L(^{-1}), phytagel 0.25%</td>
</tr>
<tr>
<td>R2</td>
<td>MS salts &amp; vitamins + kinetin 1 mg L(^{-1}), IAA 4 mg L(^{-1}), sucrose 30 g L(^{-1}), sorbitol 30 g L(^{-1}), abscisic acid 10 mg L(^{-1}), phytagel 0.25%</td>
<td>MS salts &amp; vitamins + kinetin 1 mg L(^{-1}), IAA 4 mg L(^{-1}), sucrose 30 g L(^{-1}), sorbitol 30 g L(^{-1}), phytagel 0.25%</td>
</tr>
<tr>
<td>R3</td>
<td>MS salts &amp; vitamins + kinetin 3 mg L(^{-1}), NAA 1 mg L(^{-1}), sucrose 30 g L(^{-1}), sorbitol 30 g L(^{-1}), abscisic acid 10 mg L(^{-1}), phytagel 0.25%</td>
<td>MS salts &amp; vitamins + kinetin 3 mg L(^{-1}), NAA 1 mg L(^{-1}), sucrose 30 g L(^{-1}), sorbitol 30 g L(^{-1}), phytagel 0.25%</td>
</tr>
<tr>
<td>R4</td>
<td>MS salts &amp; vitamins + maltose 30 g L(^{-1}), sorbitol 30 g L(^{-1}), abscisic acid 10 mg L(^{-1}), phytagel 0.25%</td>
<td>MS salts &amp; vitamins + maltose 30 g L(^{-1}), sorbitol 30 g L(^{-1}), phytagel 0.25%</td>
</tr>
<tr>
<td>R5</td>
<td>MS salts &amp; vitamins + kinetin 1 mg L(^{-1}), IAA 4 mg L(^{-1}), maltose 30 g L(^{-1}), sorbitol 30 g L(^{-1}), abscisic acid 10 mg L(^{-1}), phytagel 0.25%</td>
<td>MS salts &amp; vitamins + kinetin 1 mg L(^{-1}), IAA 4 mg L(^{-1}), maltose 30 g L(^{-1}), sorbitol 30 g L(^{-1}), phytagel 0.25%</td>
</tr>
<tr>
<td>R6</td>
<td>MS salts &amp; vitamins + Kinetin 3 mg L(^{-1}), NAA 1 mg L(^{-1}), maltose 30 g L(^{-1}), sorbitol 30 g L(^{-1}), abscisic acid 10 mg L(^{-1}), phytagel 0.25%</td>
<td>MS salts &amp; vitamins + Kinetin 3 mg L(^{-1}), NAA 1 mg L(^{-1}), maltose 30 g L(^{-1}), sorbitol 30 g L(^{-1}), phytagel 0.25%</td>
</tr>
</tbody>
</table>

pH of all the media was adjusted to 5.8 and autoclaved at 100 kPa for 15-20 minutes.
Table 2.4. Growth regulators and their stock preparation

<table>
<thead>
<tr>
<th>Growth regulator</th>
<th>Stock Conc.</th>
<th>Preparation*</th>
<th>Storage</th>
<th>Sterilization†</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-D</td>
<td>1,000 ppm</td>
<td>Dissolved 100 mg in 5 ml ethanol, while stirring added water (initially drop by drop). Made volume with water to 100 ml.</td>
<td>4 °C</td>
<td>CA</td>
</tr>
<tr>
<td>IAA</td>
<td>1,000 ppm</td>
<td>Dissolved 100 mg IAA in 10 ml water, then made up the volume with water to 100 ml.</td>
<td>Below 0° C</td>
<td>CA / FS</td>
</tr>
<tr>
<td>NAA</td>
<td>1,000 ppm</td>
<td>Dissolved 100 mg NAA in 5 ml of 1 N NaOH, then made the volume with water to 100 ml.</td>
<td>4 °C</td>
<td>CA</td>
</tr>
<tr>
<td>BAP</td>
<td>1,000 ppm</td>
<td>Dissolved 100 mg BAP in 5 ml of 1 N NaOH, then made up the volume with water to 100 ml.</td>
<td>4 °C</td>
<td>CA / FS</td>
</tr>
<tr>
<td>Kinetin</td>
<td>1,000 ppm</td>
<td>Dissolved 100 mg Kinetin in 5 ml of 1 N NaOH, then made the volume with water to 100 ml.</td>
<td>Below 0° C</td>
<td>CA / FS</td>
</tr>
<tr>
<td>ABA</td>
<td>10,000 ppm</td>
<td>Dissolved 100 mg abscisic acid in 5 ml of 1 N NaOH, then made up the volume with water to 100 ml</td>
<td>Below 0° C</td>
<td>CA / FS</td>
</tr>
</tbody>
</table>

* used double distilled deionized water

† CA = co-autoclaved with the medium
FS = filter sterilized and then added to the medium

Table 2.5. Root proliferation medium

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS salts and vitamins</td>
<td>½ strength</td>
</tr>
<tr>
<td>Sucrose</td>
<td>2.0 %</td>
</tr>
<tr>
<td>Phytagel</td>
<td>0.2%</td>
</tr>
<tr>
<td>pH</td>
<td>5.7-5.8</td>
</tr>
</tbody>
</table>
Table 2.6. Yoshida’s Culture Solution (Yoshida et al., 1976)

<table>
<thead>
<tr>
<th>Component</th>
<th>Stock solution (g L⁻¹)</th>
<th>Amount used per 4 liter preparation (in ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macro-nutrients:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>914.0</td>
<td>5.0</td>
</tr>
<tr>
<td>NH₂PO₄•2H₂O</td>
<td>403.0</td>
<td>5.0</td>
</tr>
<tr>
<td>K₂SO₄</td>
<td>714.0</td>
<td>5.0</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>886.0</td>
<td>5.0</td>
</tr>
<tr>
<td>MgSO₄•7H₂O</td>
<td>3240.0</td>
<td>5.0</td>
</tr>
<tr>
<td><strong>Micronutrients:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MnCl₂•4H₂O</td>
<td>15.0</td>
<td></td>
</tr>
<tr>
<td>(NH₄)₆Mo₇O₂₄•4H₂O</td>
<td>0.74</td>
<td></td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>9.34</td>
<td></td>
</tr>
<tr>
<td>ZnSO₄•7H₂O</td>
<td>0.35</td>
<td>5.0</td>
</tr>
<tr>
<td>CuSO₄•5H₂O</td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>FeCl₃•6H₂O</td>
<td>77.0</td>
<td></td>
</tr>
<tr>
<td>Citric acid (monohydrate)</td>
<td>119.0</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td>5.0</td>
</tr>
</tbody>
</table>

Dissolved separately; then combined with 500 ml of conc. H₂SO₄. Made up the volume to 10 L with distilled water.
methods were used and compared to establish regenerated plants. In the first method the regenerated plants were grown in a hydroponic culture. Styrofoam sheets (2.5 cm thickness) were cut according to the size of a plastic tub (dimensions LxWxH 55x35x20 cm). Holes of approx. 2 cm diameter were cut equi-distant (5 cm, center to center) in each row and column in the styro-foam sheet. Regenerated plants were fixed in the holes with the help of foam pieces cut in a square (2.5 cm²) shape. The tub was filled with a liquid culture medium, referred to as Yoshida’s culture solution (Yoshida et al., 1976; Table 2.6), such that the roots of the plants were completely immersed in the liquid. The water level was marked with the help of a marker. The tub was placed in the containment glass house and water level and pH of the medium was maintained on daily basis. The culture solution was replaced with fresh solution on weekly basis.

In the other method, the roots were dipped in 0.2% w/v aqueous solution of Dithane® and transplanted to small plastic pots containing sterilized sand. The pots were then covered with clear polyethylene plastic bags to provide high humidity conditions to the transplanted plants. Plastic bags were gradually backed-off to reduce humidity until plants were acclimatized to ambient humidity and temperature conditions. The acclimatized plants were then transferred to soil in the pots and transferred to containment glass-house for further growth and maintenance.

**TRANSFORMATION STUDIES OF RICE**

**Plasmids used for transformation**

Four different plasmids having *gusA* and *hpt* genes were used in this study. Some of the important features of these plasmids are as follows:

*pAct1-D*

This plasmid contains β-glucuronidase (*gusA*) coding sequence driven by *actin-1* promoter and *nos* terminator (Figure 2.1a; McElroy et al., 1991). This plasmid was used for optimization of physical and biological parameters affecting particle bombardment mediated gene delivery (courtesy Drs. R.A. Jefferson/Kate J. Wilson, CAMBIA, Australia).
**pTRA151**

This plasmid contains hygromycin phosphotransferase (*hpt*) coding sequence driven by CaMV35S promoter and tml terminator (Figure 2.1b; Zheng *et al.*, 1991). The *hpt* gene encodes for resistance to antibiotic hygromycin B. This plasmid (courtesy of Dr. Norimoto Murai, Louisiana State University, USA) was used as a source of *hpt* gene for making co-integrate vectors (pGH-I/pGH-II).

**pGH-I/pGH-II**

Co-integrate vectors, having both *gusA* and *hpt* genes, were specifically designed for use in this study. Hygromycin gene cassette comprising CaMV35S promoter, *hpt* coding sequence and tml terminator was taken out from plasmid pTRA151 and cloned in another plasmid pAct1-D having *gusA* gene under the control of rice *actin*-1 promoter and *nos* terminator at *Hind*III site. The recombinant plasmid was transformed into electro-competent cells of *E. coli* strain DH5α through electroporation. The transformed cells were plated on LB agar plates containing ampicillin (50 µg ml⁻¹) as the selectable marker. Plasmid DNA was isolated from the transformed colonies following alkaline lysis method. The isolated plasmid DNA was subjected to restriction analysis to confirm the cloning of *hpt* gene in pAct1-D.

**Mini-Prep - Plasmid Isolation from *E. coli* (Alkaline Lysis Method)**

A single *E. coli* colony was cultured in 3 ml liquid LB medium containing ampicillin (or appropriate antibiotic) and grown overnight at 37 °C. The culture was transferred to two Eppendorf tubes and centrifuged at 12,000 g for 1 minute. The supernatant was aspirated and the pellet was allowed to dry, preferably in an inverted position on a paper towel. Two hundred microliters (200 µl) of solution 1 was added to each of the Eppendorf tubes and the pellet was re-suspended in the solution by vigorous vortexing. Two hundred microliters (200 µl) of solution II was added to each of the Eppendorf tubes and mixed well by inverting the tube gently. The tubes were incubated for 5 minutes at room temperature. 200 µl of solution III was added to each of the Eppendorf tubes and mixed well by inverting the tube gently. The tubes were incubated for 5 minutes at room temperature. 200 µl of solution III was added to each of the Eppendorf tubes, mixed well and incubated for 5 minutes on ice. Centrifuged at 12,000 g for 5 minutes. The supernatant from both of the tubes was taken in a fresh Eppendorf tube and two volumes of absolute ethanol were added. The Eppendorf tube was kept at -20 °C for 20 minutes and then centrifuged at 12,000 g for 10 minutes. The
Figure 2.1. Physical maps of plasmids pAct1-D and pTRA151.

a. Physical map of plasmid pAct1-D

- Parent vector pBSII-KS (Bluescript) = 2.96 kbp
- Actin promoter = 2.24 kbp
- GUS gene = 1.8 kbp
- Nos terminator = 0.3 kbp
- → Direction of transcription

b. Hygromycin expression cassette (pTRA151)
supernatant was discarded and the pellet was washed with 70% ethanol. The pellet was allowed to air dry. Twenty microliters (20 µl) of sterile distilled water was added to the pellet to dissolve DNA. The quality and concentration of plasmid DNA was verified by electrophoresis of 2 µl of isolated DNA on 1% agarose gel. The plasmid DNA was stored at -20 °C.

Table 2.7. Solutions for plasmid isolation (Alkaline Lysis Method)

**Solution-I (Suspension buffer)**

<table>
<thead>
<tr>
<th>Components</th>
<th>Stock Conc.</th>
<th>Final Conc.</th>
<th>for 500 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-Cl, pH 8.0</td>
<td>50 mM</td>
<td>4.5 g</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>1 M</td>
<td>250 mM</td>
<td>12.5 ml</td>
</tr>
<tr>
<td>RNase</td>
<td>0.5 M</td>
<td>10 mM</td>
<td>10.0 ml</td>
</tr>
</tbody>
</table>

**Solution-II (Denaturation solution)**

<table>
<thead>
<tr>
<th>Components</th>
<th>Stock Conc.</th>
<th>Final Conc.</th>
<th>for 500 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH</td>
<td>5 M</td>
<td>0.2 N</td>
<td>0.8 ml</td>
</tr>
<tr>
<td>SDS</td>
<td>20 %</td>
<td>1 %</td>
<td>1.0 ml</td>
</tr>
</tbody>
</table>

*Not to be sterilized, use autoclave water.*

**Solution-III (Neutralization solution)**

<table>
<thead>
<tr>
<th>Components</th>
<th>for 100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium acetate (CH₃COOK)</td>
<td>60.0 ml</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>11.5 ml</td>
</tr>
<tr>
<td>Sterilized water</td>
<td>28.5 ml</td>
</tr>
</tbody>
</table>

**DNA Extraction from agarose gel using DNA Extraction Kit (MB1, Fermentas)**

The gel slice containing the required DNA band was excised using a clean and sharp blade such that there was a minimum amount of surrounding agarose excised with the fragment. The gel slice was transferred to a pre-weighed 1.5 ml tube. The gel slice was weighed and 3 volumes of binding solution (6 M sodium iodide) were added and incubated for 5 minutes at 55 °C to allow agarose to dissolve. Added 2 µl of the resuspended silica powder suspension
per 1 µg of DNA and incubated for 5 minutes at 55 °C. Mixed the contents by vortexing every 2 minutes to keep silica powder in suspension. Centrifuged silica powder/DNA complex for 5 seconds to form a pellet and removed supernatant. Added 500 µl of ice cold wash buffer (containing Tris, NaCl and EDTA), vortexed and centrifuged for 5 seconds and poured off the supernatant. Repeated this procedure three times. During each washing the pellet was resuspended completely. After removal of the supernatant from the last wash, the tube was centrifuged again and removed the remaining liquid with pipette. The pellet was allowed to air-dry for 10-15 minutes. DNA was eluted into water or TE buffer. The pellet was resuspended in an aliquot of sterile deionized water or TE buffer and incubated the tube at 55 °C for 5 minutes. Centrifuged the tube and removed the supernatant into a new tube avoiding the pellet. Repeated the elution with another aliquot of water or TE buffer. For the removal of small amounts of the silica powder, the tube was centrifuged again for 30 seconds in a table top centrifuge and the supernatant was transferred into a new tube. An aliquot was electrophoresed on agarose gel to determine the quality and amount of DNA by comparison with a control.

**Dephosphorylation reaction**

To 40 µl of the gel purified vector (pACT1-D) fragment, added 50 µl of TE. Added 10 µl (10X) alkaline phosphatase buffer followed by 0.5 µl alkaline phosphatase enzyme (0.22 u/µl; EC 3.1.3.1). Allowed to sit on ice at 37 °C for 30 minutes. Added 1 µl of 500 mM EDTA to the reaction mixture and placed the reaction tube in a water bath at 65 °C for 1 hour. Added equal volume i.e. 100 µl of phenol:chloroform mixture and mixed well by shaking/inverting the tube. Centrifuged at 12,000 g for 2-3 min. Removed upper phase and added 10 µl 3M NaOAc pH 7.0 and 2 volumes of absolute ethanol. Mixed and allowed to sit at -70 °C for 15 minutes. Centrifuged for 15 minutes at 4 °C, removed supernatant and washed pellet with 500 µl ice cold 70% ethanol. Again centrifuged for 10 minutes and the supernatant was removed. Allowed the pellet to dry at room temperature. Resuspended pellet in 1X TE buffer. Quality and quantity of the dephosphorylated fragment was determined by electrophoresis of the treated DNA fragment on 0.8% agarose gel.
**Ligation reaction**

For ligation of insert (hygromycin gene cassette, 1.7 kbp) and vector (pACT1-D, 8.0 kbp), two different mole ratios of the vector and insert (1:1 and 1:5) were used. (The weight ratio, of vector and insert would be approx. 4:1 and 20:1, respectively). The ligation was made in a 10 µl reaction with the following recipe:

<table>
<thead>
<tr>
<th>Mole ratio</th>
<th>1:1</th>
<th>1:5</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA vector (pACT-1D)</td>
<td>2.0 µl</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>Insert (hygromycin gene cassette)</td>
<td>1.0 µl</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>5X ligase buffer</td>
<td>2.0 µl</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>T4 DNA ligase (1 u/µl; EC 6.5.1.1)</td>
<td>1.0 µl</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Water</td>
<td>4.0 µl</td>
<td>4.8 µl</td>
</tr>
<tr>
<td>Total volume</td>
<td>10.0 µl</td>
<td>10.0 µl</td>
</tr>
</tbody>
</table>

**Preparation of Electro-competent cells of *E. coli* and Electroporation**

Competent cells of *E. coli* strain DH5α were prepared following the protocols as described in BTX Electroporator (ECM 600) Manual (BTX Inc. San Diego, California, USA). Single colony of *E. coli* strain DH5α was picked aseptically and transferred to 10 ml Luria Bertani (LB) liquid medium and grown overnight at 37 °C on a rotary shaker. Five ml of the culture was inoculated into 300 ml fresh LB medium and allowed to grow until OD600 reached between 0.5-1.0 (∼ 10^{10} cells ml^{-1}). The culture was transferred to 50 ml sterilized centrifuge tubes and placed on ice for 30 min. Centrifugation was carried out at 5,200 g at 4 °C for 15 min. Supernatant was discarded and the pellet was gently dissolved in 50 ml of cold deionized water. The cells were centrifuged at 5,200 g at 4 °C for 15 min. Supernatant was discarded and the pellet was dissolved gently in 25 ml cold deionized water. The tubes were centrifuged at 5,200 g for 15 min. and the supernatant was discarded. The pellet was again dissolved gently in 25 ml cold deionized water and the tubes were centrifuged at 5,200 g for 15 min. and the supernatant was discarded. The cells were finally washed in 10% glycerol,
centrifuged at 4,000 g at 4 °C for 15 min. and the pellet was redissolved gently in 200 µl of 10% glycerol. Aliquots of 40 µl were dispensed into 0.5 ml tubes, frozen in liquid nitrogen and stored at -80 °C.

**Electroporation of *E. coli* cells**

Electroporation was carried out in a BTX Electroporator Model ECM 600. The competent cells were removed from -80 °C freezer and allowed to thaw on ice. Two micro-liters (2 µl) of the plasmid DNA/ligation product was mixed with 40 µl of competent cells and transferred to pre-chilled electroporation cuvette (2 mm gap). Parameters for electroporation were set to the following conditions:

- **Voltage mode**: Select high voltage mode
- **Set charging voltage**: 2.4 kV
- **Set resistance**: 129 Ω

The cuvette was placed in the electroporation chamber and pulse was applied. Immediately after the application of pulse, 1 ml of liquid LB medium was added into the cuvette. The cells were transferred to a 15 ml polypropylene tube and allowed to grow for 1 hour at 37 °C in a shaking incubator. One hundred microliters (100 µl) of the above culture was spread on LB agar plates containing ampicillin (50 µg ml⁻¹). The plates were incubated at 37 °C for 16 hours.

Table 2.8. Luria-Bertani (LB) medium (Gerhardt *et al.*, 1994)

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantity per liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>5 g</td>
</tr>
<tr>
<td>Bacto agar</td>
<td>15 g</td>
</tr>
</tbody>
</table>

Adjust pH to 7.0 using NaOH and sterilize by autoclaving.

**Preparation of plant material for bombardment**

Two different types of tissues were used as the target material to see which is more appropriate. These included 28 day old scutellum-derived primary calli originating from mature seeds using callus induction medium (Table 2.2). Embryogenic calli were carefully
selected under the stereo-microscope. Approximately 30-40 embryogenic calli of 3-4 mm in size were arranged on bombardment medium.

The second type of target tissues were embryos excised from mature seeds. Mature seeds were sterilized by methods as described previously. Seeds were soaked in sterile water overnight. Embryos were excised under a stereo-microscope using a sharp scalpel. Approximately 50-60 embryos were arranged on the bombardment medium.

Both type of target tissue were bombarded using 5 µg plasmid DNA (pAct1-D) coated on 3 mg gold particles of 1.0 µm size, with rupture discs of 7,500 kPa (1,100 psi) and keeping the target distance of 9 cm.

**Preparation of DNA coated bullets and bombardment**
Particle bombardment studies were carried out using Particle Delivery System (PDS-1000/He; Bio-Rad Laboratories, Hercules, California, USA). Preparation/sterilization of micro-projectiles, precipitation of plasmid DNA on the micro-projectiles and bombardment procedure was followed as described by the manufacturer.

**Consumable Preparation**
Before use of the PDS-1000/He, appropriate rupture discs, the macro-carriers, macro-carrier holders and the stopping screens were sterilized by soaking them in 70% ethanol for 15 minutes followed by drying in a clean and sterile environment.

**Cleaning the PDS-1000/He and Accessories**
The sample holding plate and rupture disc retaining cap were rinse in 70% ethanol. Similarly the vacuum chamber of PDS-1000/He was swabbed with 70% ethanol. Sufficient time was allowed to let these accessories dry before further use.

**Micro-carrier Preparation**
Sixty mg gold particles were weighed in a microtube and added 1 ml 100% ethanol. The contents were sonicated for 5-10 seconds in an ultrasonic cleaner (Branson Ultrasonics Corporation, Danbury CT, USA) and then centrifuged at 12,500 g in a microfuge (Microfuge
E, Beckman Coulter, High Wycombe, UK) for 2-3 minutes. Sonication and vortex mixing was repeated three times and the contents were finally centrifuged at 12,500 g for 30 seconds. Supernatant was removed and discarded. Added 1 ml of sterile water and re-suspended gold particles by vortexing. The contents were centrifuged for 30 seconds and the supernatant was removed and discarded. Repeated this step once again and finally added 1 ml of sterile distilled water to the gold particles. Dispensed 50 µl aliquots of the final suspension into pre-sterilized microtubes while vortexing the suspension continuously. Stored the micro-carrier preps at 4 °C or room temperature.

Precipitation of DNA
To 50 µl aliquot of gold micro-carrier suspension in water, added in order under continuous vortexing, 5-10 µl of DNA (or as required) (conc. 1 µg/µl), 50 µl of 2.5 M CaCl₂ and 20 µl of 0.1 M spermidine (free base, tissue culture grade) and continued vortexing for three minutes. Centrifuged the tube at 12,500 g for 5 seconds and removed as much supernatant as possible. Washed the micro-carriers with 250 µl of cold (-20°C) 100% ethanol (HPLC or spectrophotometric grade) by vortexing briefly, centrifuged at 12,500 g for 2-3 seconds and discarded supernatant. Resuspended micro-carriers in 60 µl of 100 % ethanol by vortexing at low speed for 3-5 seconds. Macro-carriers were fixed into macro-carrier holders and 8 µl of DNA coated micro-carriers were dispensed onto the macro-carrier by drawing and loading the prep as quickly as possible (as the gold particles are heavy and settle very quickly in the microtube). The loaded micro-carriers were allowed to dry completely inside the laminar air flow cabinet before further use.

Bombardment
The main valve of the helium cylinder was opened. Appropriate helium pressure for bombardment was adjusted (should be adjusted approx. 690 kPa (100 psi) above the rupture disc pressure rating). Placed a rupture disc in the rupture disc retaining cap and screwed it up on the gas acceleration tube and slightly tightened with the help of a wrench (provided for this purpose). A stopping screen and the macro-carrier holder, loaded with DNA-coated micro-carriers, were placed in the respective slots provided for this purpose. The target tissue was placed on the target platform and the bombardment chamber was closed. The main
switch was turned “ON” and vacuum switch to turned to “VAC” position. The vacuum pump was turned on to create a vacuum in the bombardment chamber. Once the desired vacuum was generated, the vacuum switch was turned to “HOLD” position. The “FIRE” switch was pressed. The helium started getting accumulated in the gas acceleration tube. The pressure gauge read the pressure in the gas acceleration tube. The FIRE switch was kept pressed until the rupture disc ruptured with a blast. The FIRE switch was immediately released to allow the sterile-air to enter the chamber by turning the vacuum switch to “VENT” position. When the vacuum gauge reads zero, bombardment chamber was opened and the bombarded sample was removed.

**Histochemical GUS assay**

GUS assay was performed as described by Jefferson *et al.* (1987). Methanol was added to the buffer solution at a final concentration of 20% to suppress endogenous β–glucuronidase activity following Kosugi *et al.* (1990). Transient GUS expression studies were made 24-48 hours after bombardment of explants by overnight incubation in the GUS staining solution at 37 °C. Number of blue expression units (foci) were counted under the stereo-microscope.

Table 2.9. GUS Staining Solution*

<table>
<thead>
<tr>
<th>Components</th>
<th>(per 100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, cyclohexyl-ammonium salt</td>
<td>88.9 mg</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>10.0 mg</td>
</tr>
<tr>
<td>NaH₂PO₄, (0.2 M stock)</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Methanol</td>
<td>20.0 % (final volume)</td>
</tr>
<tr>
<td>pH</td>
<td>7.0-8.0</td>
</tr>
</tbody>
</table>

*Filter sterilized through 0.22 μ sterile filter and stored at -20 °C in an amber bottle.

**Optimization of different parameters affecting bombardment efficiency**

Particle Delivery System (PDS-1000/He) was used for these transformation studies. Different biological and physical parameters were studied for optimizing conditions for high efficiency delivery of DNA to the target tissues.
Effect of particle size, helium pressure and target distance on bombardment efficiency

Particle size, helium pressure and target distance can have drastic effects on transformation efficiency. In order to optimize these parameters, plasmid pAct1-D or pGH-1/pGH-II were used. The DNA 5 µg (conc. 1 µg/µl) was used to coat 3 mg gold particles. Gold particles of two different sizes i.e. 1.0 and 1.6 µm were used to coat plasmid DNA. Ruptures discs of 7,500 kPa (1,100 psi) and 10,500 kPa (1,550 psi) corresponding to equivalent bombardment pressure (± 5 %) were used. Approx. 50-60 mature Basmati rice embryos were arranged on bombardment medium with scutellum facing upward. These embryos were bombarded at different distances i.e. 3, 6, 9 and 12 cm.

Effect of particle and DNA loads

Different particle and DNA loads and particle/DNA combinations were used to define suitable particle and DNA loads and their ratio. Initially, 3 mg gold particles of 1.0 µm size were used with three different DNA loads i.e. 3, 6 and 9 µg of plasmid DNA (pAct1-D) referred to as combinations A, B and C, respectively. Based on these initial experiments, some other combinations were also tried like increasing the particle loads (from 3 to 4 or 5 mg) and DNA loads (up to 12 and 15 µg) while keeping the particle to DNA ratios at 1:2 and 1:3. Details of different particle/DNA combinations are given in Table 2.10.

Table 2.10. Different combinations of particle and DNA loads used for bombardment

<table>
<thead>
<tr>
<th>Combination</th>
<th>*Particle load (mg)</th>
<th>**DNA load (µg)</th>
<th>Particle:DNA ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3</td>
<td>3</td>
<td>1 : 1</td>
</tr>
<tr>
<td>B</td>
<td>3</td>
<td>6</td>
<td>1 : 2</td>
</tr>
<tr>
<td>C</td>
<td>3</td>
<td>9</td>
<td>1 : 3</td>
</tr>
<tr>
<td>D</td>
<td>4</td>
<td>8</td>
<td>1 : 2</td>
</tr>
<tr>
<td>E</td>
<td>4</td>
<td>12</td>
<td>1 : 3</td>
</tr>
<tr>
<td>F</td>
<td>5</td>
<td>10</td>
<td>1 : 2</td>
</tr>
<tr>
<td>G</td>
<td>5</td>
<td>15</td>
<td>1 : 3</td>
</tr>
</tbody>
</table>

* gold particles, 1 µm size  
** plasmid DNA
**Effect of number of shots on transient GUS expression**

Number of shots can have a significant positive and negative effect on transient as well as stable transformation efficiency. Therefore, the target tissues were bombarded either once or twice. The conditions for bombardment were same as optimized in the above experiments i.e. 9 µg of DNA was used to coat gold particles (1.0 µm), and the embryos were bombarded at a distance of 6 and 9 cm with 7,500 kPa helium pressure. Effect of number of shots on the target tissues was studied in duplicate.

**Effect of osmotic treatment**

Media having different concentrations of sugars/sugar alcohols were used to define a suitable medium for bombardment. Callus induction medium containing 3% sucrose was used as a control. Two other media having the same composition but additional 0.25 M and 0.4 M mannitol, respectively (Table 2.11) were compared with control for transient GUS expression in mature rice embryos. Bombardment conditions were same as optimized and described in the preceding sections.

**Determination of optimum concentration of Hygromycin B for selection of transformed cells**

Primary embryogenic calli (28 days old) developed from mature seeds of B-370 were placed on callus induction medium having different concentrations of hygromycin B i.e. 0, 25, 50 and 100 mg L\(^{-1}\) (Table 2.11). Data regarding the effectiveness of hygromycin B for killing the cells was recorded after 30 days.

Table 2.11. Media used for bombardment, selection of transformed cells and germination of seeds

<table>
<thead>
<tr>
<th>Media*</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bombardment medium</strong></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>MS salts and vitamins having 2,4-D 2.0/4.0 mg L(^{-1}) + sucrose 30 g L(^{-1}) + phytigel 0.3%</td>
</tr>
<tr>
<td>0.25 M</td>
<td>MS medium having 2,4-D 2.0/4.0 mg L(^{-1}) + sucrose 30 g L(^{-1}) + mannitol + phytigel 0.3%</td>
</tr>
<tr>
<td>0.4 M</td>
<td>MS medium having 2,4-D 2.0/4.0 mg L(^{-1}) + sucrose 30 g L(^{-1}) + mannitol + phytigel 0.3%</td>
</tr>
<tr>
<td>Selection medium</td>
<td>MS medium having 2,4-D 2.0/4.0 mg L(^{-1}) + sucrose 30 g L(^{-1}) +</td>
</tr>
<tr>
<td><strong>Germination medium</strong></td>
<td>hygromycin B 0-100 mg L⁻¹ + phytage 0.25%</td>
</tr>
<tr>
<td>-----------------------</td>
<td>-------------------------------------------</td>
</tr>
<tr>
<td></td>
<td>MS medium having 2,4-D 2.0/4.0 mg L⁻¹ + sucrose 30 g L⁻¹ + phytage 0.25%</td>
</tr>
</tbody>
</table>

*pH of all the media was adjusted to 5.8 prior to autoclaving.

**Plant regeneration from hygromycin resistant calli**

Selected calli obtained from pGH-1/pGH-II bombarded embryos were transferred to optimized pre-regeneration medium (Table 2.3) containing 50 mg L⁻¹ hygromycin B. Approx. 10-12 hygromycin resistant calli were transferred to the plates (100 x 20 mm, disposable sterile) containing 25-30 ml pre-regeneration medium (PRM-1). After 10-12 days, the calli were further transferred to plant regeneration medium (PRM-2; Table 2.3). After two weeks, the calli producing plantlets were further transferred to fresh PRM-2 medium. The regenerated plantlets were transferred to root proliferation medium without hygromycin B.

**Establishment of putative transgenic plants in hydroponics/soil**

The regenerated putative transgenic plants were established in the soil following methods as described previously. Regenerated putative transgenic plants were acclimatized and established first in sand and then transferred to soil in earthen pots and kept in containment glass-house for further growth and maintenance.

**Genomic DNA isolation from putative transgenic plants**

For genomic DNA isolation, method described by Iqbal et al. (1997) was used. 20 mL 2X cetyltrimethylammonium bromide (CTAB; Table 2.12) and 100 μL of 2-mercapto-ethanol was mixed in a 50 mL polypropylene tube. The tubes were placed in a water bath at 65 °C for 30 minutes. Fresh leaves of the plant were collected in liquid nitrogen. The sample (1-2 g) was ground to fine powder in a pre-cooled pestle mortar. The ground leaf samples were transferred to 50 ml sterile polypropylene tube containing hot CTAB solution and incubated at 65 °C for 30 minutes with occasional swirling. After incubation, equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed gently. The tubes were centrifuged at 11,000 g for 10 minutes at room temperature and the supernatant was taken into a fresh 50 ml polypropylene tube and 0.6 volume of cold isopropanol (kept at -20 °C) was added to it. The tubes were incubated at -20 °C for one hour. After the incubation, the tubes were
centrifuged at 11,000 g for 10 minutes. The supernatant was discarded and the pellet was washed with 70% ethanol, air dried and resuspended in 1 ml TE buffer. DNA was treated with RNase (10 mg/ml; EC 3.1.27.5) at 37 °C for 1 hour. RNase was removed after adding and mixing gently with equal volume of chloroform : isoamyl alcohol (24:1) followed by spinning for 10 minutes at 12,000 g in a microcentrifuge. The supernatant was transferred to a new 15 ml polypropylene tube. DNA was precipitated after adding 1/10 volume of 3 M NaOAc and 2 volumes of absolute ethanol. DNA pellet was collected after spinning at 12,000 g for 10 minutes followed by washing with 70% ethanol. DNA pellet was air-dried and resuspended in TE buffer. In order to estimate the DNA concentration, 10 µl of purified DNA was diluted in 900 µl of deionized water and optical density (OD) was measured at 260 and 280 nm on a UV/VIS spectrophotometer. An OD_{260} of 1 corresponds to a dsDNA concentration of 50 µg/ml. The ratio 260/280 gives an indication of the purity of the DNA as it should be between 1.8 to 2.0 (Sambrook et al., 1989).

Table 2.12. Reagents used for genomic DNA isolation and agarose gel electrophoresis

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantity</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2X CTAB</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTAB</td>
<td>20 g</td>
<td>Dissolved and made the final volume to 1 L with ddH2O.</td>
</tr>
<tr>
<td>EDTA, 0.5 M (pH 8.0)</td>
<td>40 ml</td>
<td></td>
</tr>
<tr>
<td>Tris HCl (pH 8.0)</td>
<td>100 ml</td>
<td></td>
</tr>
<tr>
<td>NaCl, 5 M</td>
<td>280 ml</td>
<td></td>
</tr>
<tr>
<td>PVP (polyvinylpyrrolidone)</td>
<td>10 g</td>
<td></td>
</tr>
<tr>
<td><strong>3 M Na acetate (pH 5.2)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium acetate,3H₂O</td>
<td>40.8 g</td>
<td>Adjusted pH with glacial acetic acid to 5.2 and made the volume up to 100 ml, autoclaved and stored at room temperature</td>
</tr>
<tr>
<td>Water</td>
<td>80 ml water</td>
<td></td>
</tr>
<tr>
<td><strong>RNase solution</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pancreatic RNase</td>
<td>5-10 mg</td>
<td>Dissolved 5-10 mg RNase per ml of TE buffer. Boiled the solution for 5 minutes</td>
</tr>
</tbody>
</table>
minutes to destroy any DNase and allowed to cool slowly at room temperature for at least 5 hours. Stored at 4 °C or prepared aliquots and stored at -20 °C.

<table>
<thead>
<tr>
<th>Buffers and solutions</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Contents</th>
<th>Quantity</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TE Buffer, pH 8.0</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trizma base</td>
<td>1.21 g</td>
<td>Dissolved in water, made the volume to 1000 ml, adjusted pH to 8.0 using HCl, and sterilized by autoclaving.</td>
</tr>
<tr>
<td>Na-EDTA</td>
<td>0.3722 g</td>
<td></td>
</tr>
</tbody>
</table>

| **50X Tris-acetate EDTA buffer (TAE)** |
| Tris base | 242.0 g | Made up the final volume to 1000 ml with distilled water. |
| Glacial acetic acid | 57.1 ml | |
| 0.5 M EDTA (pH 8.0) | 200.0 ml | |

| **6X Gel loading buffer** |
| Bromophenol blue (10%) | 1.0 ml | Mixed and stored at -20 °C in aliquots. |
| Xylene cyanole FF (10%) | 1.0 ml | |
| Glycerol | 5.0 ml | |
| TAE (10X) | 1.0 ml | |
| Water | 2.0 ml | |

| **Ethidium Bromide Solution** |
| Ethidium bromide | 1 g | Dissolved using a magnetic stirrer, made the volume to 100 ml and stored in dark or wrapped the bottle in aluminium foil. |
| Water | 90 ml | |
Molecular analysis of putative transgenic plants

The regenerated plants established in soil were subjected to molecular analysis for the detection of the transferred gene(s). For this purpose, polymerase chain reaction (PCR) was used. DNA from putative transgenic plants was isolated following protocols as described by Iqbal et al. (1997). PCR reaction was performed in 25 µl of reaction mixture (as shown in Table 2.13). The reaction mixture contained 50-100 ng DNA from the putative transgenic plants and the un-transformed plants (negative control) and RG100 (Ghareyazie et al., 1997) and either gusA or hpt primers. The reaction mixture was subjected to 1 cycle of 94 °C for 5 minutes followed by 35 cycles of 94 °C for 1 min. (denaturation), 60 °C for 1 min. (annealing) and 72 °C for 3 min. (extension); followed by 1 cycle of 5 min. at 72 °C to complete the reaction. Following controls were included in PCR analysis of putative transgenic rice plants. i) water control or no DNA control to ensure that there is no DNA contamination in the reagents; ii) negative control containing the DNA from a non-transgenic plant; iii) positive or plasmid DNA control used to establish that the primers are functional and that the amplified product is that of expected size; and iv) internal control (Ghareyazie et al., 1997) i.e. inclusion of a pair of primers in addition to the primer pair which amplifies the target sequence. RG100 pair of primers can amplify a single band from any DNA extracted from rice. This control shows that whether the PCR reaction was successfully performed or it was a failure and thus helps in making a confident decision that negative lines are really negative.

After amplification, 10 µl of each of the PCR product was run through 1.8 % agarose gel containing 0.5 µg/µl ethidium bromide at 100 V for 3-4 hours. The size of the PCR products was visualized under UV light and photographs were taken using Eagle Eye photodocumentation system (Stratagene, La Jolla, CA, USA).

Table 2.13. Components of PCR reaction

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR reaction mixture</td>
<td></td>
</tr>
<tr>
<td>PCR buffer (10 X)</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>dNTPs (1 mM)</td>
<td>3.0 µl</td>
</tr>
<tr>
<td>RG100-F primer (20 ng/µl)</td>
<td>1.0 µl</td>
</tr>
</tbody>
</table>
DEVELOPMENT OF BACTERIAL BLIGHT RESISTANT RICE

Plant materials
Mature embryos of five different Basmati rice varieties namely B-370, B-2000, B-Pak, B-385 and S-Basmati were used in this study. Methods for excision and culture of embryos are already discussed in the preceding sections.

Plasmids used for transformation
The cloned Xa21 gene was acquired from University of California, Davis, USA through Pakistan Agricultural Research Council (PARC), Islamabad, Pakistan. The physical map of the plasmid pC822 having cloned Xa21 gene is shown in Figure 2.2a. The 9.6 kbp Xa21 locus is cloned at Kpn1 sites in the parent vector. Another vector named pZS1 (Figure 2.2b) containing the hygromycin resistance (hpt) gene as a selectable marker under the regulatory control of 35S promoter was used in this study. Both the vectors were used in 3:1 mole ratio for co-transformation of Xa21 and hpt genes.

Transformation/selection of transformed cells/regeneration of putative bacterial blight resistant plants and their establishment in soil
Mature embryos of five different Basmati rice varieties were used in these transformation experiments. The methods for transformation, selection of transformed cells, regeneration of putative transgenic plants from selected calli and their establishment in soil were same as described previously.
Figure 2.2. Physical maps of plasmids used for stable transformation of rice. a) A partial map of plasmid pC822 showing different regions within the Xa21 gene. U1 and U1 are the regions showing the primers used to amplify the Xa21 gene fragment while a 3.8 kbp EcoRV region was used as a probe for southern analysis; b) physical map of plasmid pZS1 having a plant selectable marker (hpt) gene under the regulatory control of 2X CaMV35S promoter and CaMV termintor.
Molecular analysis of putative bacterial blight resistant transgenic plants

The regenerated plants established in soil were subjected to molecular analysis for the detection of the \textit{Xa21} and \textit{hpt} gene(s). For this purpose PCR method was used for initial screening and progeny analysis while southern analysis was used to further validate the PCR results as well as to get information on the integration of foreign gene(s) at different loci.

\textit{PCR analysis of putative transgenic plants}

PCR can be used as a routine analytical tool for quick analysis of putative transgenic plants for the presence of foreign genes. Putative transgenic rice plants were first screened by polymerase chain reaction (PCR) using \textit{Xa21} specific primers (Table 2.14). DNA from putative transgenic plants was isolated following protocols as described by Iqbal \textit{et al.} (1997). PCR reaction was performed in 25 µl of reaction mixture as described previously (Table 2.13) containing 50-100 ng DNA from the putative transgenic plants and the un-transformed plants (negative control) and 40 ng of \textit{hpt} and/or \textit{Xa21} primers with or without 20 ng each of RG100 primers (Table 2.14). The reaction mixture was subjected to 1 cycle of 94 °C for 5 minutes followed by 35 cycles of 94 °C for 1 min. (denaturation), 60 °C for 1 min. (annealing) and 72 °C for 3 min. (extension); followed by 1 cycle of 5 min. at 72 °C to complete the reaction. A number of different controls were included in PCR analysis of putative transgenic rice plants to authenticate PCR results as discussed and described previously.

After amplification, 10 µl of each of the PCR product was electrophoresed through 1.0 % agarose gel containing 0.5 µg/µl ethidium bromide at 100 V for 3-4 hours. The size of the PCR products was visualized under UV light and photographs were taken.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gusA-F</td>
<td>GCCATTTGAAGCCGATGTCACGCC</td>
</tr>
<tr>
<td>gusA-R</td>
<td>GTATCGGTGTAGCGTGCAGAAC</td>
</tr>
<tr>
<td>Hpt-F</td>
<td>AGAATCTCGTGCTTTTCAGCTTCGA</td>
</tr>
<tr>
<td>Hpt-R</td>
<td>TCAAGACCAATGCGGAGCATATAC</td>
</tr>
</tbody>
</table>
Southern Analysis

Ten micrograms of (10 µg) genomic DNA from putative transgenic (T₀) lines and non-transformed control was digested with appropriate restriction enzymes. Digested and undigested DNA samples were fractionated through 0.8% (w/v) agarose gel. The DNA was transferred to nylon membrane according to Southern (1975). The gel was placed in 500 ml of 0.25 N HCl (Table 2.15) in a tray and placed on a shaker at low speed for 10 min. The gel was rinsed with distilled water before transferring it to the alkali transfer (AT) buffer (0.4 M NaOH; Table 2.15) for 20 minutes to denature the DNA. Transferred the gel to fresh AT buffer and equilibrated for another 20 minutes. For alkali transfer, filled the tray, of an appropriate size, with AT buffer, placed a bridge of Whatman 3 mm filter paper, soaked in AT buffer. Placed a clean glass plate across the tray and covered the glass plate with a wet paper so that the ends are dipped in the buffer solution. Placed the gel on the wet paper and removed any trapped air bubbles beneath the paper. Cut a sheet of blotting membrane (Hybond N⁺) of the size of the gel and placed on top of the gel. Covered the membrane filter with two sheets of Whatman 3mm paper, soaked in AT buffer, of the same dimensions as the nylon membrane. Stacked absorbent paper towels (approx. 5 cm high) on top of the 3 mm paper and placed a glass plate on top of the paper towels. Placed ~ 1 kg weight on top and allowed the transfer to proceed overnight. After blotting, washed the membrane briefly in 2XSSC (Table 2.15) to remove any adhering agarose. For hybridization, added 40 ml of hybridization buffer (Table 2.15) in a hybridization box. Boiled 75 µl sheared salmon sperm DNA (ssDNA, 10 mg/ml) in a microcentrifuge tube for 5-10 minutes, in a boiling water bath. Added sssDNA to the hybridization buffer and mixed well. Added the filters to the hybridization boxes and pre-hybridized for at-least one hour at 65 °C in a chamber shaker. After 1 hour, added [α³²P]-labelled probe consisting of the nick translated coding sequence of the Xa21 gene and allowed to hybridize overnight at the same temperature as for pre-
hybridization. Following day, washed the filters in a plastic tray with shaking as follows: 2XSSC, 0.1% SDS, room temperature, two times, each for 10 minutes; followed by 1XSSC, 0.1% SDS at hybridization temperature for 15 minutes. Then filters were washed with 0.5X SSC, 0.1% SDS at 65 °C for 10 minutes. The filters were removed, wrapped in Saran wrap and exposed to x-ray film for 2-3 days at -70 °C.

Table 2.15. Solutions for Southern Hybridization

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantity</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>0.25 N HCl</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 N HCl</td>
<td>20.83 ml</td>
<td>Adjust volume to 1 liter with distilled water.</td>
</tr>
<tr>
<td>distilled water</td>
<td>700 ml</td>
<td></td>
</tr>
</tbody>
</table>

**Alkali transfer buffer (0.4 M NaOH)**

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantity</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH pellets</td>
<td>16 g</td>
<td>Adjust volume to 1 liter with distilled water.</td>
</tr>
<tr>
<td>Distilled water</td>
<td>900 ml</td>
<td></td>
</tr>
</tbody>
</table>

**20X SSC (stock)**

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantity</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>175.3 g</td>
<td>Dissolved in approx. 800 ml ddH2O, adjust pH to 7.0 with hydrochloric acid (HCl) and made final volume to 1 L.</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>88.2 g</td>
<td></td>
</tr>
<tr>
<td>Nanopure water</td>
<td>800.0 ml</td>
<td></td>
</tr>
</tbody>
</table>

**Hybridization buffer**

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantity</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium phosphate buffer (0.5 M stock), pH 7.2</td>
<td>5.0 ml</td>
<td>Make volume to 20 ml with deionized water</td>
</tr>
<tr>
<td>Sodium chloride (5 M stock)</td>
<td>1.0 ml</td>
<td></td>
</tr>
<tr>
<td>SDS</td>
<td>2.8 g</td>
<td></td>
</tr>
</tbody>
</table>

**Progeny analysis**

Seeds of T₀ transgenic rice were collected and analysed for presence and expression of foreign genes. Ten seeds of each transgenic line along with non-transformed control were surface sterilized and plated on MSO medium (MS medium without growth regulators; Table 2.11) either without hygromycin B or containing 25 mg L⁻¹ hygromycin B. Seed viability was
determined by the number of seeds that germinated on medium without hygromycin. Similarly, the number of seeds that germinated and formed plants on MSO medium supplemented with hygromycin B were counted. The plants germinated on these plates were transferred to soil in the pots and grown to maturity inside the containment glass-house.

Polymerase chain reaction was also used to detect the presence of \textit{Xa21} gene in the T\textsubscript{1} plants. For this purpose, DNA isolated from T\textsubscript{1} transgenic lines was subjected to PCR analysis using \textit{Xa21} specific primers (Table 2.14). Similarly, DNA extracted from the T\textsubscript{2} plants was subjected to PCR analysis to look for presence/absence of \textit{Xa21}/\textit{hpt} genes.

\textbf{Screening against BB resistance}

Screening of T\textsubscript{1} plants was conducted under the containment glass-house conditions. T\textsubscript{1} seeds were collected from the primary transformants. Ten T\textsubscript{1} seeds of each line were sown in separate earthen pots, containing soil. At three-leaf stage, two plants of each of these transgenic lines along with non-transformed control were transplanted to earthen pots so that each line contained three pots having two plants each. Plants aged sixty days were used to screen for bacterial blight resistance.

Two different local isolates of \textit{X. oryzae. pv. oryzae} namely \textit{Xoo} 1.2.1 and \textit{Xoo} 3.2.2 were used to screen rice transgenic lines for bacterial blight resistance. These isolates were obtained from the Nuclear Institute for Agriculture and Biology, Faisalabad. The inoculum of both the isolates was prepared following methods as described by Tu \textit{et al.} (1998) by incubating the bacteria in Wakimoto’s medium (Table 2.16) for 72 hours at 30 °C followed by suspension of pure culture in sterile distilled water such that cell density of about $10^9$ cells per ml was achieved. The transgenic T\textsubscript{1} plants along with their respective non-transgenic controls were grown in the containment glass-house. A temperature of 30 °C and 85% relative humidity (RH) during the day time while 25 °C and 90 % RH at night were maintained inside the containment glass-house throughout the screening duration. Five to six leaves of each plant were inoculated, at the maximum tillering stage, with the above two isolates of \textit{Xoo} using the leaf clipping method (Kaufman \textit{et al.}, 1973). For this purpose, scissors dipped in the bacterial suspension was used for clipping off the leaves. The inoculated plants were surveyed every day to monitor the symptom development. Plant
reaction was scored after 14 days of inoculation. Disease reactions were characterized as resistant (R), moderately resistant (MR), or susceptible (S) based on leaf lesion length of 0-3 cm, 3-6 cm or > 6 cm, respectively.

Table 2.16. Wakimoto’s medium

<table>
<thead>
<tr>
<th>Components</th>
<th>Quantity</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato</td>
<td>200 g</td>
<td>Dissolved sucrose, peptone, sodium phosphate and calcium nitrate in 200 ml water and mixed with the broth from 200 g of boiled potatoes, made volume to 1 liter with ddH₂O and adjusted pH to 7.0.</td>
</tr>
<tr>
<td>Sucrose</td>
<td>15.0 g</td>
<td></td>
</tr>
<tr>
<td>Peptone</td>
<td>5.0 g</td>
<td></td>
</tr>
<tr>
<td>Na₂HPO₄·2H₂O</td>
<td>2.0 g</td>
<td></td>
</tr>
<tr>
<td>Ca(NO₃)₂</td>
<td>0.5 g</td>
<td></td>
</tr>
</tbody>
</table>

**Field Performance of Transgenic BB resistant Basmati Lines**

Limited field trials were conducted at the designated field area inside the premises of the National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad. The field experiments were performed following a randomized complete block (RCB) design with three replications. Seed of some of the selected S-Basmati transgenic lines were germinated in the glasshouse and transplanted into the field by hand 30 days after seeding. Seedlings were planted in plots measuring 1.8 x 1.8 m with plant to plant distance of 20 cm. All plots received a pre-plant application of NP fertilizer @ 100 and 80 kg ha⁻¹. Standard agronomic practices were used throughout the rice growing period. Data regarding different traits like days to flowering, plant height, number of fertile tillers per plant; and grain characteristics like grain length and grain width were recorded. Days to flowering was measured as number of days from sowing to the time when 50% of the panicles emerged. Plant height was measured on the main culm or the tallest tiller at or following anthesis from the ground level to the tip of the panicle in centimeters (cm). Total number of fertile tillers was counted on each plant at the time of maturity. Grain yield per plant was determined by weighing hand-threshed, clean and dried grains harvested from panicles from each plant. Grain length (n = 10) was measured in millimeters as the distance from the base of the lower-
most sterile lemma to the tip (apiculus) of the fertile lemma or palea, whichever was longer. Width of grain ($n = 10$) was measured in millimeters as the distance across the fertile lemma and the palea at the widest point.
CHAPTER 3

IN-VITRO STUDIES

Cell and tissue culture is a prerequisite for any genetic manipulation work. Considerable progress has made in tissue culture and whole plant regeneration of rice during the past few years; however, little work has been done on Basmati rice (Grewal et al., 2005). As a prerequisite, availability of a highly efficient plant regeneration system from in-vitro cultures is essential before embarking on genetic transformation studies. Hence a reproducible and an efficient plant regeneration system from callus cultures of different Basmati rice cultivars was developed by exploiting different growth regulator combinations, carbon sources etc. for practical utilization of this technology in genetic improvement of Basmati rice.

Callus induction

Five different Basmati rice varieties Basmati 370 (B-370), Basmati-2000 (B-2000), Basmati-Pak (B-Pak), Basmati-385 (B-385) and Super Basmati (S-Basmati) were tested for callus induction and subsequent plant regeneration ability. Ten different treatments (T1-T10) based on MS (Murashige and Skoog, 1962) medium supplemented with different concentrations of 2,4-D (0.0 - 4.0 mg L\(^{-1}\)) and combination of 2,4-D (0.0 – 4.0 mg L\(^{-1}\)) and BAP (0.5 mg L\(^{-1}\)) as shown in Table 3.1 were used to define suitable medium for high frequency embryogenic callus induction. The data for callus induction was recorded after 28 days of culture of mature seeds. The mean differences among the ten different treatments, tested by Duncan’s multiple range test (DMRT) for embryogenic callus induction frequency (ECIF), are presented in Table 3.1. Callus was induced from all the rice varieties at varying frequency. In general, all of the tested genotypes performed well in their response to callus induction (Figure 3.1 a-c). Two types of callus were produced in all the varieties as shown in Figure 3.1 d. One type was pale yellow to white, compact and nodular termed as embryogenic callus. The other type of callus was yellowish brown, watery and some times mucilaginous and considered as non-embryogenic. No callus was induced from any of the five varieties in callus induction medium (CIM) without 2,4-D or CIM having BAP only. B-Pak exhibited highest (90.4 %) while B-385 showed lowest (63.6 %) ECIF.
In B-370, significant difference among different treatments was observed (Table 3.1). Highest ECIF (76.3 %) was observed in T4 followed by T10 (68.3 %), T9 (66.2 %) and T5 (62.3 %) respectively, which differed non-significantly from T4. No callus was induced in callus induction medium without growth regulators (T1) or medium having BAP alone (T6). Increasing the 2,4-D concentration from 0.5 to 4.0 mg L⁻¹ either alone or combination with BAP generally increased ECIF, except T5 where a decrease in ECIF was recorded. Therefore T4 was found to be optimum medium for embryogenic callus induction in B-370.

Highest ECIF in B-2000 was observed in T5 (66.6%) which significantly differed from other treatments (Table 3.1). This was followed by T10 (60.2 %), T4 (58.7 %) and T9 (58.4 %) respectively, which did not differ significantly from each other. Increasing the 2,4-D concentration from 0.5 to 4.0 mg L⁻¹ either alone or combination with BAP increased ECIF. Therefore, T5 was found to be optimum medium for embryogenic callus induction in B-2000.

In B-Pak, non-significant difference at p=0.05 was observed among three treatments i.e. T5, T4 and T10. Highest ECIF (90.4 %) was observed in T5 which was followed by T4 (88.3 %), T10 (85.8 %) and T9 (80.3 %) respectively. No callus was induced in callus induction medium without growth regulators (T1) or medium having BAP alone (T6). Increasing the 2,4-D concentration from 0.5 to 4.0 mg L⁻¹ either alone or in combination with BAP increased ECIF. From these results it can be concluded that T5 or T4 are optimum media for embryogenic callus induction in B-2000.

Highest ECIF in B-385 was recorded in T5 (63.6 %) which differed non-significantly from T4 (61.1 %) at 5% level of significance. No callus was induced in callus induction medium without growth regulators (T1) or medium having BAP alone (T6). Increasing the 2,4-D concentration increased ECIF. From these results it can be concluded that T5 or T4 are optimum media for embryogenic callus induction in B-385.
Table 3.1. Effect of different combinations of growth regulators (2,4-D and BAP) in Murashige and Skoog (MS) medium on embryogenic callus induction frequency (ECIF) from five different Basmati rice varieties.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>2,4-D/BAP mg l(^{-1})</th>
<th>Embryogenic callus induction frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B-370</td>
<td>B-2000</td>
</tr>
<tr>
<td>T1</td>
<td>0.0/0.0</td>
<td>0.00 ± 0.0 e</td>
</tr>
<tr>
<td>T2</td>
<td>0.5/0.0</td>
<td>18.48 ± 1.7 e</td>
</tr>
<tr>
<td>T3</td>
<td>1.0/0.0</td>
<td>51.03 ± 1.0 c</td>
</tr>
<tr>
<td>T4</td>
<td>2.0/0.0</td>
<td>76.29 ± 8.9 a</td>
</tr>
<tr>
<td>T5</td>
<td>4.0/0.0</td>
<td>62.33 ± 1.0 b</td>
</tr>
<tr>
<td>T6</td>
<td>0.0/0.5</td>
<td>0.00 ± 0.0 f</td>
</tr>
<tr>
<td>T7</td>
<td>0.5/0.5</td>
<td>13.30 ± 1.4 e</td>
</tr>
<tr>
<td>T8</td>
<td>1.0/0.5</td>
<td>36.19 ± 6.0 d</td>
</tr>
<tr>
<td>T9</td>
<td>2.0/0.5</td>
<td>66.24 ± 3.8 b</td>
</tr>
<tr>
<td>T10</td>
<td>4.0/0.5</td>
<td>68.27 ± 1.4 b</td>
</tr>
<tr>
<td>c.v.</td>
<td></td>
<td>9.5%</td>
</tr>
</tbody>
</table>

Means within a column followed by a common letter are not significantly different at 5% level by DMRT.
Each value is a mean of three replicates.
\(\pm\) standard error between replicates.
c.v. = coefficient of variation
In S-Basmati, highest embryogenic callus induction frequency was recorded in T5 (71.7 %) followed by T4 (67.1 %). Non-significant difference, at 5 % level of significance, was observed between T5 and T4. Media combinations T9 (51.8 %) and T10 (42.2 %) having 2.0 and 4.0 mg L\(^{-1}\) 2,4-D supplemented with 0.5 mg L\(^{-1}\) BAP, respectively followed T5 and T4. This showed that supplementation of BAP lowered embryogenic callus induction in S-Basmati. No callus was induced in callus induction medium without growth regulators (T1) or medium having BAP alone (T6). Increasing the 2,4-D concentration increased ECIF except T10 where a decrease in ECIF was observed when 2,4-D was increased from 2.0 to 4.0 mg L\(^{-1}\) in callus induction medium having 0.5 mg L\(^{-1}\) BAP. From these results it can be concluded that T5 or T4 are optimum media for embryogenic callus induction in S-Basmati.

From these results it can be concluded that increasing the 2,4-D concentration in the callus induction medium generally increased ECIF as shown in the trend lines for ECIF of five different rice varieties on media having different concentrations of 2,4-D (Figure 3.2 a) except in B-370 in the treatment T5 where increasing the 2,4-D concentration decreased ECIF from 76.3 % in T4 (2 mg L\(^{-1}\) 2,4-D) to 62.33 % in T5 (4 mg L\(^{-1}\) 2,4-D). Similarly, increasing 2,4-D concentration in the callus induction medium supplemented with BAP (Figure 3.2 b) generally increased ECIF as shown in the trend lines for ECIF of five different rice varieties on media having different concentrations of 2,4-D and BAP (Figure 3.2 b) except in S-Basmati where increasing the 2,4-D concentration from 2 mg L\(^{-1}\) to 4 mg L\(^{-1}\) decreased ECIF from 51.8 % (T9) to 42.2 % (T10). This study shows that 2,4-D alone was sufficient to induce embryogenic callus in all of the varieties and there was generally a negative effect of BAP supplementation in the callus induction medium except in some instances like T10 in B-370; T2 and T3 in B-2000; T3 in B-Pak and T7 in B-385.

T4 and T5 having 2.0 and 4.0 mg L\(^{-1}\) 2,4-D, respectively proved to be the best media for embryogenic callus induction in B-Pak, B-385 and S-Basmati and no significant differences were observed between these two treatments (Table 3.1), therefore T4 (having 2 mg L\(^{-1}\) 2,4-D) was preferred in all the subsequent studies. Similarly T4 proved to be the most suitable medium for B-370 while T5 exhibited highest embryogenic callus induction in B-2000,
Figure 3.1. Mature seeds of B-370 placed on callus induction medium (a); callus proliferation of B-370 from mature seeds 14 d after culture (b); Embryogenic callus induction from mature seed of B-370 (c); Calli induced from mature seeds of B-385 showing embryogenic (E) and non-embryogenic (NE) sectors (b); Globular and notch-shaped somatic embryos in S-Basmati cultures (e); Plant regeneration from embryogenic calli of B-370 on plant regeneration medium after 4 and 8 weeks of culture (f)
Figure 3.2. General trend of embryogenic callus induction from five different basmati rice varieties on MS medium supplemented with (a) different concentrations of 2,4-D and (b) different concentrations of 2,4-D and BAP (0.5 mg L\(^{-1}\)).
therefore, T4 and T5 were used for further studies on B-370 and B-2000.

**Plant regeneration from calli**

Six different MS based media combinations (R1 to R6) as shown in Table 3.2 were used either without growth regulators (R1 and R4) or supplemented with different combinations of kinetin, IAA and NAA and (R2, R3, R5 and R6) either having sucrose or maltose as the carbon source. Regeneration was achieved in two steps. The calli were first placed on pre-regeneration medium (PRM-1) for induction of somatic embryos and later transferred to plant regeneration medium (PRM-2) for regeneration of complete plants. PRM-1 differed from PRM-2 only in having abscisic acid (10 mg L⁻¹).

Significant differences (at p = 0.05) in plant regeneration frequency were observed within the different treatments. The mean differences among the six different treatments on plant regeneration frequency from Basmati rice cultivars, tested by DMRT, are presented in Table 3.2. Plant regeneration was achieved in all the five Basmati varieties with varying frequency. Highest plant regeneration frequency of 74.5% was achieved in S-Basmati followed by 70.4 % in B-Pak, 66.8 % in B-370, 58.0 % in B-2000 and 44.2 % in B-385 (Table 3.2). Plant regeneration efficiency remained low in treatments R1 and R4 (having no growth regulators) in all the varieties tested indicating that auxin and cytokinin combination was essentially required for induction of somatic embryos and subsequent plant regeneration. Treatments R3 (having kinetin, NAA and sucrose) and R6 (having kinetin, NAA and maltose) proved better for plant regeneration than R2 (having kinetin, IAA and sucrose) and R5 (having kinetin, IAA and maltose) in B-2000, B-385 and S-Basmati, respectively while no significant difference was observed in B-370 and B-Pak.

This indicated that medium supplemented with kinetin + NAA was better for somatic embryo induction and plant regeneration in B-2000, B-385 and S-Basmati; while for B-370 and B-Pak, both the media having either kinetin + NAA or kinetin + IAA did not differ significantly. Plant regeneration frequency was generally higher in all the varieties on regeneration media containing maltose except B-385 where a non-significantly higher regeneration frequency was obtained in media containing sucrose. Maltose significantly
Table 3.2: Effect of different pre-regeneration (PRM-1) and regeneration (PRM-2) media components on plant regeneration frequency (%) in different Basmati rice varieties.

<table>
<thead>
<tr>
<th>Variety</th>
<th>Treatment Number*</th>
<th>Number of calli</th>
<th>Calli producing plants</th>
<th>Number of plants recovered</th>
<th>Regeneration frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-370</td>
<td>R1</td>
<td>106</td>
<td>21</td>
<td>42</td>
<td>19.81 ± 0.32 c</td>
</tr>
<tr>
<td></td>
<td>R2</td>
<td>123</td>
<td>70</td>
<td>118</td>
<td>57.72 ± 7.95 b</td>
</tr>
<tr>
<td></td>
<td>R3</td>
<td>123</td>
<td>76</td>
<td>156</td>
<td>61.83 ± 1.16 ab</td>
</tr>
<tr>
<td></td>
<td>R4</td>
<td>128</td>
<td>27</td>
<td>38</td>
<td>21.16 ± 1.49 c</td>
</tr>
<tr>
<td></td>
<td>R5</td>
<td>127</td>
<td>73</td>
<td>118</td>
<td>57.58 ± 4.29 b</td>
</tr>
<tr>
<td></td>
<td>R6</td>
<td>124</td>
<td>83</td>
<td>125</td>
<td>66.80 ± 3.18 a</td>
</tr>
<tr>
<td>B-2000</td>
<td>R1</td>
<td>134</td>
<td>17</td>
<td>20</td>
<td>12.63 ± 1.78 e</td>
</tr>
<tr>
<td></td>
<td>R2</td>
<td>125</td>
<td>28</td>
<td>40</td>
<td>22.39 ± 0.92 d</td>
</tr>
<tr>
<td></td>
<td>R3</td>
<td>129</td>
<td>52</td>
<td>62</td>
<td>40.34 ± 2.30 b</td>
</tr>
<tr>
<td></td>
<td>R4</td>
<td>114</td>
<td>23</td>
<td>33</td>
<td>20.31 ± 1.44 d</td>
</tr>
<tr>
<td></td>
<td>R5</td>
<td>120</td>
<td>38</td>
<td>48</td>
<td>31.64 ± 2.17 c</td>
</tr>
<tr>
<td></td>
<td>R6</td>
<td>125</td>
<td>72</td>
<td>92</td>
<td>58.03 ± 7.25 a</td>
</tr>
<tr>
<td>B-Pak</td>
<td>R1</td>
<td>134</td>
<td>41</td>
<td>59</td>
<td>30.53 ± 2.58 c</td>
</tr>
<tr>
<td></td>
<td>R2</td>
<td>120</td>
<td>62</td>
<td>96</td>
<td>51.80 ± 2.22 b</td>
</tr>
<tr>
<td></td>
<td>R3</td>
<td>127</td>
<td>68</td>
<td>92</td>
<td>53.56 ± 5.24 b</td>
</tr>
<tr>
<td></td>
<td>R4</td>
<td>121</td>
<td>41</td>
<td>68</td>
<td>33.81 ± 2.09 c</td>
</tr>
<tr>
<td></td>
<td>R5</td>
<td>121</td>
<td>73</td>
<td>128</td>
<td>60.41 ± 2.38 ab</td>
</tr>
<tr>
<td></td>
<td>R6</td>
<td>125</td>
<td>87</td>
<td>136</td>
<td>70.39 ± 11.27 a</td>
</tr>
<tr>
<td>B-385</td>
<td>R1</td>
<td>120</td>
<td>9</td>
<td>12</td>
<td>7.47 ± 2.29 c</td>
</tr>
<tr>
<td></td>
<td>R2</td>
<td>134</td>
<td>47</td>
<td>62</td>
<td>35.16 ± 1.66 b</td>
</tr>
<tr>
<td></td>
<td>R3</td>
<td>127</td>
<td>56</td>
<td>95</td>
<td>44.15 ± 2.16 b</td>
</tr>
<tr>
<td></td>
<td>R4</td>
<td>127</td>
<td>14</td>
<td>18</td>
<td>11.04 ± 1.49 c</td>
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<tr>
<td></td>
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<td>41</td>
<td>62</td>
<td>36.52 ± 4.75 b</td>
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<tr>
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<td>107</td>
<td>46</td>
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<td>43.05 ± 1.30 a</td>
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<tr>
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<td>143</td>
<td>19</td>
<td>32</td>
<td>13.31 ± 1.29 c</td>
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<tr>
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<td>64</td>
<td>116</td>
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<tr>
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<td>R5</td>
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<td>59</td>
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<td>R6</td>
<td>123</td>
<td>92</td>
<td>178</td>
<td>74.75 ± 3.14 a</td>
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</table>

Means for each variety followed by a common letter are not significantly different at 5% level by DMRT.

*Treatment #   Medium composition

R1  MS + sucrose 30 g L⁻¹, sorbitol 30 g L⁻¹, phytagel 0.25%
R2  MS + kinetin 1 mg L⁻¹, IAA 4 mg L⁻¹, sucrose 30 g L⁻¹, sorbitol 30 g L⁻¹, phytagel 0.25%
R3  MS + kinetin 3 mg L⁻¹, NAA 1 mg L⁻¹, sucrose 30 g L⁻¹, sorbitol 30 g L⁻¹, phytagel 0.25%
R4  MS + maltose 30 g L⁻¹, sorbitol 30 g L⁻¹, phytagel 0.25%
R5  MS + kinetin 1 mg L⁻¹, IAA 4 mg L⁻¹, maltose 30 g L⁻¹, sorbitol 30 g L⁻¹, phytagel 0.25%
R6  MS + kinetin 3 mg L⁻¹, NAA 1 mg L⁻¹, maltose 30 g L⁻¹, sorbitol 30 g L⁻¹, phytagel 0.25%
enhanced regeneration efficiency in B-2000 and B-Pak while no significant difference was observed in B-370, B-385 and S-Basmati.

In all the Basmati varieties, somatic embryogenesis occurred at varying frequency. It was observed that the number of somatic embryos was more when the cultures were incubated initially in the dark as compared to the cultures that were kept under 16/8 hour light/dark period. The average number of somatic embryos formed per callus was highest (12.0) in S-Basmati while lowest (0.3) in B-385. Figure 3.1e shows the development of globular as well as scutellar notch-stage embryoids in S-Basmati callus after 12 days of culture in dark. These embryoids later developed a well-defined scutellum and coleoptile. The embryoids started germinating rapidly after shifting on the plant regeneration (PRM-2) medium (Figure 3.1f).

From the results presented in Table 3.2, it is clear that treatment number R6 (having kinetin 3 mg L\(^{-1}\), NAA 1 mg L\(^{-1}\), maltose 3 %) produced significantly higher regeneration frequency (RF) in B-2000 and B-Pak. In B-370 and S-Basmati, although R6 gave the highest RF but differed non-significantly from R3 (having kinetin 3 mg L\(^{-1}\), NAA 1 mg L\(^{-1}\), sucrose 3 %). In B-385, however, highest RF was recorded in R3 which differed non-significantly from R6. Overall highest number of plants were recovered from all the five Basmati varieties from treatment number R6 (593 plants) followed by R3 (572 plants) compared to R2 and R3 (432 and 446 plants, respectively). From these results, it can be concluded that R6 proved to be the best medium for high frequency plant regeneration in all the varieties except for B-385 where R3 showed slightly better results.

**Establishment of Regenerated Plants in Hydroponics/Soil**

Two different methods for establishing the regenerated plants were evaluated i.e. hydroponic culture and planting directly in the sand/soil. From the plant regeneration experiments, more than two thousand plants were regenerated, however, only 100 plants were removed (approximately 20 plants of each variety) and used for further studies.

These plants were divided into two groups of 50 each. Of the fifty plants transferred to sterilized sand, 46 plants were successfully established and later transplanted to soil in the earthen pots. Similarly, 41 out of 50 plants survived in hydroponic culture and all of them
Figure 3.3 Tissue culture derived rice plants of different Basmati varieties; established in the hydroponics (a); in the soil (b); regenerated plants transferred to soil in earthen pots (c); regenerated plants were phenotypically normal and fertile (d).
were successfully established in the soil. The regenerated plants transferred to soil (Figure 3.3), either directly or via hydroponic culture, showed a normal phenotype and set seeds.

**Discussion**

Production of embryogenic calli with high regeneration efficiency is a pre-requisite for the practical utilization of tissue culture technology for improvement of rice through genetic engineering. Indica rice species are less responsive not only in embryogenic callus induction but also problematical in subsequent plant regeneration (Ge *et al*., 2006). Therefore, extensive research has been made to improve plant regeneration efficiencies by manipulating different factors which include plant growth regulators (Ge *et al*., 2006); carbohydrate source (Lee *et al*., 2002; Grewal *et al*., 2005); osmotic conditioning / partial desiccation (Kavi-Kishor, 1987; Tsukahara and Hirosawa, 1992; Wang *et al*., 1999; Chand and Sahrawat, 2001; Saharan *et al*., 2004; Geng *et al*., 2008); and other supplements like casein hydrolysate (Khalida and Al-Forkan, 2006), L-tryptophan (Sahrawat and Chand, 2004), L-proline (Datta *et al*., 1992; Chowdhry *et al*., 1993) etc.

Extensive efforts have been made to identify suitable explants in rice for embryogenic callus induction and optimization of suitable culture conditions. Although mature seeds are less suitable for embryogenic callus induction compared to immature embryos which have large number of actively dividing cells but the former have a distinct advantage because these are readily available all the year round and can be conveniently stored (Ge *et al*., 2006). In these experiments, by defining a suitable medium composition, high efficiency embryogenic callus induction frequencies were achieved from mature seeds of five different Basmati rice varieties.

It has been proposed that growth regulators play an important role in callus induction and subsequent growth. Auxins like 2,4-D are indispensable for induction and proliferation of callus while supplementation of cytokinins may increase the growth rate of pro-embryogenic masses (Kommamine *et al*., 1992). In this study, five different Basmati rice varieties were tested for embryogenic callus induction frequency (ECIF) on modified MS medium containing various concentrations of 2,4-D (0, 1, 2 and 4 mg L⁻¹) either alone or in
combination with 0.5 mg L\(^{-1}\) BAP. Embryogenic callus induction frequency ranged from 0.0-76.3 % for B-370; 0.0-66.6 % for B-2000; 0.0–90.4 % for B-385 and 0.0-71.7% for S-Basmati (Table 3.1). These results showed that 2,4-D alone was sufficient to induce embryogenic callus in all of the varieties and there was generally a negative effect of BAP supplementation in the callus induction medium. It has been extensively reported that 2,4-D alone is sufficient to induce and sustain growth of embryogenic callus (Lee et al., 2002; Ozawa et al., 2003). However, there are a number of reports showing that combination of auxin with cytokinin was more effective for embryogenic callus induction (Fan et al., 2002; Wang et al., 2004; Ge et al., 2006; Zaidi et al., 2006). The results of this study are supported by the findings of Rashid et al. (2001) which showed that 2,4-D alone was sufficient for callus induction in Super Basmati and that inclusion of BAP resulted in significant decrease in callus induction frequency.

Plant regeneration was achieved in all the five Basmati rice varieties. Six different combinations of regeneration media were tested. A combination of auxin with cytokinin in the regeneration medium was found to be important for high frequency plant regeneration from embryogenic calli as indicated by low frequency plant regeneration in media types R1 and R4 (having no growth regulators). Moreover, it was observed that inclusion of ABA (10 mg L\(^{-1}\)) in the pre-regeneration medium was essential for somatic embryo induction. Sorbitol was used as an osmoticum which also played a significant role for enhancing regeneration from Basmati calli. Plant regeneration frequency varied between 44.2 - 74.5 % (Table 3.2). Maximum shoot regeneration was achieved in S- Basmati. Plant regeneration medium R6 (MS basal medium supplemented with kinetin 3 mg L\(^{-1}\), NAA 1 mg L\(^{-1}\), maltose 3% and phytagel 0.25%) proved to be the best medium for high frequency plant regeneration except for B-385 where R3 showed non-significantly higher regeneration frequency. These results have demonstrated that the composition of regeneration medium is critical in achieving high frequency plant regeneration from embryogenic calli of Basmati rice cultivars. Although regeneration was achieved in regeneration media without growth regulators but the frequency was low as compared to regeneration media having appropriate concentrations of cytokinins and auxins.
Higuchi and Maeda (1991) achieved enhancement of regeneration frequency by high osmolarity of the growth medium. The results presented here indicate that addition of sorbitol in the plant regeneration medium contributed significantly in the somatic embryo induction and subsequent plant regeneration. Rashid et al. (2004) reported that inclusion of sorbitol in the plant regeneration medium significantly contributed to higher plant regeneration efficiencies in Basmati rice. Similarly, Lai and Liu (1988) observed that inclusion of mannitol was one of the important factors for high efficiency regeneration from rice calli.

Carbon source is also an important factor which can significantly contribute to regeneration efficiencies. Plant regeneration frequency was generally higher in all the varieties on regeneration media containing maltose except B-385 where a non-significantly higher regeneration frequency was obtained in media containing sucrose. Maltose significantly enhanced regeneration efficiency in B-2000 and B-Pak while no significant difference was observed in B-370, B-385 and S-Basmati. These findings are in conformation with the results reported by Zaidi et al. (2006) which showed that maltose was the most effective carbon source producing maximum number of plants from the rice calli compared to sucrose and glucose. The research findings presented here shows that maltose produced better results; however, the difference was not as great in magnitude as reported by other workers. Ghosh-Biswas and Zapata (1993) reported 8-12 fold higher plant regeneration efficiency in maltose than sucrose for IR43.

These results have demonstrated that the compositions of callus induction and plant regeneration media are critical in achieving high frequency embryogenic callus and subsequent plant regeneration from embryogenic calli of Basmati rice cultivars. These studies will pave the way for the improvement of Basmati rice through genetic engineering.
CHAPTER 4
OPTIMIZATION OF PARTICLE BOMBARDMENT CONDITIONS

Direct DNA transfer methods for genetic transformation of plants depend entirely on physical or chemical principles to deliver DNA into the plant cells (Altpeter et al., 2005). A number of different direct DNA transfer methods have been described by various workers including particle bombardment (Klein et al. 1987; Christou et al. 1992; Finer et al., 1992), polyethylene-glycol mediated transformation of protoplasts (Negrutiu et al. 1987; Datta et al. 1990), micro-injection (Crossway et al. 1986), electroporation (Shillito et al. 1985; Fromm et al. 1986) and transformation using silicon-carbide whiskers (Frame et al. 1994; Asad et al., 2008). However, particle bombardment has been used as the method of choice for developing commercial transgenic crops (Altpeter et al., 2005). The delivery of transgenes into embryonic tissues mediated by particle bombardment continues to be the principle direct DNA transfer technique in plant biotechnology (James 2003).

Particle bombardment technology has proved to be an effective and versatile method of gene delivery over the past few years (Altpeter et al., 2005). It has demonstrated several advantages, over other gene transfer techniques, like i) diverse cell types can be used as targets, ii) minimal expression cassettes can be used without the requirement of a vector backbone, iii) multigene transformation is achievable, iv) high molecular weight DNA delivery into plant cells is possible, and v) it is one of the most convenient method for organelle transformation.

The present chapter deals with the development of reproducible procedures for particle-bombardment mediated transformation of Basmati rice by optimization of different biological and physical parameters which can have a dramatic effect on gene delivery to the target cells.
CONSTRUCTION OF CO-INTEGRATE VECTORS

Co-integrate vectors having both \textit{gusA} and \textit{hpt} genes were designed specifically for purpose of optimization and stable transformation studies. Hygromycin gene cassette comprising CaMV35S promoter, hygromycin coding sequence and \textit{tml} terminator was taken out from plasmid pTRA151 by \textit{HindIII} (EC 3.1.21.4) digestion. Similarly the plasmid pAct1-D having \textit{gusA} gene under the control of rice \textit{actin-1} promoter and \textit{nos} terminator was digested with \textit{HindIII} as shown in Figure 4.1a. Both the vectors (pAct1-D) and the insert (1.7 kbp \textit{hpt} gene cassette) fragments were purified by gel purification as shown in Figure 4.1b. These fragments were ligated and transformed into \textit{E. coli}. Figure 4.2 shows the gel electrophoresis of the plasmid DNA, isolated from six independent colonies of \textit{E. coli} transformed with the ligation mixture; following digestion with \textit{PstI} (EC 3.1.21.4). Each colony is represented by two lanes as having un-digested and \textit{PstI} digested plasmid DNA, respectively. The plasmid pAct1-D has a single \textit{PstI} site which is close to \textit{HindIII} site as shown in the physical map of the plasmid (Figure 2.1a). Similarly \textit{hpt} gene cassette has only one \textit{PstI} site (near the 5’ end of CaMV35S promoter) next to the \textit{HindIII} site (Figure 2.1b). Successful cloning of \textit{hpt} gene in pAct1-D should result in the development of two co-integrate vectors (as shown in Figure 4.3) depending on the orientation of the \textit{hpt} gene cassette. Upon digestion with \textit{PstI}, the co-integrate vector of orientation type I should produce two fragments of approx. 1.7 and 8.0 kbp while the vector having type II orientation should also produce two fragments of ~ 9.7 kbp and a very small fragment of few base pairs (as the two \textit{PstI} sites, one in parent vector and the other in the \textit{hpt} gene cassette are very close, as shown in the Figure 4.3). Colonies numbering 1, 2 and 3 produced two fragments of 1.7 and 8.0 kbp (Figure 4.2; lanes 3, 5 and 7, respectively) indicating that the isolated plasmid is of type-I orientation (Figure 4.3). The colonies numbering 4, 5 and 6 (Figure 4.2; lanes 9, 11 and 13, respectively) produced two fragments of ~ 9.7 kbp and second one of a very small size which could not detected on the gel due to its small size. This confirmed that these three colonies contained the recombinant plasmid of orientation type-II (Figure 4.3). The resulting co-integrate vectors were named as pGH-I and pGH-II based on the orientation type-I and II, respectively. These vectors were used in the subsequent transformation experiments along with other vectors.
Figure 4.1 Agarose gel electrophoresis of plasmid pAct1-D and pTRA151 after restriction digestion with HindIII. (a) Arrows indicate vector (8.0 kbp) and insert (1.7 kbp). (b) Same vector and insert fragments after gel purification.

Figure 4.2 Restriction analysis of recombinant plasmid DNA (minipreps) isolated from six different E. coli colonies following transformation with ligation product. Lane 1, 1 kbp DNA ladder as molecular weight marker; Lanes 2, 4, 6, 8, 10 and 12 show undigested plasmid DNA while Lanes 3, 5, 7, 9, 11 and 13 show PstI digested plasmid DNA.
Figure 4.3. Physical map of the co-integrate vectors pGH-I and pGH-II.
OPTIMIZATION OF PARAMETERS AFFECTING BOMBARDMENT EFFICIENCY

Effect of type/physiological and developmental stage of the target tissue:
Embryogenic calli of rice have been extensively used in particle bombardment experiments, however, there are no reports on the use of mature embryos as the target material. In order to define suitable bombardment conditions for this explant, Basmati-370 (B-370) was selected for studying various factors which can affect transformation efficiency. In this study, two types of tissues were used as targets for foreign DNA delivery which included 28 days old primary embryogenic calli and mature embryos. Both 28 days old primary embryogenic calli and mature rice embryos showed equally good results in the transient GUS expression studies. A large number of blue foci were observed after bombardment of primary embryogenic calli as well as mature rice embryos as shown in Figure 4.4. Primary calli were easy to get and maintain and gave a very good response in transient GUS studies. However, the selection efficiency was not as good as desired. The transformed calli could be differentiated from non-transformed cells as relatively fast growing white to pale yellow in colour on the selection medium after two weeks of culture. However, when these calli were sub-cultured to fresh medium some of the sectors again turned necrotic which indicated that there was a need for another selection passage. It was observed that the calli which lived on selection medium for a longer time had a reduced ability to regenerate plants. A more effective selection should be the one which gives a clear and early indication of the transformed nature of the calli being selected. Therefore, embryos separated or excised from mature seeds were tried as an alternate target material. It was observed that when mature embryos were bombarded with plasmid pGH-I/pGH-II, having gusA and hpt genes, callus mostly originated only from the transformed sectors of the scutellum surface (Figure 4.10 c-d) and not from the whole scutellum tissue. Thus the originating callus can be easily and effectively selected during two passages on the selection medium. Encouraging results obtained in the initial experiments with mature rice embryos, giving equally good transient GUS expression in comparison with 28 days primary embryogenic calli but providing better selection efficiency, prompted us to use this explant for detailed studies. For all subsequent optimization work, mature embryos of B-370 and the plasmids pGH-I/pGH-II were used.
Figure 4.4. Particle-bombardment of Basmati-370 mature embryos and embryogenic calli with pGH-I/pGH-II (having *gusA* & hygromycin resistance genes) for transient GUS expression studies. a) Excision of mature embryos from over-night soaked seeds; b) close-up of an excised mature embryo of B-370; c) arrangement of mature rice embryos of B-370 on bombardment medium; d-f) mature embryos bombarded under different bombardment conditions, stained with GUS substrate 24 hours after the bombardment showing transient GUS expression; g) un-bombarded and pGH-I bombarded primary embryogenic calli stained with GUS substrate 24 hours after the bombardment; bombarded calli (right) showing transient GUS expression.
Effect of particle type/size, helium pressure and target distance on bombardment efficiency:

Efficiency of DNA delivery to target cells depends largely upon the size of micro-particles and the force with which these DNA coated particles are pushed towards the target cells. The depth of penetration of these particles into the target tissues can be controlled by varying particle size, helium pressure and the target distance. The target distance also defines the target area i.e. less the target distance, the narrower is the target area and greater the distance, wider is the target area. So these three parameters are important in determining optimum conditions for efficient DNA delivery to target cells/tissues.

In these experiments, mature embryos were bombarded with gold particles of two different sizes i.e. 1.0 and 1.6 µm at different helium pressures of 7,500 and 10,500 kPa (1,100 and 1,550 psi, respectively) and at a variable target distance of 3, 6, 9 and 12 cm. Figure 4.5 shows the results of varying particle size, helium pressure and target distance on transient GUS expression in mature B-370 embryos. The values shown in Figure 4.5 are means of three replicates. In this experiment, DNA and particle load were kept constant at 5 µg and 0.5 mg per bombardment following manufacturer’s instructions. Vacuum, macro-carrier flight distance and the gap between the rupture disc and macro-carrier were also kept constant at 25 inches of Hg, 8 mm and ¼ inch, respectively. Highest number of blue foci (350.7 ± 43.6) was observed when the embryos were bombarded at a distance of 6 cm with 1.0 µm gold particles and a pressure of 7,500 kPa. The lowest transient GUS expression (7.0 ± 2.6) was recorded when the embryos were bombarded with 1.6 µm gold particles at 10,500 kPa pressure and 3 cm target distance. The results shown in Figure 4.5 indicate that bombardment at a distance of 3 cm resulted in poor transient GUS expression, and therefore, it is not a suitable distance for bombardment. At this distance, high helium pressure (10,500 kPa) or bigger particle size (1.6 µm) further reduced the number of blue foci. This decreased transient GUS expression may be a result of tissue damage due to high momentum with which the particles hit the tissues and a higher number of particles hitting per unit area.

At a distance of 6 cm, the highest number of blue foci (350.7 ± 43.6) was observed when the embryos were bombarded with 1.0 µm particles at a pressure of 7,500 kPa. When the
Figure 4.5. Effect of particle size, helium pressure and target distance on transient GUS expression in mature embryos of B-370.
pressure or particle size was increased, the transient GUS expression decreased. This may be
due to increased tissue damage as described in previous para. Although highest number of
blue foci was obtained at 6 cm of bombardment distance with 1.0 µm particles and 7,500 kPa
helium pressure, however, more consistent results were obtained when the embryos were
bombarded at a distance of 9 cm at both pressures and particle sizes. At 9 cm target distance,
highest number of blue foci (319.0 ± 47.3) were recorded at 10,500 kPa helium pressure with
1.0 µm gold particles followed by 7,500 kPa pressure and 1.0 µm gold (312.0 ± 52.1). This
indicates that a distance of 9 cm results in more uniform dispersion of particles over the
target area and may also reduce the tissue damage especially when higher pressure (10,500
kPa) or bigger particles (1.6 µm) are used.

Transient GUS expression was significantly reduced as the target distance was increased to
12 cm. At this distance, 1.6 µm particles when bombarded at 10,500 kPa produced more
number of blue foci compared to 7,500 kPa or 1.0 µm particles at both 7,500 and 10,500 kPa
helium pressure. This indicates that at this distance, the particles disperse to a larger area and
some of the DNA coated particles may have dispersed beyond the target area thus the target
tissues were hit but fewer DNA coated particles. Another reason may be that the particles did
not hit the target tissues with enough force to penetrate the tissues because of longer distance.

From these results it can be concluded that particle size of 1.0 µm is more suitable than 1.6
µm for rice mature embryos while a helium pressure of 7,500 kPa at 6 cm or either 7,500 or
10,500 kPa at a target distance of 9 cm can be used for high efficiency DNA delivery to these
target tissues.

Effect of particle and DNA loads:
Different DNA to particle ratios and loads (combinations A-G as shown in Figure 4.6) were
used to determine suitable combination for high efficiency DNA delivery to target tissues.
DNA loads from 3 to 15 µg were used to coat 3-5 mg gold particles in three different
particle:DNA ratios i.e. 1:1, 1:2 and 1:3, respectively. Figure 4.6 shows the effect of different
DNA particle ratios on transient GUS expression in mature rice embryos. Gold particles of
1.0 µm size were used in this experiment. Each DNA-gold prep was used for six
Figure 4.6. Effect of different combinations (A-G) of DNA and particle loads on transient GUS expression in mature embryos of B-370.

<table>
<thead>
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<th>Particle load (mg)</th>
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<th>Particle:DNA ratio</th>
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bombardments. Bombardment conditions were as follows: helium pressure, 7,500 kPa; target distance 6 cm; vacuum, macro-carrier flight distance and the gap between rupture disc and macro-carrier were kept constant at 685 mm (27 inches) of Hg, 8 mm and 6.5 mm, respectively. Maximum number of blue loci (405.3 ± 32.1) were observed in combination C where 9 µg DNA was used with 3 mg gold. The lowest numbers of blue foci (101.0 ± 19.0) were recorded in combination A where 3 µg DNA was used with 3 mg gold particles. While keeping particle load at 3 mg, and decreasing the DNA load from 9 µg (as in combination C) to 6 µg and 3 µg (combination B and A, respectively) a reduction in the transient GUS expression was observed. This indicates that in combination A and B, DNA becomes the limiting factor and results in reduced transient GUS expression. Keeping the ratio of DNA to particles at 2 and 3, the loads of both DNA and particles were increased (combinations D, E, F and G) to see their effect on transient GUS expression. It was noted that as the DNA and particle load was increased (from 6 or 9 µg DNA and 3 mg particles; combinations B and C) to 8 or 12 µg DNA with 4 mg particles, there was a reduction in transient GUS expression. The number of blue foci further decreased as the DNA and particle load was further increased to 10 or 15 µg with 5 mg particles. From these results it is clear that increasing the number of DNA coated particles per shot did not enhance the transient GUS expression. This reduction in transient GUS expression may be attributed to higher degree of damage to the tissues as a result of hitting higher number of DNA coated particles per unit area. From these experiments, it can be concluded that a particle load of 3 mg (0.5 mg/shot) is sufficient to produce high level of transient GUS expression when used in conjunction with 9 µg DNA (1:3 particle-DNA ratio).

Effect of number of shots on transient GUS expression:
After optimizing some of the important physical parameters described in the above two experiments, the effect of number of bombardments on transient GUS expression in rice mature embryos was studied. The conditions for bombardment were same as optimized in the two above mentioned experiments i.e. 9 µg DNA was used to coat 1.0 µm gold particles and the embryos were bombarded at a distance of 6 and 9 cm with 7,500 kPa helium pressure. Vacuum, macro-carrier flight distance and the gap between rupture disc and macro-carrier were kept constant at 685 mm of Hg, 8 mm and 6.5 mm, respectively. Figure 4.7 shows the
Figure 4.7. Effect of number of bombardments on transient GUS expression in mature embryos of B-370.
results of number of bombardments on transient GUS expression in mature rice embryos. The values are means of two replicates. It was observed that single shot resulted in higher number of blue foci (476.5 ± 44.5) compared to double shot (321.0 ± 60.8) when the target distance was 6 cm. However, when embryos were bombarded twice at 9 cm target distance, a considerable increase in the number of blue foci was observed compared to single shot at the same distance. The reduced GUS expression at 6 cm with double shot may again be attributed to the tissue damage as a result of higher number of particles hitting per unit area. At 9 cm, however, the particles disperse uniformly to a wider area and thus causing less injury. From these results, it is clear that embryos can be bombarded twice at a distance of 9 cm to produce high level of transient GUS expression. Therefore, all subsequent experiments were performed at 9 cm target distance with each plate bombarded twice.

Effect of osmotic treatment on transient GUS expression of bombarded mature rice embryos:

In order to evaluate the effect of osmotic pre-treatment on transient expression as well as stable transformation of foreign genes, several experiments were conducted by varying osmoticum of the bombardment medium. Callus induction medium having 2,4-D (2.0 mg L⁻¹) and 3% sucrose was used as a control. Two other media having additional 0.25 and 0.4 M mannitol, respectively were also used and compared to see its effect on transient GUS expression in rice embryos. Figure 4.8 shows the results of the osmotic treatment on transient GUS expression in mature rice embryos. Bombardment medium containing 3% sucrose was used as control. Significant differences were observed between the control and the osmotic treatments. Bombardment medium having 0.25 M mannitol produced the highest number of blue foci (900.0 ± 47.5) followed by 0.4 M mannitol (832.0 ± 39.0) while least GUS expression (313.0 ± 20.2) was recorded in the control (3% sucrose). Hence there was a 2.7 to 2.9 fold increase in transient GUS expression to controls in 0.4 M and 0.25 M mannitol containing bombardment media, respectively.

OPTIMIZATION FOR HYGROMYCIN B SELECTION

Effective and efficient selection of transformed cells from within a large population of non-transformed cells is one of the most important steps in developing high efficiency system for
Figure 4.8. Effect of osmotic pre-treatment on transient GUS expression in mature embryos of B-370.

<table>
<thead>
<tr>
<th>Hygromycin B concentration (mg l$^{-1}$)</th>
<th>0</th>
<th>25</th>
<th>50</th>
<th>75</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 % sucrose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25 M mannitol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.4 M mannitol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 % sucrose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.9. Determination of optimum concentration of Hygromycin B for selection of transformed cells / calli of B-370. Approx. 20 primary embryogenic calli (28-days old) of B-370 were plated on callus induction medium (in triplicate) supplemented with 0, 25, 50, 75 and 100 mg l$^{-1}$ hygromycin B.
transformation of crop plants. In the preliminary experiments, primary embryogenic callus was used as the target material for biolistic transformation. Twenty to twenty-five primary calli (3-5 mm diameter) of B-370 were plated in triplicate on the respective callus induction media containing 0, 25, 50, 75 and 100 mg L\(^{-1}\) hygromycin B. Figure 4.9 shows the effect of different concentrations of hygromycin B on the growth of primary embryogenic calli of B-370, 30 days after placement on the respective media. It was observed that hygromycin B at 25 mg L\(^{-1}\) did not completely check the growth of calli and some sectors kept on growing even after 30 days of selection. At 50 mg L\(^{-1}\), after some initial growth, however, the calli turned brown and no further growth was observed. Higher hygromycin B concentrations (75 and 100 mg L\(^{-1}\)) proved to be more lethal as the callus growth was checked right from the initial days of plating. From these results it is concluded that a concentration of 50 mg L\(^{-1}\) hygromycin B is sufficient to kill non-transformed cells/calli. Similar studies were also conducted using mature rice embryos. Mature embryos of B-370 were plated on the optimized callus induction medium containing the same concentration of hygromycin B as described for primary calli. At 25 mg L\(^{-1}\) hygromycin B, some embryos started forming callus but at a very low frequency, whereas at 50 mg L\(^{-1}\) and higher concentrations of hygromycin B, no callus induction was observed and the embryos turned brown and necrotic. This indicated that 50 mg L\(^{-1}\) hygromycin is optimum concentration to check the growth of non-transformed calli.

PLANT REGENERATION FROM HYGROMYCIN RESISTANT CALLI

In eight different bombardment experiments, a total of 1,740 mature rice embryos of B-370 were bombarded with plasmid pGH-1/pGH-II having \textit{gusA} and \textit{hpt} genes. Following bombardment, these embryos were placed on selection medium (optimized callus induction medium containing 50 mg L\(^{-1}\) hygromycin B). Most of the bombarded embryos when placed on selection medium (Figure 10a) turned brown and necrotic after 7-8 days of culture while few embryos showed transformed sectors from which hygromycin resistant callus originated as shown in Figure 4.10b-d. After two weeks, these small hygromycin resistant calli were separated and placed on fresh selection medium. Results of these bombardment experiments are given in Table 4.1. A total of 66 calli survived the second round of selection. Some of
Table 4.1. Summary of bombardment, selection and regeneration from mature embryos of B-370.

<table>
<thead>
<tr>
<th>Rice variety</th>
<th>Expt. #</th>
<th>No. of embryos bombarded*</th>
<th>Total # of plates</th>
<th>Single shot</th>
<th>Double shot</th>
<th>Plasmid Used</th>
<th>No. of Selected Calli**</th>
<th>No. of regenerated plants***</th>
<th>No. of Hyg/GUS+ plants</th>
<th>Transformation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-370</td>
<td>1</td>
<td>368</td>
<td>6</td>
<td>6</td>
<td>-</td>
<td>pGH-I</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>0.54</td>
</tr>
<tr>
<td>&quot;</td>
<td>2</td>
<td>350</td>
<td>6</td>
<td>6</td>
<td>-</td>
<td>pGH-II</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>0.29</td>
</tr>
<tr>
<td>&quot;</td>
<td>3</td>
<td>326</td>
<td>6</td>
<td>6</td>
<td>-</td>
<td>pGH-I</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>0.92</td>
</tr>
<tr>
<td>&quot;</td>
<td>4</td>
<td>332</td>
<td>6</td>
<td>6</td>
<td>-</td>
<td>pGH-II</td>
<td>12</td>
<td>4</td>
<td>3</td>
<td>0.90</td>
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<td>&quot;</td>
<td>5</td>
<td>240</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>pGH-I</td>
<td>8</td>
<td>3</td>
<td>3</td>
<td>1.67</td>
</tr>
<tr>
<td>&quot;</td>
<td>6</td>
<td>235</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>pGH-II</td>
<td>9</td>
<td>4</td>
<td>4</td>
<td>2.13</td>
</tr>
<tr>
<td>&quot;</td>
<td>7</td>
<td>243</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>pGH-I</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>1.65</td>
</tr>
<tr>
<td>&quot;</td>
<td>8</td>
<td>240</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>pGH-II</td>
<td>12</td>
<td>5</td>
<td>5</td>
<td>2.08</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>1740</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>66</td>
<td>27</td>
<td>26</td>
<td>1.41</td>
</tr>
</tbody>
</table>

* Bombardment medium consisted of optimized callus induction medium containing MS salts and vitamins supplemented with 2.0 mgL-1 2,4-D, 3% sucrose and 0.25 M mannitol.

** Selection medium consisted of optimized callus induction medium containing MS salts and vitamins supplemented with 2.0 mg L-1 2,4-D, 3% sucrose and 50 mg L-1 hygromycin B.

*** Regeneration medium contained MS salts and vitamins supplemented with kinetin 3 mg L-1, NAA 1 mg L-1, maltose 30 g L-1, sorbitol 30 g L-1 and phytagel 0.25%
Figure 4.10. Selection of pGH-I bombarded mature embryos of B-370 and regeneration of plants from selected calli; a) selection of transformed embryos on selection medium containing hygromycin B; b) some of the bombarded embryos showing the development of hygromycin resistant callus while others are killed by the antibiotic; c-d) proliferation of hygromycin resistant callus from scutellar surface of bombarded mature embryos of B-370 on selection medium; e) one of the actively growing hygromycin resistant B-370 callus stained with GUS substrate showing GUS activity (left) while an un-bombarded callus (right) showing no GUS activity; f-h) Plant regeneration from hygromycin resistant calli.
these calli stained for GUS activity showed intense GUS staining as shown in Figure 4.10e. These hygromycin resistant calli were first placed on optimized pre-regeneration medium (PRM-1) for somatic embryo induction and then on the regeneration medium (PRM-2) for complete plant regeneration. Upon transfer to pre-regeneration medium, the calli developed somatic embryos within 10-12 days of culture. These somatic embryos germinated, upon transfer to regeneration medium (Figure 4.10f-h), to complete plants with simultaneous development of shoot and root. From the 66 hygromycin resistant calli, only 27 plants could be regenerated. These plants were then shifted to root proliferation medium (½ strength MS medium without growth regulators, containing 2 % sucrose) for vigorous root development.

ESTABLISHMENT OF PUTATIVE TRANSGENIC PLANTS IN HYDROPONICS/ SOIL

All the twenty seven regenerated plants were removed from the root proliferation medium and their roots were washed in running tap water, briefly dipped in 0.2 % w/v aqueous solution of Dithane® and planted in sterilized sand. Of the twenty seven plants shifted to pots, 26 plants survived and were successfully transferred to soil in the pots. These established plants were transferred to containment glass-house for further growth and maintenance.

MOLECULAR ANALYSIS OF PUTATIVE TRANSGENIC PLANTS

Genomic DNA was isolated from all the 26 putative transgenic B-370 plants and subjected to PCR analysis. Figure 4.11 shows PCR analysis of 12 different putative transgenic plants with gusA primers which amplified a correct sized (1,201 bp) fragment from the internal region of gusA gene. No amplification was observed in non-transformed control and water control (no DNA control). This indicated that all these plants were positive for gusA gene. In another reaction, genomic DNA from 14 putative B-370 plants transformed with pGH-1 were subjected to PCR analysis using Hpt-F and CaMV35-R primers (Figure 4.12) which amplified ~ 900 bp fragment (between 3’ region of the 35S promoter and 5’ region of the hpt gene). Thirteen plants showed amplification of a specific sized fragment while one plant (Figure 4.12; lane 12) did not show any amplification. This plant was, therefore, adjudged as negative for the transgene.
Figure 4.11. PCR analysis of putative transgenic B-370 lines transformed with pGH-I/pGH-II with gusA specific primers. Lane M = 1 kbp DNA ladder (Fermentas cat # SM0311); Lane +, positive control (pGH-I); Lane W, water control; Lane -, negative control (DNA from a non-transformed control plant); Lanes 1-12, different putative transgenic B-370 rice plants regenerated on selection media.

Figure 4.12. PCR analysis of putative transgenic B-370 lines transformed with pGH-1 with CaMV35S-F and hpt-R specific primers. Lane M = 1 kbp DNA ladder (Fermentas cat # SM0311); Lane +, positive control (pGH-1); Lane W, water control; Lane -, negative control (DNA from a non-transformed control plant); Lanes 1-14, different putative transgenic B-370 rice plants regenerated on selection media.
However, to confirm these results, histochemical GUS assay was also performed on the leaves. Leaves from all the plants showed GUS expression except the plant which was found to be negative in PCR. The transgenic plants recovered showed expression of both gusA and hpt genes. The transgenic Basmati rice plants showed normal phenotype and were fertile. An optimized system for gene delivery to Basmati rice has therefore been developed for recovery of transgenic plants within a short period of time. Moreover, these optimized parameters for particle-bombardment mediated gene delivery into mature embryos provided us with a simple procedure applicable to other Basmati rice varieties studied and discussed in Chapter 3. This study has paved the way to transform other agronomically important genes into Basmati rice for its improvement.

**DISCUSSION**

Transgenic technology is becoming an indispensable part of plant breeding and is believed to be an efficient approach for improving agronomically important traits of crop plants. In this study, particle bombardment was employed as a technique to explore the potential of this technology for accomplishing gene transfer to Basmati rice varieties. Since there is no universal transformation system which is readily applicable to transformation of all indica rice varieties, therefore, it was imperative to evolve an efficient system of Basmati rice transformation which can be routinely used to improve quality traits of this important commodity.

To define optimum conditions for high efficiency gene delivery through Biolistic approach, different parameters were studied which can affect bombardment efficiency. Target tissue can play a very vital role both in terms of efficiency with which gene(s) can be delivered to the target cells as well as in the subsequent events of selection and transgenic plant recovery. In this study, it has been demonstrated that both primary calli (28 days old) and mature embryos gave equally good results as far as transient GUS expression is concerned. A number of researchers have demonstrated the recovery of transgenic rice plants from embryogenic calli. Li *et al.* (1993) exhibited recovery of hundreds of transgenic plants from bombarded embryogenic calli of japonica (Taipie 309) and indica (Tetep) rice species. Similarly, Chen *et al.* (1998) showed that by
optimizing the age of the tissue at the time of gene transfer and applying an improved selection procedure, transgenic Taipie 309 plants were recovered in 8 weeks from the time of gene transfer, at an average frequency of 22.3 ± 9.7 per 100 calli. However, in the work presented here, it can be concluded that the selection efficiency of embryogenic calli is far less than the mature embryos. It was observed that hygromycin resistant calli originated mostly from the transformed sectors of the scutellum surface giving a very early and clear indication of its transgenic nature. It was further noted that when these calli were separated from the mother explant and further subcultured on fresh selection medium, most of them exhibited a fast growth rate. On the other hand, it was difficult to judge transgenic nature of the hygromycin resistant calli that originated from the bombarded primary calli as siblings. Since there are no previous reports on the use of excised mature embryos of rice as the target tissues, therefore these offer a useful explant source for potential use in particle-bombardment mediated gene delivery in rice.

Different physical factors affecting bombardment efficiency were also optimized during the course of this study. Smaller particles (1.0 μm) produced more number of blue foci on average compared to bigger particles (1.6 μm) at 3, 6 and 9 cm target distance. The lower transient GUS expression with 1.6 μm particles may be the result of more damage to the tissue at these distances. However, higher transient GUS activity was recorded at a target distance 12 cm. This indicates that at 12 cm target distance, the force of penetration becomes a limiting factor for smaller (1.0 μm) particles. At this distance, however, the bigger particles have enough force of penetration thereby giving a higher transient GUS activity. These results are in agreement with those reported by Ramesh and Gupta (2005). They showed that out of the various combinations of helium pressure and target distances used in their experiments for particle bombardment mediated transformation of rice embryogenic calli, maximum transient GUS activity was observed at 7,500 kPa (1,100 psi) pressure and 9 cm target distance. High frequency of transient GUS expression at a helium pressure of 7,500 kPa (1,100 psi) and a target distance of 9 cm was also found to be optimum in a number of other studies (Jain et al., 1996; Minhas et al., 1996; Zhang et al., 1996; Chen et al., 1998, Jiang et al., 2000; Anoop and Gupta., 2004). Chernobrovkina et al. (2007) showed the effect of varying bombardment conditions on GFP expression in
barley embryos. They demonstrated that maximum expression was achieved with 1 μm gold particles at the helium pressure of 61.24–74.85 atm, and at a target distance of 9 cm.

Similarly it was found that DNA to particle ratio was another important parameter affecting bombardment efficiency. Higher DNA to particle ratio decreased the transient GUS expression in the bombarded mature rice embryos. This may be the result of agglomeration of gold particles at higher DNA concentration as seen during loading the macro-carriers. Highest transient GUS expression was recorded at 9 μg DNA with 3 mg gold particles (3:1 DNA-particle ratio). These results are in agreement with the results reported earlier by Ratnayaka and Oard (1995). They used different amounts of DNA to precipitate the gold particles and demonstrated that highest transient GUS activity was observed at 10 μg plasmid DNA. They also showed that higher amounts of plasmid DNA (i.e. 25 and 50 μg) dramatically reduced transient GUS expression in rice suspension cells. The reduction in transformation rates may also be an effect of toxic DNA levels (Klein et al., 1988).

These research findings showed that osmotic treatment significantly enhanced transient GUS expression compared to the controls. Osmotic treatment with 0.2 M mannitol gave highest GUS expression followed by 0.4 M mannitol, and yielded as much as 2.7 to 2.9 fold increase, respectively in transient GUS expression over the control. These results are in accordance with the results reported by Zhang et al., 1996 and Ghosh-Biswas and Potrykus, 1997. Zhang et al. (1996) demonstrated 2.3 and 3.6 fold increase in transient GUS expression in IR64 and an advanced indica breeding line IR57311-95-2-3, respectively. They used 3% mannitol, 3% sorbitol in addition to 3% sucrose in the medium for osmotic treatment. Ghosh-Biswas and Potrykus (1997) reported 2.5-2.9 fold increase in the recovery of hygromycin resistant rice (IR43) calli after osmotic treatment using 14% maltose over control (3% maltose). Similarly Chen et al. (1998) showed significant enhancement of transient GUS activity by placing rice embryogenic calli at 0.6 M carbohydrate before and after the bombardment. It has been proposed that osmotic treatment enhances transient as well as stable transformation by facilitating plasmolysis of target cells. Cells in the plasmolysed state are less likely to extrude their protoplasm.
following bombardment by the micro-particles (Armaleo et al. 1990) thus resulting in higher survival rates of the targeted cells after the bombardment.

Resistance to antibiotic hygromycin B is routinely used as selection criterion for selecting transformed rice cells (Abedinia et al., 2004; Sripriya et al. 2008). Sensitivity of Basmati rice calli to hygromycin B was assessed before transformation experiments. Hygromycin at 50 mg L\(^{-1}\) was found to completely arrest the growth of un-transformed cells. These results are in agreement as those reported by various other workers (Lin et al., 1995; Duan et al., 1996; Sivamani et al., 1996; Zhang et al., 1996; Wu et al., 1997; Chen et al., 1998; Abedinia et al., 2004; Sripriya et al., 2008).

Out of the 1,740 embryos bombarded with co-integrate plasmid pGH-1/pGH-II, a total of 26 plants were successfully established in soil. These plants were analysed by PCR and histological GUS assay. Out of the 26 plants analysed by PCR and GUS assay, only one plant failed to amplify \(hpt\) gene from the genomic DNA subjected to PCR. Leaves of this plant also showed no GUS activity. Therefore an over all transformation efficiency of 1.5% was achieved in these experiments. Moreover, these plants were phenotypically normal and set seeds. Various studies on rice transformation have shown that indica rices exhibit low transformation efficiencies compared to highly responsive japonica rice cultivars. Sivamani et al. (1996) recovered 10-20 plants per 500 bombarded embryogenic calli with an overall transformation efficiency of 2-4%. On the other hand, Chen et al. (1998) obtained much higher transformation efficiency (upto 22%) of embryogenic calli of a japonica rice cv. Taipie 309. Cho et al. (2004) reported particle-bombardment mediated transformation of highly regenerative green tissue obtained from mature seeds of Taipie 309 with an efficiency of 6.5%.

In conclusion, these studies resulted in the development of a simple and reproducible protocol for transformation of Basmati rice varieties. This study will pave the way for genetic improvement of Basmati rice varieties especially for those characters which are otherwise difficult to achieve through conventional breeding methods.
CHAPTER 5

DEVELOPMENT OF BACTERIAL BLIGHT RESISTANT RICE

Bacterial blight caused by Xanthomonas oryzae pv. oryzae (Xoo) is one of the most devastating diseases of rice worldwide (Yu et al. 2008). Yield losses due to this disease can reach up to 50% (Adhikari et al. 1995). In Pakistan, the incidence of this disease is increasing in famous rice growing areas of Pakistan (Khan et al., 2000a) and no natural resistance to this disease could be found in Basmati cultivars (Khan et al., 2000b). Breeding bacterial blight resistant rice is therefore highly desirable. Conventional breeding is the most obvious choice to evolve a bacterial blight resistant rice variety, however, it involves lengthy crossing and back-crossing procedures and sometimes may also result in the incorporation of undesirable genetic components which are difficult to remove from the parental background. Genetic engineering on the other hand provides us with an opportunity to transfer either one or more cloned genes to the regenerable tissues from which resistant plants can be developed, hence providing us with relatively controlled engineering for improvement of desired traits.

Xa21 was the first resistance gene to be cloned (Song et al., 1995; Wang et al., 1996) and transferred into rice conferring high level of resistance to the bacterial blight pathogen (Tu et al., 1998; Zhang et al., 1998; Tang et al., 1999). The Xa21 gene not only conferred high level of resistance to the transgenic plants but these transgenic lines also exhibited excellent field performance (Tu et al. 2000). Therefore, Xa21 seems to be the best candidate gene so far to produce bacterial blight-resistant transgenic plants (Bajaj and Mohanty, 2005).

The present study was, therefore, aimed at establishing genetically engineered resistance in Basmati rice against bacterial leaf blight through transformation of Xa21 gene and evaluation of transgenic plants against the local isolates of bacterial blight pathogen X. oryzae pv. oryzae (Xoo).
TRANSFORMATION, SELECTION AND REGENERATION OF PLANTS

Mature embryos of five different Basmati rice varieties [Basmati-370 (B-370), Basmati-2000 (B-2000), Basmati-Pak (B-Pak), Basmati-385 (B-385) and Super Basmati (S-Basmati)] were co-bombarded with two different plasmids pC822 (having Xa21 gene) and pZS1 (having hpt as selectable marker gene) using BioRad Particle Delivery System (PDS-1000/He) following conditions as optimized and described in Chapter 4. The embryos were arranged on the bombardment medium, as shown in Figure 5.1a, three to four hours prior to bombardment. These embryos were bombarded using 1.0 µm gold particles at helium pressure of 7,500 kPa (1,100 psi) and target distance of 9 cm. Other conditions like vacuum, macro-carrier flight distance and rupture-disc to macro-carrier distance were kept constant at 685 mm of Hg, 8.0 mm, and 6.4 mm, respectively. Each plate was bombarded twice. The bombarded embryos were kept on the bombardment medium for 24 hours before placing them on the selection medium. Results of different transformations experiments are shown in Table 5.1. More than 2,800 mature embryos of different Basmati rice varieties were bombarded, and placed on selection medium, however, most of the bombarded embryos turned brown and necrotic indicating that these were not transformed. Only few embryos started producing small calli from the transformed sectors of the scutellar tissues after 7-8 days of culture. Figure 5.1 shows different steps in the development of bacterial blight resistant Basmati rice.

Highest transformation efficiency (2.38 %) was obtained in S-Basmati where 21 hygromycin resistant calli were recovered out of the 884 bombarded embryos while lowest transformation efficiency (1.12 %) was observed in B-385 where only 5 hygromycin resistant calli could be recovered from 522 bombarded embryos.

In B-370, 350 mature embryos were bombarded in two different experiments. Small calli started originating from the bombarded scutellar surface. These calli were separated from the mother tissue and allowed to grow further for second round of selection. However, only few calli showed vigorous growth while others either turned brown or exhibited a slow growth rate. These slow growing calli or the calli which turned brown were considered as non-transformed. Identification of transformed calli on the basis of
Table 5.1. Summary of bombardment, selection and regeneration of five different Basmati rice varieties from mature embryos bombarded with pC822 (having cloned \textit{Xa21} gene) and pZS1 (having \textit{hpt} gene).

<table>
<thead>
<tr>
<th>Rice variety</th>
<th>No. of embryos bombarded</th>
<th>Total No. of plates*</th>
<th>No. of selected calli</th>
<th>No. of regenerated plants</th>
<th>No. of Hyg(^{+}) plants</th>
<th>Mean Transformation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-370</td>
<td>184</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>1.71</td>
</tr>
<tr>
<td></td>
<td>166</td>
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<td>3</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>B-2000</td>
<td>175</td>
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<td>3</td>
<td>3</td>
<td>1.31</td>
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<tr>
<td></td>
<td>180</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>B-385</td>
<td>182</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1.12</td>
</tr>
<tr>
<td></td>
<td>172</td>
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<td>2</td>
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<td>3</td>
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</tr>
<tr>
<td></td>
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<td>1</td>
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</tr>
<tr>
<td>B-Pak</td>
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<td>4</td>
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<tr>
<td></td>
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<td>3</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>S-Basmati</td>
<td>186</td>
<td>3</td>
<td>6</td>
<td>8</td>
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<td></td>
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<tr>
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<td>4</td>
<td>5</td>
<td>5</td>
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</tr>
<tr>
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<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>2,801</td>
<td>48</td>
<td>54</td>
<td>50</td>
<td>49</td>
<td>1.75</td>
</tr>
</tbody>
</table>

* The plates were bombarded twice following optimized bombardment conditions.
Figure 5.1. Different steps in the development of bacterial blight resistant basmati rice. a) Mature embryos of B-370 arranged on bombardment medium 3-4 hours prior to bombardment; b) hygromycin resistant callus produced from bombarded embryo of S-Basmati; c) vigorous proliferation of hygromycin resistant callus of S-Basmati on selection medium; d) induction of somatic embryos on hygromycin resistant callus of B-370; e and f) plant regeneration from selected hygR calli of B-385; g) vigorous root formation in B-2000 putative transgenic plants in root induction medium; h) regenerated plants of Super Basmati and B-385 established in soil and kept in containment glass-house for growth, analysis and seed collection.
vigorous growth on hygromycin containing medium was the key factor for obtaining high
selection efficiency. From the 350 bombarded embryos, only 7 hygromycin resistant calli
were produced. These selected calli upon transfer to regeneration medium produced
only six plants.

In B-2000, only 10 hygromycin resistant calli were recovered from 533 mature embryos
that were bombarded in three independent experiments. These selected calli produced
seven putative transgenic plants. Similarly in other rice varieties like B-385 and B-Pak,
only 5 and 9 hygromycin resistant calli were recovered from 522 and 512 bombarded
embryos, respectively. From these selected calli, only 6 plants could be regenerated in
case of B-385 while 9 plants were recovered from selected B-Pak calli. In Super Basmati,
884 mature embryos were bombarded in five different experiments. Twenty one
vigorously growing hygromycin resistant calli were recovered which produced 22 plants
upon transfer to regeneration medium.

MOLECULAR ANALYSIS OF TRANSGENIC PLANTS
Two different approaches were adopted to provide proof of stable integration of foreign
genes in the transgenic plants which included PCR and Southern analysis.

PCR analysis
These transformation experiments were conducted using a co-transformation strategy i.e.
using two separate plasmids bearing Xa21 and hpt gene, respectively. Therefore the
initial screening of the putative transgenic plants was made through PCR. Genomic DNA
isolated from all the fifty regenerated putative transgenic plants was first subjected to
PCR analysis for the detection of hpt gene using hygromycin specific primers (Table
2.13). Figures 5.2 – 5.4 show PCR analysis of different Basmati rice varieties for the
detection of hpt gene in the primary transformants (T0 plants). Genomic DNA from all
the six putative transgenic B-370 plants subjected to PCR amplification showed
amplification of expected 0.9 kbp band as shown in Figure 5.2a. Similarly, specific
amplification of hpt gene fragment (0.9 kbp) was observed in all the nine regenerated
plants of B-Pak (Figure 5.2b), seven regenerated plants of B-2000 (Figure 5.3a), and six
Figure 5.2 Agarose gel electrophoresis of the PCR amplified products showing the amplification of the internal fragment of hpt gene from putative (T₀) transgenic plants of B-370 (a) and B-Pak (b) regenerated from hygromycin resistant calli after bombardment of mature embryos with plasmids pZS1 and pC822. a) M, 1 kbp DNA ladder; lane 1, plasmid (pZS1) control; lanes 2-7, DNA from six different putative (T₀) transgenic B-370 plants; b) M, 1 kbp DNA ladder; lane 1, plasmid (pZS1) control; lane 2, DNA from a non-transformed plant (negative control); lanes 3-11, DNA from nine different putative transgenic (T₀) B-Pak plants
Figure 5.3. Agarose gel electrophoresis of PCR amplified products from genomic DNA isolated from seven putative transgenic B-2000 plants (a), and six different putative (T₀) transgenic B-385 plants (b) showing amplification of correct sized (900 bp) fragment of the internal region of hpt gene. a) M, 1 kbp DNA ladder; lanes 1-7, DNA from seven different putative transgenic B-2000 lines; lane 8, DNA from a non-transgenic control; lane 9, plasmid pZS1. b) lane M shows 1 kbp DNA ladder as marker; lane 1, plasmid control (pZS1); lanes 2-7, DNA from six different putative transgenic B-385 plants.
Figure 5.4. (a & b) Agarose gel electrophoresis of the PCR amplified products showing the amplification of the internal fragment of *hpt* gene from putative transgenic (T₀) plants of S-Basmati regenerated from hygromycin resistant calli after bombardment of mature embryos with plasmids pZS1 and pC822. **a)** Lane M, 1 kbp DNA ladder; lane 1, plasmid (pZS1) control; lanes 2-13, DNA from twelve different putative transgenic (T₀) S-Basmati plants; **b)** Lane M, 1 kbp DNA ladder; lanes 1-10, DNA from ten different putative transgenic (T₀) S-Basmati plants; lane 11, negative control (DNA from a non-transformed control plant); and lane 12, plasmid (pZS1) control.
putative transgenic plants of B-385 (Figure 5.3b). In S-Basmati, a total of 22 plants were regenerated from hygromycin resistant calli. Genomic DNA from all of these putative transgenic plants was subjected to PCR analysis. Results of the PCR analysis of putative transgenic S-Basmati are shown in Figure 5.4. Only one plant represented by lane 10 in Figure 5.4a could not amplify the hygromycin specific fragment indicating that it was negative for the $hpt$ gene. However all other twenty one plants showed a correct sized (0.9 kbp) hygromycin specific fragment as shown in Figures 5.4a & 5.4b.

The second round of screening of the regenerated plants was done by PCR using $Xa21$ specific primers (Table 2.13). Figure 5.5 shows the PCR analysis of 27 different transgenic lines of B-370, B-2000, B-Pak and B-385 for the detection of $Xa21$ gene. In B-370, all the six lines amplified the correct sized (1.4 kbp) fragment of the $Xa21$ gene (Figure 5.5 a). Similarly, all the seven lines of B-2000; eight lines of B-Pak and six lines of B-385 amplified the correct sized fragment as shown in Figure 5.5 b-d. No amplification was observed in the non-transformed control. This indicated that all these lines contained $Xa21$ gene. Similarly, PCR analysis of transgenic S-Basmati lines was also conducted for the detection of $Xa21$ gene in these lines. Figure 5.6 shows PCR analysis of eleven different S-Basmati lines, which contained $hpt$ gene (as determined by PCR analysis), with $Xa21$ specific primers. The agarose gel electrophoresis of the amplified products showed amplification of the expected 1.4 kbp fragment of the $Xa21$ gene from all the eleven S-Basmati transgenic lines. No amplification was observed in the negative as well water controls (Figure 5.6, lanes 2 & 3, respectively). Similarly in another PCR reaction, seven T$_0$ putative transgenic Super Basmati plants were analysed for $Xa21$ gene. In only one plant represented, by lane 6 - Figure 5.7, no amplification of the respective $Xa21$ specific fragment could be observed indicating that this plant was negative for $Xa21$ gene.

To further authenticate PCR results, conditions were optimized for multiplex PCR using three different sets of primers which amplified three different targets in the same PCR reaction. These included RG100, $hpt$ and $Xa21$ pairs of primers. Figure 5.8 shows the results of multiplex PCR analysis of seven S-Basmati transgenic plants (those represented
Figure 5.5. Agarose gel electrophoresis of PCR amplified products showing the amplification of 1.4 kbp fragment of \textit{Xa21} gene from the genomic DNA of different transgenic lines of a) B-370; b) B-2000; c) B-Pak and d) B-385. M shows molecular weight marker (1 kbp DNA ladder); + represents plasmid (pC822) control; and - refers to DNA from a non-transformed control plant; lanes represented by numerical values are different transgenic lines.
Figure 5.6. PCR analysis of putative transgenic S-Basmati plants with Xa21 specific primers. M, 1 kbp ladder; lane 1, positive control (pC822); lane 2, water control; lane 3, Negative control (DNA from a non-transformed plant); lanes 4-14, DNA from putative transgenic plants.

Figure 5.7. PCR analysis for Xa21 gene in the T₀ transgenic plants of S-Basmati. Lane M, 1 kbp DNA ladder; lane 1, positive control (pC822); lane 2 DNA from a non-transformed control (Negative control); lane 3, water control; lanes 4-10, DNA from seven different T₀ transgenic lines.
Figure 5.8. Agarose gel electrophoresis of multiplex PCR amplified products showing the amplification of Xa21, hpt and RG100 locus from the genomic DNA isolated from seven different S-Basmati transgenic (T0) lines transformed with Xa21 and hpt genes. Lanes M represent 1 kbp DNA ladder as molecular weight marker while lane 1 represent water control; lane 2, plasmid pC822; lane 3, DNA from a non-transformed plant (negative control); lane 4, plasmid pZS1; lanes 5–11, DNA from seven different putative transgenic (T0) S-Basmati plants, respectively.
by lanes 4-10 in Figure 5.6). The results show that bands of expected sizes (0.7 kbp of RG100; 1.4 kbp of Xa21 and 0.9 kbp of hpt, respectively) were amplified from all the plants which were previously identified positive for hpt and Xa21 genes in independent PCR analysis experiments. This further confirmed the presence of hpt and Xa21 genes in these transgenic lines.

The PCR analysis showed that out of 50 regenerated putative transgenic plants, 49 plants were positive for hpt gene. When these 49 hygromycin positive plants were further analysed for the presence of Xa21 gene, it was found that 48 plants were positive for Xa21 gene. These results showed that 48 transgenic plants had both hpt as well as Xa21 genes.

Each transgenic plant was given a line number based on variety name followed by the experiment number from which the plants were regenerated and the plant number. Line numbers starting with 37 refers to transgenic lines of Basmati-370, whereas 20, P, 38 and S refers to Basmati-2000, Basmati-Pak, Basmati-385 and Super-Basmati, respectively. In these experiments, six B-370 transgenic plants were regenerated from two different experiments. Four plants were recovered from experiment 1, therefore these were named as 37-1-01, 37-1-02, 37-1-03 and 37-1-04. Similarly, from experiment 2, two transgenic plants were recovered which were named as 37-2-01 and 37-2-02. Same pattern of naming was used in transgenic lines of other varieties.

**Southern Analysis**

Southern analysis was conducted for different transgenic Basmati lines which were found to be positive for hpt as well as Xa21 gene (as determined by PCR). Ten microgram undigested and EcoRV (EC 3.1.21.4) or BamHI (EC 3.1.21.4) digested genomic DNA from ten transgenic (T0) lines of S-Basmati and a non-transformed control was transferred to nylon membrane following protocols as described by Sambrook et al. (1989) and hybridized to a radio-labelled 1.3 kbp HindIII-EcoRV fragment of the Xa21 as the gene probe. Figure 5.9 shows the Southern hybridization of DNA from ten
Figure 5.9. Southern analysis of putative S-Basmati rice plants transformed with \( Xa21 \) gene; +, pC822; -, DNA from a non-transformed control plant; w, water control; 1-10, represent DNA from 10 different putative transgenic S-Basmati rice lines, where each set of three lanes represent undigested DNA, \( EcoRV \) and \( BamH1 \) digest, respectively.

Figure 5.10. Southern analysis of 11 putative transgenic lines of S-Basmati transformed with \( Xa21 \) gene. Lane 1, plasmid control (\( HindIII-EcoRV \) 1.3 kbp fragment); Lane 2, DNA from a non-transformed plant; lanes 3-13, DNA from 11 different putative transgenic S-Basmati digested with \( HindIII-EcoRV \).
Figure 5.11. Southern analysis of nine putative transgenic lines of different Basmati rice varieties (B-370, B-2000, B-Pak and B-385) transformed with \textit{Xa21} gene. + represents plasmid control (\textit{Hind}III-\textit{Eco}RV, 1.3 kbp fragment); - shows DNA from a non-transformed plant; The other lanes represent genomic DNA, digested with \textit{Hind}III-\textit{Eco}RV, from nine different transgenic lines where 37-1-01; 37-2-01 are B-370 lines, 20-1-02 and 20-2-01 are B-2000 lines; P-1-01, P-2-03 and P-3-03 are B-Pak while 38-1-01 and 38-2-03 are the two B-385 transgenic lines, respectively.
different S-Basmati plants using *Xa21* as the gene probe. Figure 5.9 shows that *Xa21* gene has been integrated into the high molecular weight DNA as represented by the each of the first lanes in the set of three representing different S-Basmati transgenic lines. In the *EcoRV* digest, a common band appeared at the same position in almost all the samples because two sites of *EcoRV* are located within the *Xa21* coding sequence. However, some bands below and above this specific band were seen which indicates re-arrangements. *BamH1* digests however, showed variation in the number of bands and 2-5 bands could be seen in the autoradiogram indicating that *Xa21* gene may have integrated at 2-5 different loci.

In another Southern hybridization experiment (Figure 5.10), DNA from eleven different putative transgenic S-Basmati plants was double-digested with *HindIII* and *EcoRV*, and hybridized to a radio-labelled 1.3 kbp *HindIII-EcoRV* fragment of the *Xa21* as the gene probe. Ten plants showed specific hybridization with corresponding 1.3 kbp bands from the genomic DNA of the transgenic rice lines indicating that the *Xa21* gene has been incorporated in these rice lines. One plant (S-3-03) did not show any hybridization with the *Xa21* gene probe. This showed that plant line S-3-03 was negative for *Xa21* gene. Although approximately equal amounts of DNA was loaded for each rice line, however, it can be seen that the band intensity varied between the different lanes which may indicate difference in number of copies of *Xa21* gene that may have been inserted in the plant genome. In lanes 10 and 12, a band of less than 1.3 kbp could be seen which indicates that re-arrangement might have occurred before the integration of *Xa21* in the plant genome of the respective transgenic lines. Similarly, Southern analysis was performed for nine other transgenic lines representing two lines each from B-370, B-2000 and B-385 while three transgenic lines of B-Pak. Genomic DNA from nine different transgenic lines, as shown in Figure 5.11, was double-digested with *HindIII* and *EcoRV* and hybridized to a radio-labelled 1.3 kbp *HindIII-EcoRV* fragment of the *Xa21* as the gene probe. All the plants showed specific hybridization with corresponding 1.3 kbp
bands from the genomic DNA of the transgenic rice lines indicating that the \textit{Xa21} gene has been incorporated in these rice lines.

\textbf{SCREENING OF T\textsubscript{1} GENERATION}

\textbf{Screening of transgenic (T\textsubscript{1}) plants for hygromycin resistance}

In order to check the presence of a functional \textit{hpt} transgenes in the T\textsubscript{1} generation, a simple germination test was performed. T\textsubscript{1} seeds from six selected lines along with non-transformed controls were placed on MS-0 medium either without hygromycin B or having 50 mg l\textsuperscript{-1} hygromycin B. The results of these experiments are shown in Table 5.2 and Figure 5.12. The results show that 83-100 % seeds of different transgenic lines along with non-transformed controls germinated on MS-O medium having no hygromycin B. Whereas, on MS medium supplemented with 50 mg l\textsuperscript{-1} hygromycin B, no germination was observed in any of the controls, whereas 86 - 90 % seeds of different transgenic lines germinated on the said medium indicating that the \textit{hpt} gene was functional in all these T\textsubscript{1} transgenic lines.

\textbf{Screening of transgenic (T\textsubscript{1}) plants for bacterial blight resistance}

The reaction of the transgenic T\textsubscript{1} plants to the bacterial blight (BB) pathogen was evaluated by inoculation with two different local isolates of \textit{X. oryzae pv. oryzae} (\textit{Xoo}). Six to eight leaves of each of the two plants per pot (Figure 5.13), representing a transgenic line /non-transformed control were inoculated with \textit{Xoo} isolates , \textit{Xoo} 1.2.1 or \textit{Xoo} 3.2.2, using leaf clipping method (Kauffman \textit{et al.}, 1973). Plant reaction was scored 14 days after the inoculation. Lesion lengths of each of the inoculated leaf were scored and the disease reaction was characterized as resistant, moderately resistant or susceptible based on the lesion length of 0-3 cm, 3-6 cm and >6 cm, respectively. The results of bacterial blight (BB) reaction of transgenic rice lines and their corresponding controls inoculated with two local isolates of \textit{Xoo} are presented in Table 5.3. All the Basmati varieties (non-transformed controls) were found to be susceptible to both the local isolates.
Table 5.2. Germination of seeds collected from transgenic (T₀) and un-transformed (control) plants on MS-O medium (MS salts & vitamins without growth regulators) without hygromycin B or having 50 mg l⁻¹ hygromycin B. Respective controls of each variety were also included.

<table>
<thead>
<tr>
<th>Line No.</th>
<th>Germination on MS-O medium without Hygromycin B</th>
<th>Germination on MS-O medium having 50 mg l⁻¹ hygromycin B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of seeds</td>
<td>No. of germinating seeds</td>
</tr>
<tr>
<td>B370-C</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>B2000-C</td>
<td>21</td>
<td>20</td>
</tr>
<tr>
<td>B-Pak-C</td>
<td>24</td>
<td>22</td>
</tr>
<tr>
<td>B385-C</td>
<td>20</td>
<td>18</td>
</tr>
<tr>
<td>S-Bas-C</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>37-1-01</td>
<td>21</td>
<td>20</td>
</tr>
<tr>
<td>20-3-01</td>
<td>25</td>
<td>23</td>
</tr>
<tr>
<td>P-2-03</td>
<td>24</td>
<td>22</td>
</tr>
<tr>
<td>38-1-02</td>
<td>24</td>
<td>23</td>
</tr>
<tr>
<td>S-1-02</td>
<td>18</td>
<td>15</td>
</tr>
<tr>
<td>S-3-05</td>
<td>20</td>
<td>19</td>
</tr>
</tbody>
</table>
Figure 5.12. Hygromycin sensitivity test of T₁ seeds of transgenic Basmati line (S-3-05) along with non-transformed controls. a & c are non-transformed S-Basmati seeds placed on MS-O medium without hygromycin B and having 50 mg l⁻¹ hygromycin B, respectively; b & d, represent T₁ seeds of S-Basmati transgenic line (S-3-05) placed on MS-O medium without hygromycin B and having 50 mg l⁻¹ hygromycin B, respectively.
Initial symptoms developed at the point of inoculation within 4-5 days in the form of leaf curling. It was observed that in case of transgenic lines showing high level of resistance, the lesions progressed uniformly downward from the point of inoculation whereas in case of moderately resistant or susceptible plant lines, the lesions progressed more rapidly along the leaf margins. Figure 5.14 shows the BB reaction of different transgenic lines and the respective controls following inoculation with two local isolates of Xoo. The transgenic plants showed a variable reaction to Xoo inoculation. In response to Xoo isolate 1.2.1, thirty-four transgenic lines showed a resistant (R) reaction and produced lesions of \( \leq 3.0 \) cm length while eleven lines showed moderately resistant (MR) reaction (lesion length ranging from 3.12 – 5.65 cm). Four lines (37-2-02, 20-1-01, S-3-03 and S-4-01) showed a susceptible reaction producing lesions ranging from 6.98 – 17.85 cm). Similarly in response to Xoo isolate 3.2.2, thirty-eight transgenic lines produced lesions of \( \leq 3.0 \) cm, while 8 lines showed a moderately susceptible reaction (lesion length ranging from 3.15 – 4.80 cm) and only 3 transgenic lines showed a susceptible reaction producing lesions in the range of 7.68 – 16.08 cm compared to larger lesion lengths ranging from 14.43 - 17.43 cm in the non-transformed control plants. Three transgenic lines (37-2-02, 20-1-01 and S-3-03) were found to be susceptible to isolate 3.2.2. The lesion length in two of these transgenic lines (i.e. 20-1-01 and S-3-03) was comparable to an extent as that of control plants. One of these plant lines (i.e. S-3-03) was actually negative for Xa21 gene as adjudged by PCR and southern analysis. On the other hand, the plant line 20-1-01 showed a susceptible reaction to both the local isolates of Xoo, indicating that the expression of the transgene in this plant was completely missing.

In B-370, significant differences (at \( p=0.05 \)) in disease reaction were observed (Table 5.3) between transgenic lines and the controls towards both the Xoo isolates. Only one line (37-2-02) showed a susceptible reaction while other lines either showed a resistant or moderately resistant reaction. Line numbers 37-1-01 and 37-1-03 exhibited high level of resistance to both the isolates of Xoo and developed 22-27 times shorter lesions (ranging from 0.57 – 0.8 cm) compared to the controls (14.43 – 17.58 cm). In B-2000, five out of seven transgenic lines showed a resistant reaction with significantly reduced lesion lengths (ranging from 0.8 – 2.92 cm) in reaction to both Xoo isolates. Transgenic line 20-
Table 5.3. BB reactions of transgenic Basmati rice lines and their corresponding controls inoculated with two local isolates (Xoo 1.2.1 and 3.2.2) of Xoo. Disease reactions are characterized as resistant (R), moderately resistant (MR), or susceptible (S) based on leaf lesion length of 0-3 cm, 3-6 cm or > 6 cm, respectively.

<table>
<thead>
<tr>
<th>Rice Variety</th>
<th>Line Number</th>
<th>Reaction to Isolate 1.2.1</th>
<th>Reaction to Isolate 3.2.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-370</td>
<td>Control</td>
<td>Lesion length (cm) Reaction</td>
<td>Lesion length (cm) Reaction</td>
</tr>
<tr>
<td></td>
<td>17.58 ± 1.17 a</td>
<td>S</td>
<td>14.43 ± 1.37 a</td>
</tr>
<tr>
<td></td>
<td>37-1-01</td>
<td>0.65 ± 0.21 d</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>37-1-02</td>
<td>1.15 ± 0.23 d</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>37-1-03</td>
<td>0.80 ± 0.36 d</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>37-1-04</td>
<td>2.87 ± 0.36 c</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>37-2-01</td>
<td>3.37 ± 0.31 c</td>
<td>MR</td>
</tr>
<tr>
<td></td>
<td>37-2-02</td>
<td>7.42 ± 1.23 b</td>
<td>S</td>
</tr>
<tr>
<td>B-2000</td>
<td>Control</td>
<td>16.73 ± 2.50 a</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>20-1-01</td>
<td>17.85 ± 2.34 a</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>20-1-02</td>
<td>0.88 ± 0.30 c</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>20-1-03</td>
<td>2.83 ± 0.47 b</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>20-2-01</td>
<td>2.70 ± 0.37 b</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>20-2-02</td>
<td>3.67 ± 0.63 b</td>
<td>MR</td>
</tr>
<tr>
<td></td>
<td>20-3-01</td>
<td>1.58 ± 0.38 c</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>20-3-02</td>
<td>2.23 ± 0.30 bc</td>
<td>R</td>
</tr>
<tr>
<td>B-Pak</td>
<td>Control</td>
<td>16.20 ± 1.37 a</td>
<td>S</td>
</tr>
<tr>
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<td>P-1-01</td>
<td>2.55 ± 0.46 c</td>
<td>R</td>
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<tr>
<td></td>
<td>P-1-02</td>
<td>1.52 ± 0.44 d</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>P-2-01</td>
<td>5.52 ± 1.33 b</td>
<td>MR</td>
</tr>
<tr>
<td></td>
<td>P-2-02</td>
<td>2.95 ± 0.39 c</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>P-2-03</td>
<td>2.63 ± 0.34 c</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>P-3-01</td>
<td>1.22 ± 0.48 d</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>P-3-02</td>
<td>0.90 ± 0.32 d</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>P-3-03</td>
<td>4.40 ± 0.44 b</td>
<td>MR</td>
</tr>
<tr>
<td></td>
<td>P-3-04</td>
<td>1.88 ± 0.46 cd</td>
<td>R</td>
</tr>
<tr>
<td>B-385</td>
<td>Control</td>
<td>18.83 ± 1.35 a</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>38-1-01</td>
<td>0.63 ± 0.23 e</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>38-1-02</td>
<td>1.42 ± 0.34 de</td>
<td>R</td>
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<td>5.67 ± 1.11 b</td>
<td>MR</td>
</tr>
<tr>
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<td>38-2-02</td>
<td>2.32 ± 0.69 d</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>38-2-03</td>
<td>4.15 ± 0.67 c</td>
<td>MR</td>
</tr>
<tr>
<td></td>
<td>38-3-01</td>
<td>0.78 ± 0.42 e</td>
<td>R</td>
</tr>
<tr>
<td>S-Basmati</td>
<td>Control</td>
<td>17.82 ± 0.88 a</td>
<td>S</td>
</tr>
<tr>
<td></td>
<td>S-1-01</td>
<td>0.92 ± 0.45 i</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>S-1-02</td>
<td>0.53 ± 0.23 j</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>S-1-03</td>
<td>2.72 ± 0.67 f</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>S-1-04</td>
<td>1.98 ± 0.44 gh</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>S-1-05</td>
<td>2.53 ± 0.50 fg</td>
<td>R</td>
</tr>
<tr>
<td></td>
<td>S-1-06</td>
<td>3.12 ± 0.40 e</td>
<td>MR</td>
</tr>
<tr>
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</tr>
<tr>
<td>S-1-07</td>
<td>0.67 ± 0.29 ij</td>
<td>R</td>
<td>0.43 ± 0.14 g</td>
</tr>
<tr>
<td>S-2-01</td>
<td>2.17 ± 0.54 g</td>
<td>R</td>
<td>1.27 ± 0.32 fg</td>
</tr>
<tr>
<td>S-2-02</td>
<td>3.72 ± 0.78 de</td>
<td>MR</td>
<td>1.93 ± 0.24 ef</td>
</tr>
<tr>
<td>S-2-03</td>
<td>5.65 ± 0.73 c</td>
<td>MR</td>
<td>3.28 ± 0.63 cd</td>
</tr>
<tr>
<td>S-2-04</td>
<td>0.43 ± 0.14 j</td>
<td>R</td>
<td>0.50 ± 0.20 g</td>
</tr>
<tr>
<td>S-3-01</td>
<td>2.62 ± 0.49 f</td>
<td>R</td>
<td>2.27 ± 0.32 e</td>
</tr>
<tr>
<td>S-3-02</td>
<td>4.22 ± 0.68 d</td>
<td>MR</td>
<td>2.38 ± 0.52 de</td>
</tr>
<tr>
<td>S-3-03</td>
<td>16.18 ± 1.42 a</td>
<td>S</td>
<td>16.08 ± 0.85 a</td>
</tr>
<tr>
<td>S-3-04</td>
<td>1.28 ± 0.42 hi</td>
<td>R</td>
<td>0.98 ± 0.44 g</td>
</tr>
<tr>
<td>S-3-05</td>
<td>2.85 ± 0.29 f</td>
<td>R</td>
<td>2.10 ± 0.35 e</td>
</tr>
<tr>
<td>S-4-01</td>
<td>6.98 ± 1.25 b</td>
<td>S</td>
<td>4.80 ± 0.91 b</td>
</tr>
<tr>
<td>S-4-02</td>
<td>2.87 ± 0.47 f</td>
<td>R</td>
<td>2.28 ± 0.51 e</td>
</tr>
<tr>
<td>S-4-03</td>
<td>4.27 ± 0.66 d</td>
<td>MR</td>
<td>3.62 ± 0.47 c</td>
</tr>
<tr>
<td>S-5-01</td>
<td>0.73 ± 0.32 i</td>
<td>R</td>
<td>0.57 ± 0.29 g</td>
</tr>
<tr>
<td>S-5-02</td>
<td>3.00 ± 0.55 ef</td>
<td>R</td>
<td>2.70 ± 0.54 d</td>
</tr>
</tbody>
</table>

For each rice variety, means within a column followed by a common letter are not significantly different at 5% level by DMRT.

Each value is the mean of three replicates.

± refers to standard deviation between the replicates.
Figure 5.13. $T_1$ generation of transgenic S-Basmati rice lines transformed with $Xa21$ gene along with non-transformed controls being raised in the containment glass house at NIBGE for screening for bacterial blight resistance.

Figure 5.14. Screening transgenic S-Basmati lines against two local isolates of Bacterial blight pathogen, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Picture shows the screening of three transgenic SB lines along with the un-transformed control. Each line is represented by a set of four leaves, of them two leaves each were inoculated with local *Xoo* isolates 1.2.1 and 3.2.2. Picture was taken after 14 days of *Xoo* inoculation.
1-02 exhibited a high level of resistance to both the isolates. One line (20-2-02) showed a moderately resistant reaction while line 20-1-01 was found to be susceptible to both the isolates and produced lesions (ranging from 14.97 – 17.85 cm) comparable with that of non-transformed control (16.73 – 17.43 cm). In B-Pak, significant differences (at p=0.05) in disease reaction against both the Xoo isolates were observed between the transgenic lines and the control plants. Seven B-Pak transgenic lines showed resistant reaction (lesion lengths ranging from 0.58 – 2.97 cm) to both the Xoo isolates, however, lines P-1-02, P-3-01 and P-3-02 were found to be more resistant than other B-Pak transgenic lines producing lesions ranging in length from 0.58 – 1.52 cm. Two lines (P-2-01 and P-3-03) were found to be moderately susceptible to both isolates 1.2.1 and 3.2.2 and produced lesion in the range of 3.87 – 5.52 cm while none of the transgenic lines showed susceptible reaction. Similarly in B-385, significant differences in disease reaction were observed between the transgenic lines and the control plants in response to Xoo inoculation. Four transgenic lines (38-1-01, 38-1-02, 38-2-02 and 38-3-01) showed a resistant reaction to both isolates and produced lesion lengths ranging from 0.55 – 2.32 cm. Two lines (38-2-01 and 38-2-03) were found to be moderately resistant to isolate 1.2.1 while only one line was found to be moderately resistant to isolate 3.2.2. In S-Basmati, except line S-3-03, all transgenic lines produced significantly reduced lesion lengths compared to the controls in response to the both Xoo isolates. However, transgenic lines showed a variable response in reaction to both the isolates. A line resistant to one isolate was found to be moderately resistant to the other or moderately resistant to one isolate and susceptible to the other. In response to Xoo isolate 1.2.1, fourteen S-Basmati transgenic lines showed resistant reaction with average lesion lengths ranging from 0.43 – 3.0 cm, five showed moderately resistant reactions producing lesions ranging in length from 3.12 – 5.65 cm while two lines (S-3-03 and S-4-01) showed a susceptible reaction with lesions as large as 6.98 – 16.18 cm in contrast to the average lesion length (17.82 cm) produced in the non-transformed control. In response to isolate 3.2.2, seventeen transgenic lines showed a resistant reaction with average lesion length ranging from 0.43 – 2.70 cm, three lines exhibited a moderately resistant reaction (3.28 – 4.8 cm) while only one line (S-3-03) was found to be susceptible with 16.08 cm
large lesions compared to the non-transformed control with average lesion length of 16.55 cm. This line was found to be susceptible to both Xoo isolates.

These results indicated that the Xa21 functioned well in the most of the transgenic T1 plants, however, it was silenced or inactivated in one of the B-2000 transgenic line (20-1-01) during its inheritance from the T0 generation to the next. Another S-Basmati transgenic line (S-3-03) which showed susceptible reaction to both Xoo isolates was actually negative for Xa21 gene as indicated by both PCR and southern analysis.

**ANALYSIS OF THE T2 GENERATION OF THE TRANSGENIC PLANTS**

Due to limited space available for field testing, it was not possible to grow all the transgenic lines in the field. Therefore only six promising S-Basmati transgenic lines showing high level of bacterial blight resistance in the T1 generation were selected (as shown in Table 5.4) and grown along with a non-transformed control in a replicated field trial. Since the transformation was carried out using two separate plasmids bearing hpt and Xa21 genes, therefore, there was a probability that hpt and Xa21 might segregate in the T2 generation. The probability of such segregation would depend, however, on the number of loci at which the integration of these genes had taken place and how tightly these genes are linked. In a search for identifying marker free transgenic lines, DNA from the T2 plants was subjected to PCR analysis for both Xa21 and hpt genes. Figures 5.15 and 5.16 show the analysis of 32 different plants representing six different promising Super Basmati transgenic lines as shown in Table 5.4. One to two plants were selected at random, representing one plant line, from each block of a replicated limited field trial of transgenic Super Basmati. These results show that out of the 32 plants analysed by PCR, 27 plants contained both hpt and Xa21 genes while three plants represented by Figure 5.16, lane 14, 17 and 22, respectively contained only the hpt gene as no amplification of Xa21 specific gene fragment could be obtained in these plants. Similarly, two plants contained only Xa21 gene as no amplification of hpt gene was recorded (Figure 5.15, lanes 10 and 11). These results were further confirmed by repeating the PCR reaction, but same results were obtained. These results confirmed that two plants represented by
Figure 5.15. PCR detection of hpt gene with hygromycin specific primers in 32 different T2 plants of transgenic S-Basmati lines. Lanes M represent 1 kbp DNA ladder as molecular weight marker; + represent plasmid (pZS1) control; - represents no DNA control; lanes 1-32 are respective DNA's from 32 transgenic plants.
Figure 5.16. PCR detection of Xa21 gene in 32 different T2 plants of transgenic S-Basmati lines. Lanes M, represent 1 kbp DNA ladder as molecular weight marker; + represent plasmid (pC822) control; - represents no DNA control; lanes 1-32 are respective DNA’s from 32 transgenic plants.
Figure 5.15, lanes 10 and 11 were marker free lines containing only the bacterial blight resistance (*Xa21*) gene. So there is a high probability of finding more number of marker free plants if the whole population is screened.

**FIELD PERFORMANCE OF TRANSGENIC BB RESISTANT BASMATI LINES**

Limited field trials of some of the selected S-Basmati transgenic lines (S-1-01, S-1-02, S-1-07, S-2-04, S-3-04 and S-5-01) were conducted at the experimental area inside the premises of NIBGE, Faisalabad (Figure 5.17). Sixty-four T$_2$ seedlings of each selected transgenic line along with non-transformed controls were transplanted in each block with a plant to plant distance of 25 cm in three replicates. Data for plant height, number of fertile tillers per plant and grain yield per plant was collected from 10 plants randomly selected from each of the replicated plots representing a transgenic line/non-transformed control in a randomized complete block (RCB) design having three replicates. Data regarding grain-length and grain-width was collected from 10 seeds of one plant each from the three replicated plots representing a transgenic line / non-transformed control in the limited field trials. The data regarding these characters is projected in Table 5.4. These results show that there was a significant reduction in plant height in all the transgenic lines compared to the control. The mean plant height recorded for the control plants was 122.4 cm which was significantly higher ($p = < 0.05$) compared to different transgenic lines ranging in plant height from 84.0 to 95.2 cm. Three lines (S-1-02, S-2-04 and S-5-01) showed a significant increase in the number of fertile tillers compared to the controls while no significant increase or decrease in the number of fertile tillers per plant was observed in three lines i.e. S-1-01, S-1-07 and S-3-04. Transgenic lines took significantly longer time to flower compared to the non-transformed controls. The transgenic lines took 8-12 days longer to flower compared to the non-transformed controls. The mean flowering time of transgenic line S-1-02 was 85.3 days compared to 73.3 days in the non-transformed control. There was no significant difference (at $p=0.05$) in the grain yield per plant between the transgenic lines and the non-transformed control. However, there was a lot of variation in grain yield from plant to plant as indicated by the standard deviations from the means in some of the transgenic lines like S-1-02, S-1-07.
Table 5.4. Field performance of transgenic S-Basmati rice lines

<table>
<thead>
<tr>
<th>Line number</th>
<th>Plant height* (cm)</th>
<th>Number of fertile tillers per plant*</th>
<th>Days to flowering*</th>
<th>Grain yield per plant* (g)</th>
<th>Grain Length (mm) †</th>
<th>Grain Width (mm) †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>122.4 ± 4.34 a</td>
<td>21.4 ± 3.84 b</td>
<td>73.3 ± 1.26 c</td>
<td>21.94 ± 4.42 a</td>
<td>8.12 ± 0.36 a</td>
<td>1.70 ± 0.05 b</td>
</tr>
<tr>
<td>S-1-01</td>
<td>91.4 ± 2.31 b</td>
<td>25.2 ± 5.76 b</td>
<td>82.3 ± 0.50 a</td>
<td>25.82 ± 5.90 a</td>
<td>7.92 ± 0.39 a</td>
<td>1.69 ± 0.07 b</td>
</tr>
<tr>
<td>S-1-02</td>
<td>95.2 ± 3.83 b</td>
<td>28.8 ± 9.01 a</td>
<td>85.3 ± 0.96 ab</td>
<td>26.26 ± 10.2 a</td>
<td>7.87 ± 0.14 a</td>
<td>1.76 ± 0.05 ab</td>
</tr>
<tr>
<td>S-1-07</td>
<td>92.6 ± 10.0 b</td>
<td>20.4 ± 7.09 b</td>
<td>80.8 ± 3.30 bc</td>
<td>26.08 ± 14.3 a</td>
<td>8.00 ± 0.21 a</td>
<td>1.83 ± 0.13 a</td>
</tr>
<tr>
<td>S-2-04</td>
<td>84.0 ± 5.52 c</td>
<td>34.8 ± 6.83 a</td>
<td>83.3 ± 1.89 a</td>
<td>17.10 ± 4.05 ab</td>
<td>7.75 ± 0.30 ab</td>
<td>1.78 ± 0.10 a</td>
</tr>
<tr>
<td>S-3-04</td>
<td>87.6 ± 5.18 bc</td>
<td>26.0 ± 8.46 ab</td>
<td>82.8 ± 1.26 a</td>
<td>32.60 ± 16.38 a</td>
<td>7.83 ± 0.42 a</td>
<td>1.63 ± 0.16 bc</td>
</tr>
<tr>
<td>S-5-01</td>
<td>88.0 ± 3.08 b</td>
<td>26.2 ± 5.40 a</td>
<td>81.0 ± 2.45 b</td>
<td>20.92 ± 5.77 a</td>
<td>7.80 ± 0.32 a</td>
<td>1.70 ± 0.10 b</td>
</tr>
</tbody>
</table>

Means within a column followed by a common letter are not significantly different at 5% level by DMRT.
*Based on data collected from 10 plants randomly selected from each plot representing a transgenic line/non-transformed control in a Randomized Complete Block (RCB) Design having three replicates.
†Based on data collected from 10 seeds of plant representing a transgenic line / non-transformed control in a limited field trial having three replicates.
Figure 5.17. Limited field trials of some of the selected S-Basmati transgenic lines along with non-transformed controls conducted at NIBGE, Faisalabad.
and S-3-04. No significant difference (p > 0.05) in grain-length was observed in the transgenic grains compared to the grains from the non-transformed control plants. However, grain-width in two of the transgenic lines S-1-07 and S-2-04 was significantly reduced compared to the seeds from the control plants.

In summary, transgenic Basmati lines were developed which showed resistance to two different local isolates of bacterial blight pathogen (*X. oryzae pv. oryzae*). The transgenic lines showed a significant reduction in plant height compared to the control plants. This short stature of transgenic Basmati rice is an important development as yield losses due to lodging has been one of the major problems in Basmati rice cultivation. There was no significant difference in the grain yield and grain-length of the transgenic plants compared to control. Any decrease in grain yield or grain length would have been highly undesirable. The only drawback observed in the these transgenic lines was that all the transgenic plants took 8-12 days longer to flower compared to the non-transformed controls. Field data of the subsequent generations, however, will help to determine the consistently of these characteristics in the next generations.

**DISCUSSION**

One of the key challenges in agriculture worldwide is to control the significant yield losses caused by pests and pathogens. Bacterial blight is one of the most destructive bacterial diseases of rice worldwide (Yu *et al.*, 2008). Therefore, incorporation of resistance genes form resistant sources either through conventional breeding or genetic transformation is highly desirable. *Xa21*, identified from a wild rice species called *O. longistaminata*, is the first resistance gene to be cloned (Song *et al.*, 1995) and transferred in any cereal crop plants (Wang *et al.*, 1996). Due to its wide-spectrum resistance to the bacterial blight pathogen, it is of great significance in breeding rice for resistance against this dreadful disease (Huang *et al.*, 2005).

Khan *et al.* (2000b) observed that none of the 39 local rice varieties they tested were resistant to bacterial blight. Akhtar *et al.* (2003) noted that Super basmati, which is the most widely cultivated Basmati rice variety in the Punjab province, was highly
susceptible to bacterial blight. Noor et al. (2006) reported that Super Basmati was susceptible to all the exotic strains of *X. oryzae* pv. *oryzae* they tested. They further noticed that Basmati-385 and Basmati-2000 were susceptible to only couple of exotic strains while showing resistance to most of them. These studies indicate that engineering bacterial blight resistance in Basmati rice is highly desirable.

Most of the *Xa21* transgenic Basmati lines developed during the course of this study have shown high level of resistance against two local isolates of the bacterial blight pathogen (*Xoo*). Thirty-four transgenic lines produced lesions of ≤ 3.0 cm, while 11 lines showed a moderately susceptible reaction and only four transgenic lines showed a susceptible reaction compared to larger lesion ranging from 16.20 - 18.83 cm in the non-transformed control plants against the *Xoo* isolate 1.2.1. Similarly thirty-six transgenic lines produced lesions of ≤ 3.0 cm, while 8 lines showed a moderately susceptible reaction and only 3 transgenic lines showed a susceptible reaction compared to larger lesion ranging from 14.43 - 17.43 cm in the non-transformed control plants against the *Xoo* isolate 3.2.2. Two transgenic lines (20-1-01 and S-3-03) were found to be highly susceptible to both the *Xoo* isolates to the extent as that of control plants. One of these plant lines (i.e. S-3-03) was actually negative for *Xa21* gene. On the other hand, the plant line # 20-1-01 showed a highly susceptible reaction to both the local isolates of *Xoo*, indicating that the expression of the transgene in this plant was completely missing. These results are in agreement with those reported by Tu et al. (1998). They developed *Xa21* transgenic IR72 lines and observed highly reduced lesions of less than 3.1 cm in transgenic bacterial blight resistant plants and a *Xa21* donor (IRBB21), compared to the larger lesions of 13.3 – 20.3 cm in the control plants.

In another study reported by Jiang et al. (2004), *Xa21* and a fused Bt cry1Ab/1Ac were introduced into an elite indica cytoplasm male sterile (CMS) restorer line ‘Minghui 63’. They demonstrated that the average lesion lengths of BB in the pyramided line ‘Minghui 63’/Bt & *Xa21* and in ‘Minghui 63’/*Xa21* was 5.8 cm compared to 21.4 cm in ‘Minghui 63’ controls. Similarly, Maruthasalam et al. (2007) pyramided Pusa Basmati1 line with *chi11, tlp*, and *Xa21* and showed an enhanced resistance to both sheath blight and
bacterial blight. They showed that apart from the reduction in the number of infection cushions, the leaves of transgenic plants also exhibited reduced lesion length with characteristic browning around the lesions, which clearly showed effective restriction of pathogenic invasion.

These studies have shown that transgenic Basmati rice lines transformed with Xa21 gene exhibited variation in plant height, days to flowering and the number of fertile tillers per plant while no significant difference in grain yields was observed compared to the control plants. No significant difference was observed in the grain yield and other grain characteristics of the transgenic Basmati rice lines compared to the non-transformed controls. However, few lines showed reduced grain-width compared to the controls. Tu et al. (2000) observed that some of the transgenic IR72 lines transformed with Xa21 gene gave excellent field performance. They found that yield performance of one of the transgenic line T103-10 was comparable with that of control. Similarly Datta et al. (2007) saw no significant variation in grain yields, 100-seed weight and total biomass of the golden rice transgenic lines as compared to the control plants. However, few studies indicated that transgenic lines exhibited lower grain yields compared to their respective controls. Kim et al. (2008) observed significant reduction in yield components of three Korean rice varieties transformed with cry1Ab gene compared to those of the parental varieties.

A significant reduction in plant height of the transgenic lines was detected ranging from 84.0 – 95.2 cm which was 22-32% less than the respective controls. Similar results have also been reported by other workers. Bashir et al. (2004) noticed significant reduction in plant height of the transgenic rice lines L-5, L-13 and L-6-411 compared to the controls. Kim et al. (2008) observed highly significant reduction in plant height in few insect resistant transgenic lines of three Korean rice varieties as much as 65% compared to the controls, however, some transgenic lines showed normal plant height.

Transgenic Basmati lines took 8-12 days longer to flower compared to the control plants. Similar behaviour of delayed flowering time has also been reported in several other
studies (Tu et al., 2000; Bashir et al., 2004). Three of these transgenic lines also showed significant increase in the number of fertile tillers. Shu et al. (2002) also noticed tremendous variation in number of productive tillers in the T₀ and T₁ transgenic plants of nine different rice varieties. One of these transgenic Basmati-2000 lines (i.e. 20-1-01) exhibited a highly susceptible reaction to both the isolates comparable to that of the non-transformed control. A similar transgene inactivation of the Xa21 expression in few transgenic IR72 plants was reported by Tu et al. (1998). These variations in phenotypic and agronomic characters may be a result of somaclonal variations, insertion mutagenesis, transgene induced endogenous gene silencing (Larkin and Scowcroft, 1981; Matzke et al., 1993; Kathuria et al., 2007).

This study shows that transgenic approach could be used as a powerful tool in the rice breeding program. Transgenic rice with bacterial blight resistance would have a large economic impact. It can be effectively cultivated in those areas where bacterial blight is emerging as a major threat to rice production.
Transgenic technology provides us with powerful tools for developing transgenic plants with improved traits that are otherwise difficult to achieve through conventional breeding. It offers unique opportunities to introduce novel genetic traits from diverse biological organisms and to create desired phenotypes from known genes (Zhong, 2001). The application and potential use of transgenic technology in crop improvement has been very well demonstrated in recent commercialization of varieties and hybrids with novel and improved traits such as insect and disease resistance (During, 1996; Jouanin et al., 1998) and tolerance to herbicides (Tsaftaris, 1996). Transgenic technology is, therefore, becoming an essential part of plant breeding (Zhong, 2001).

While making a transgene to work out for a desired phenotype is a fundamental challenge, there are certain pre-requisites which are critical for the success of transgenic breeding. These include availability of reproducible and efficient tissue culture system for the subject crop, accessibility to cloned genes for transformation and an appropriate gene transfer system. The availability of a highly efficient and robust tissue culture method is the first and the most critical pre-requisite for developing a high efficiency transformation system (Geng et al., 2008). In indica rice, despite of the extensive efforts in the in-vitro culture, robust and widely applicable methods for embryogenic callus induction and regeneration have not been established. The success of all the published protocols for culturing indica rice is largely genotype-dependent, which limits the use of such protocols (Lin and Zhang, 2005).

It is known from literature that successful in-vitro culture of rice depends on a number of factors, such as the type and physiological status of the explant, the composition and concentration of the basal salts, the organic components and plant growth regulators in the culture medium and most importantly the genotype of the donor plant (Ge et al., 2006). Indica rice species are considered to be recalcitrant in their response to tissue culture both in terms of embryogenic callus induction and subsequent plant regeneration,
therefore, extensive research has been conducted to improve plant regeneration efficiencies by manipulating different factors such as genotypes, hormonal composition, carbohydrate sources, culture methods, and water-deficient treatment, etc. (Greg and Roberta, 1991; Masayoshi and Takayasu, 1992; Tsukahara and Hirosawa, 1992; Yoshito et al. 1994; Jain et al., 1996; Masayoshi et al., 1996; Wang et al., 1999; Asad et al., 2001; Lee et al., 2002; She et al., 2002; Grewal et al., 2005; 2006; Geng et al., 2008).

Selection of a suitable explant for in-vitro culture is an important factor contributing to the successful embryogenic callus induction and subsequent plant regeneration. Therefore, various explants like mature embryos, immature embryos, immature panicles, young inflorescences, roots, leaf bases, anthers or pollen etc., have been used by different workers to identify suitable explants in rice for embryogenic callus induction and optimization of suitable culture conditions (Chen et al., 1980; 1985; Lai and Liu, 1982; Ling et al., 1983; Abe and Futsuhara, 1985; Toriyama et al., 1986; Li et al., 1992; Ozawa et al., 2003). However, mature seeds and immature embryos have been among the most commonly used explants for rice tissue culture. In this study, mature seeds of five different Basmati rice varieties viz. Basmati-370, Basmati-2000, Basmati-385, Basmati-Pak and Super Basmati (subsequently referred to as B-370, B-2000, B-385, B-Pak and S-Basmati, respectively) were explored as the potential starting material for embryogenic callus induction. Another important factor contributing to the successful tissue culture is the use of appropriate medium and suitable concentration and/or combination of auxins and cytokinins. MS medium (Murashige and Skoog, 1962) has been the most widely used basal medium in rice tissue cultures (Ge et al., 2006). Therefore, MS medium was used in present study. In addition to the media constituents, growth regulators play a vital role in the embryogenic callus induction and its subsequent growth. Auxins are considered to be essential for induction and proliferation of callus while addition of cytokinins may promote the frequency of embryogenic callus formation (Ge et al., 2006). In these experiments, ten different treatments (T1-T10) having various concentrations of 2,4-D (0, 1, 2 and 4 mg L⁻¹) either alone or in combination with BAP (0.5 mg L⁻¹) in modified MS (Murashige and Skoog, 1962) medium were used to identify a suitable composition for embryogenic callus induction from mature seeds of aforementioned Basmati rice
varieties. Highest embryogenic callus induction frequencies of 76.3, 66.6, 90.4, 63.6 and 71.7 % were obtained from B-370; B-2000; B-Pak, B-385 and S-Basmati, respectively. These results demonstrated that 2,4-D alone was sufficient to induce embryogenic callus in all of the varieties and more often there was a negative effect of BAP supplementation in the callus induction medium. It has been extensively reported that 2,4-D alone is sufficient for induction and maintenance of embryogenic calli (Lin and Zhang, 2005; Ge et al., 2006). However, there are evidences showing that combination of auxins with cytokinins were more effective for embryogenic callus induction in other rice cultivars (Wu et al., 2002; Zaidi et al., 2006). Ge et al. (2006) observed that 2,4-D alone enabled embryogenic callus induction from rice cultivars Minghui 63, Zhenshan 97 and 93-11. However, they also found that in some other rice near-isogenic lines IRBB13, IRBB10, IRBB4, and IR24; auxins like 2,4-D, NAA, and PAA, combined with a cytokinin (kinetin) were optimum for embryogenic callus induction. The results presented here demonstrated that cytokinin (BAP) supplementation in the callus induction medium had generally a negative effect on embryogenic callus induction from Basmati rice cultivars. These findings are in conformation with the results reported by Rashid et al. (2001). They also observed that inclusion of BAP in the callus induction medium resulted in significant decrease in callus induction frequency in Super Basmati.

In the plant regeneration experiments, it was found that a combination of auxin and cytokinin was important for high frequency plant regeneration. Plant regeneration frequencies in media having no growth regulators remained low. Maximum shoot regeneration frequency (upto 74.8%) was achieved in Super Basmati. Plant regeneration medium (R6) having MS basal salts and vitamins supplemented with kinetin 3 mg L⁻¹, NAA 1 mg L⁻¹, maltose 3% and phytagel 0.25% proved to be the best medium for high frequency plant regeneration except for B-385 where R3 (having same composition as R6 except 3% sucrose instead of maltose) showed non-significantly higher regeneration frequency. Moreover, presence of ABA (10 mg L⁻¹) in the pre-regeneration medium was found to be vital for somatic embryo induction. Mariani et al. (2000) proposed that ABA is necessary for the development but is inhibitory for the germination of rice embryos. These results have demonstrated that the composition of regeneration medium is critical
in achieving high frequency plant regeneration from embryogenic calli of Basmati rice cultivars. It was observed that addition of sorbitol in the plant regeneration medium was vital for somatic embryo induction and subsequent plant regeneration. These results are in conformation of the findings of Geng et al. (2008) who observed significant increase in the plant regeneration frequency of the three elite upland rice cultivars, Handao 297, Handao 502, Handao 65 and one lowland rice cultivar Zhongzuo 93 with the addition of appropriate amounts of sorbitol in culture media. These results showed that plant regeneration frequency was significantly higher in B-2000 and B-Pak while no significant difference was observed in B-370, B-385 and Super Basmati. In B-2000 and B-Pak, 1.3 to 1.6 folds increase in plant regeneration frequency was observed on media containing maltose compared to the corresponding media containing sucrose. This shows that although maltose produced better results but the difference was not as significant as reported for rice cultivar IR43 by Ghosh-Biswas and Zapata (1993). They observed 8-12 fold increase in plant regeneration efficiency with maltose compared to sucrose. Lin and Zhang (2005) also found that maltose was a better carbon source than sucrose in both subculture and differentiation media for all the four indica rice breeding lines including Minghui 63, Zhenshan 97, W9864S and Zhong 419. Similarly, Zaidi et al. (2006) while working with indica rice cv. MDU-5 demonstrated that maltose was more effective in enhancing plant recovery from the rice calli compared to sucrose and glucose.

After the development of a simple and reproducible method for embryogenic callus induction and subsequent plant regeneration, the next step was to carry out gene transfer studies. Since there is no universal transformation system which is readily applicable to transformation of all indica rice varieties, therefore, it was imperative to evolve an efficient system of Basmati rice transformation which can be routinely used to improve quality traits of this important commodity. For this purpose particle-bombardment (Particle Delivery System (PDS-1000/He) BioRad, USA) method was used. Schopke et al. (1997) suggested that optimization of different bombardment parameters for any plant tissue is necessary. Similarly, Taylor and Vasil (1991) proposed that due to a number of variables in the PDS-1000/He, the system should be assessed for efficient delivery of foreign DNA into the target cells. Therefore, to define optimum conditions for high
efficiency gene delivery through particle-bombardment, different biological and physical
parameters which can affect bombardment efficiency were studied.

Use of appropriate target tissues can have dramatic affect on the efficiency with which
gene(s) can be delivered to the target cells. Moreover, suitable target tissue can also be
helpful in the subsequent events of selection and transgenic plant recovery. High
transformation efficiencies can be achieved if tissues are bombarded at the right
developmental stage. Moore et al. (1994) observed that actively dividing cells are more
receptive to foreign DNA integration. The results presented here indicate that both
primary calli (28 days old) and mature embryos produced high level of transient GUS
expression, indicating their suitability as the target tissues for particle bombardment.
Embryogenic calli have been extensively as the target tissues by different workers for the
recovery of transgenic rice plants. Visarada and Sarma (2004) recovered a large number
of transgenic plants from bombarded embryogenic calli of both indica (Vibhava, Seshu,
Rasi, Nagarjuna, Sonasali, Swarna, Vikas, Mahsuri) and japonica (Taipie 309) rice
species. Chen et al. (1998) showed that selection and use of appropriate stage of the
callus and applying an improved selection procedure, transgenic Taipie 309 plants were
recovered at an average frequency of 22.3 %. Results of this study show that bombarded
mature embryos offer higher selection efficiency than embryogenic calli. In the mature
embryos, hygromycin resistant calli mostly originated from the transformed sectors of the
scutellum surface while the non-transformed sectors turned brown and necrotic. These
hygromycin resistant calli showed a fast growth rate when subcultured on fresh selection
medium for the second round of selection. However in case of primary embryogenic
calli, an early and clear judgment could not be made about their transgenic nature and
some times, the originating hygromycin resistant calli also turned brown or showed signs
of necrosis during the second round of selection. No previous reports on the use of
excised mature embryos as the target material for particle bombardment mediated
transformation of rice could be found in literature. This study has successfully
demonstrated the use of mature embryos as a potential explant source for particle-
bombardment mediated gene delivery in rice offering almost all the advantages that of
immature embryos but with additional convenience of availability all round the year.
It is well established that transient expression of transgenes is comparatively easy and straightforward to obtain using biolistics, but stable integration requires a careful combination of the parameters which are suitable for a particular type of tissue (Tadesse et al., 2003). Different physical factors like particle size, helium pressure, target distance, particle/DNA loads and their ratio, number of shots and pre- and post-bombardment osmotic treatment were tested to determine the best conditions for particle bombardment. Gold particles of 1.0 µm size produced more number of blue foci on average compared to bigger particles of 1.6 µm size at the target distance of 3, 6 and 9 cm. At a higher helium pressure of 10,500 kPa (1,550 psi), 1.6 µm particles resulted in lower transient GUS expression at 3, 6 and 9 cm target distance indicating higher extent of damage to the target tissues at these distances. This could be due to the fact that bigger particles in combination with high helium pressure leads to increased tissue damage. However, a higher transient GUS activity was recorded at a target distance 12 cm which indicates that at 12 cm target distance, the force of penetration becomes a limiting factor for smaller (1.0 µm) particles. At 12 cm distance, the bigger particles have enough force of penetration thus resulting in a higher transient GUS activity. While working with particle bombardment mediated transformation of rice embryogenic calli, Ramesh and Gupta (2005) obtained maximum transient GUS activity at a combination of helium pressure and target distance of 7,500 kPa (1,100 psi) and 9 cm, respectively out of the various combinations of helium pressure and target distances they tested. Similarly, higher transient GUS expression was also recorded at a helium pressure of 7,500 kPa (1,100 psi) and a target distance of 9 cm in a number of other studies (Schopke et al., 1997; Chen et al., 1998; Ramesh and Gupta, 2005). Petrillo et al. (2008) observed higher transient GUS expression when immature embryos of maize tropical inbred lines L1345 and L3 were bombarded at 7,500 kPa (1,100 psi) compared to 4,480 kPa (650 psi) helium pressure. They also recovered a greater number of stable transgenic plants from these maize inbred lines when immature embryos were bombarded at 7,500 kPa (1,100 psi) helium pressure. This may have resulted due to the presence of cells capable of embryogenesis in deeper cell layers as proposed by Brettschneider et al. (1997). Rasco-Gaunt et al. (1999) demonstrated that at low helium pressures of 4,480, 6,250, 7,500 kPa (650, 900, 1,100 psi
respectively), the GUS expression in wheat embryos was evenly distributed which was attributed to reduced bombardment shock and tissue injuries. They observed that at high helium pressure of 8,950 and 10,500 kPa (1,300 and 1,550 psi, respectively), a small area of the target tissues was very strongly targeted and damaged. Thus the optimum target distance for a particular particle size seems to be helium pressure dependent. Taylor and Fauquet (2002) postulated that particle size is important for determining the optimum helium pressure which dramatically affects the depth of penetration of the particles. This is also supported by findings of Gharanjik et al. (2008) who observed that higher helium pressure and a shorter target distance resulted in reduced transient GUS expression in wheat tissues.

DNA to particle ratio can also have a significant effect on bombardment efficiency. Parveez et al. (1998) proposed that appropriate amount of DNA is important for efficient DNA-microcarrier binding. In this study, a decreased transient GUS expression in the bombarded mature rice embryos was observed at higher DNA to particle ratio. This may have resulted due to aggregation of gold particles at higher DNA concentration. Higher particles loads (4 and 5 mg gold) also reduced transient GUS expression owing to higher degree of damage to the target tissues. These results are in agreement with findings of Becker et al. (1994) who used GUS and bar genes for coating two different amounts of gold particles to bombard wheat immature embryos and observed that embryogenic callus formation was adversely affected by the higher density of gold particles. The results presented here showed that highest transient GUS expression at 9 µg DNA with 3 mg gold particles (3:1 DNA-particle ratio) while lowest expression was recorded when 3 µg DNA was used to coat 3 mg gold (1:1 ratio). Carsono and Yoshida (2008) observed that 1.5 µg DNA delivered per shot produced the highest number of sgfp spots in rice cultivars Fatmawati and Nipponbare. Therefore these findings are in agreement with their results. Rasco-Gaunt et al. (1999) observed no significant change in the levels of GUS expression in wheat embryos when different amounts of plasmid DNA (2.5, 5, 10 or 20 µg) was used for precipitation onto 2 mg gold particles.
To increase the number of target tissues receiving the DNA coated particles, effect of multiple bombardments (more than one shot per plate) was studied. Single shot resulted in higher number of blue foci compared to double shot at 6 cm target distance, however, at 9 cm; double shot resulted in considerable increase in the GUS expressing units. These results are in agreement with the findings of Petrillo et al. (2008) who observed that 0.9% and 2.31% of immature embryos of maize inbred lines L3 and L1345 produced transgenic plants when plates were bombarded once and twice, respectively. Wang et al. (1996) observed significant increase in transient GUS expression of rice and wheat suspension cells using double compared to single bombardment. However, Reggiardo et al. (1991) demonstrated that more than one shot per plate was injurious to barley and maize cells. Similarly, Rasco-Gaunt et al. (1999) observed no significant difference in GUS expression with single or multiple (2 or 3 times) bombardments in wheat embryos.

A medium having high osmotic strength is thought to protect target tissues during bombardment by reducing the cell turgor pressure so that chances of cell survival are higher due to reduced leakage of cell contents following bombardment (Vain et al., 1993). This plasmolysed state of the target tissues is generally obtained by placing these cells/tissues/explants on a hypertonic medium containing mannitol, sorbitol, maltose or sucrose (Brettschneider et al., 1997; Bohorova et al., 1999). These research findings showed that osmotic treatment significantly enhanced transient GUS expression compared to the controls. Osmotic treatment with 0.2 M mannitol gave highest GUS expression followed by 0.4 M mannitol, and yielded as much as 2.7 to 2.9 fold increase, respectively in transient GUS expression over the control. Vain et al. (1993) demonstrated significant increase in transient GUS expression by placing embryogenic maize cells on a medium containing 0.2 M sorbitol and 0.2 M mannitol. Similarly, Gharanjik et al. (2008) observed significant improvement of GUS expression in wheat embryos following osmotic treatment attributing to improved survival rates following particle bombardment. Rasco-Gaunt et al. (1999) showed significant improvement in transient GUS expression when wheat tissues were cultured on 6% and 9% sucrose in comparison with tissues cultured on 3% sucrose before and after the bombardment.
All transformation systems aimed at developing transgenic plants require use of selectable marker genes for selecting those cells that have integrated the foreign DNA into their plant genome and for the regeneration or recovery of transgenic plants from the small proportion of transformed cells (Miki and McHugh, 2004). Hygromycin phosphotransferase (hpt) has been one of the most widely used selectable marker genes for rice transformation (Hiei et al., 1994; Rashid et al., 1996; Martinez-Trujillo et al., 2003; Sallaud et al., 2003; Visarada and Sarma, 2004; Hiei and Komari, 2006; Swamy et al., 2006; Maruthasalam et al., 2007). During the course of this study, several experiments were conducted to determine appropriate concentration of hygromycin B for killing the non-transformed cells. Hygromycin B at 50 mg L⁻¹ in the growth medium was found to be effective for completely preventing the growth of un-transformed cells. Similar results have been reported by several other workers (Hiei and Komari, 2006; Rashid et al., 1996; Wu et al., 1997; Chen et al., 1998; Abedinia et al., 2004; Sripriya et al., 2008). However, Martinez-Trujillo et al. (2003) observed that when bombarded rice tissues were subjected to selective media containing either 50 or 80 mg L⁻¹ of hygromycin B, four times more calli survived selection at 50 mg L⁻¹ compared to 80 mg L⁻¹ of hygromycin. However, 3 times more hygromycin-resistant calli with stable GUS activity were recovered at 80 mg L⁻¹ hygromycin B. This was mainly attributed to the escapes in the media containing 50 mg L⁻¹ of hygromycin. Thus, they proposed that a higher hygromycin concentration improves the efficiency of recovering stably transformed rice calli, at least with the rice variety they used in their experiments.

During the series of experiments conducted for optimization of different physical and biological parameters for particle-bombardment mediated transformation of Basmati rice, 26 putative transgenic plants were recovered from 1,740 embryos bombarded with co-integrate plasmid(s) pGH-I/pGH-II with an over all transformation efficiency of 1.5%. Despite of several successful reports on rice transformation, producing transgenic plants from indica rice varieties is still problematic (Martinez-Trujillo et al., 2003). Moreover, the transformation efficiency for indica rice species is relatively low compared to the japonica rice species. While using an indica rice variety TN1, Sivamani et al. (1996) recovered transgenic rice plants with an overall transformation efficiency of 2-4%. Martinez-Trujillo et al. (2003) obtained transgenic rice plants of an indica rice variety
Morelos A-92 with 4.6% efficiency. However, much higher transformation efficiency (upto 22%) has been reported from a japonica rice cv. Taipie 309, (Chen et al., 1998).

These experiments resulted in the development of a simple and reproducible protocol for transformation of Basmati rice varieties. These optimized conditions were further used to develop bacterial blight resistant transgenic Basmati rice. Bacterial blight is one of the most destructive bacterial diseases of rice worldwide (Wu et al., 2008) resulting in significant yield losses in Asia and many other rice growing countries. Basmati rice, in particular, is highly susceptible to bacterial leaf blight caused by *X. oryzae* pv. *oryzae*. (Gopalakrishnan *et al.*, 2008). Khan *et al.* (2008) conducted field surveys of northern Punjab during 1997, 2002, 2006 and 2007, and reported that bacterial leaf blight epidemically damaged the crop in some of these areas. The extent of damage from bacterial blight ranged from 70-100%. They also screened forty-two entries/varieties from different institutes for resistance against bacterial blight under field conditions, however, no entry/variety was found to be resistant or tolerant to bacterial leaf blight. Similarly in another study (Yasin *et al.*, 2007), forty-nine fine grain rice varieties/lines screened for bacterial blight resistance, under artificially inoculated conditions, however, only one variety showed resistant reaction against bacterial leaf blight. Waheed *et al.* (2009) considered bacterial blight as one of the most important threats to rice production in Pakistan mainly because of lack of information regarding the pathogen and effective measures to control this disease. Lack of natural resistance in the Basmati germplasm makes this important commodity a very important candidate for engineered resistance against bacterial leaf blight. Fortunately, rice has evolved a number of resistance (*R*) genes to counteract this destructive disease. Resistant cultivars carrying a single *R* gene can be effectively utilized to control this disease (Wu *et al.*, 2008). *Xa21* gene, due to its wide-spectrum resistance to the bacterial blight pathogen, is of great significance in breeding rice for resistance against this dreadful disease (Huang *et al.*, 2005). Bacterial blight resistance genes can be transferred to Basmati from non-Basmati sources through conventional techniques but it shows complex inheritance pattern and requires strict monitoring for recovery of the desirable Basmati quality traits in the recombinants (Gopalakrishnan *et al.*, 2008). Therefore genetic transformation seems to be a logical
alternative as one or more cloned bacterial blight resistance genes can be transferred using transgenic approach. Several transgenic Basmati lines were developed using Xa21 gene during the course of this study. Most of these transgenic lines have shown high level of resistance against two local isolates of the bacterial blight pathogen (Xoo). In reaction to inoculation of a local Xoo isolate 1.2.1, thirty-four transgenic lines produced lesions of ≤ 3.0 cm, eleven lines showed a moderately susceptible reaction while four transgenic lines showed a susceptible reaction. The non-transformed lines in comparison produced larger lesion ranging from 16.20 - 18.83 cm. Similarly, in response to Xoo isolate 3.2.2 inoculation, thirty-eight transgenic lines produced lesions of ≤ 3.0 cm, 8 lines showed a moderately susceptible reaction, while 3 transgenic lines showed a susceptible reaction. The non-transformed control plants produced larger lesions ranging from 14.43 - 17.43 cm. These results are in agreement with those reported by Tu et al. (1998) for transgenic IR72 lines carrying Xa21 gene. They observed highly reduced lesions of less than 3.1 cm in transgenic bacterial blight resistant plants compared to the larger lesions of 13.3 – 20.3 cm in the control plants. The results presented here show that two transgenic lines (20-1-01 and S-3-03) failed to confer any resistance against both the Xoo isolates producing lesions as large as that of the non-transformed control plants. One of these plant lines (i.e. S-3-03) was actually negative for Xa21 gene. Another plant line 20-1-01 showed susceptible reaction to both the local Xoo isolates 1.2.1 and 3.2.2, indicating the lack of expression of the Xa21 gene in this plant. Similar results were also reported by Tu et al. (1998). They observed that Xa21 functioned well in most of the T1 transgenic IR72 lines, however, it was silenced or inactivated in few cases during its inheritance from the primary generation to the next. Narayanan et al. (2002) transformed IR50 with Xa21 via particle bombardment into an introgressed line already having Piz-5 against the rice blast disease. They clearly demonstrated that the transgenic IR50 lines showed high level of resistance against bacterial blight. Among the 20 plants analysed, 15 were completely resistant while five plants were found to be susceptible. The lesion length of 13 T1 plants and the other donor IRBB21 was 4 cm, whereas the lesion length of the control IR24 ranged from 12 to 17 cm. These studies indicated that Xa21 conferred high level resistance to the targeted plants against different Xoo strains. Swamy et al. (2006), however, found that Pusa Basmati transformed with Xa21 failed to show resistance
against all the isolates they tested. They observed that these lines often had lesions covering the entire inoculated leaf, leading to plant death at 21 days after inoculation. They proposed that Xa21 gene alone is ineffective against some virulent isolates (Mxo4, Mxo5 and Mxo6) and thus must be deployed in genetic backgrounds that contain other BB resistance genes. This may be due to the fact that the pathogen is known to exhibit pathogenic variation, with diverse pathotypes or races in different rice-growing areas (Shanti et al., 2001), therefore, information about the pathogen population structure and their virulence characteristics is therefore necessary for a successful breeding program (Leung et al., 1993).

The transgenic lines developed during the course of this study were evaluated for agronomic performance under limited field conditions. These transgenic lines showed comparable agronomic performance to that of the non-transformed controls. The transgene did not confer any negative effects on the yield as well grain characteristics. However, variation in some of the studied traits was observed. The transgenic Basmati rice lines transformed with Xa21 gene exhibited significant reduction in plant height compared to the non-transformed control. Few transgenic lines showed significant increase in the number of fertile tillers compared to the control plants. All the transgenic lines took 8-12 days longer to flower. No significant difference in grain yield/plant and grain-length was observed between the transgenic lines and the control plants. Similar results have been reported in some earlier studies. Field studies of transgenic IR72 having Xa21 gene not only showed resistance to the bacterial blight pathogen but also showed comparable yield performance to the un-transformed controls (Tu et al., 2000). Their data showed that transgenic line T103-10 grew 8.0-cm less in plant height, took 2 days longer to flower, and set 2.9 more filled-seeds per panicle. The grain yield of T103-10, however, as predicted by its yield components showed no significant difference from that of the IR72 control. Similar results are also reported by other workers. Bashir et al. (2004) detected similar height reduction and later maturity of the transgenic rice lines, because of transgene integration, compared to the non-transformed controls. In their study, a plant height of 139 cm was recorded for L-5 and L-13, as compared to 159 cm in the untransformed control. Similarly during the second year, the height of L-6-411 was on
average 122 cm as compared to 165 cm for the control. The non-transformed control took 113 and 141 days for panicle initiation and maturity respectively, from the date of sowing whereas transgenic lines L-5 and L-13 took an average of 122 and 152 days for panicle initiation and maturity, respectively. Similarly, during the second year almost the same pattern was observed for these lines. The control took 99 and 128 days for panicle initiation and maturity from the date of sowing, whereas transgenic lines took up to 12 and 13 days longer for both characters. Liu et al. (2005) observed no change for most of the agronomic characters of the transgenic maintainer lines and the relevant transgenic hybrids. Ye et al. (2007) noticed small variation in different quality traits of the transgenic rice lines transformed with pea ferritin gene. One of their transgenic line showed a higher yield potential compared to the controls while retaining the same quality traits as the wild type. In another study, Kim et al. (2008) evaluated three Korean rice varieties transformed with cry1Ab against yellow stem borer (YSB) and studied the behavior of transgenic lines under field conditions. They observed that majority of transgenic lines demonstrated 100% toxicity towards the neonate YSB larvae. The yield of some transgenic lines was similar to that of control lines. However, phenotypic variations such as stunting and lower fertility were observed in some T0 transgenic plants. The reduction in plant height and the decrease of fertility in these lines was approximately 65 and 30% of the original height and fertility, respectively. Most of the important yield components decreased in transgenic lines compared to those in parental varieties. The yield of some transgenic lines was either similar or not significantly different from the controls although the mean values of yield were lower than those in the controls. One of the transgenic Basmati-2000 lines (20-1-01) was found to be susceptible to both the Xoo isolates 1.2.1 and 3.2.2 producing lesions as large as that of the non-transformed control. It appeared that Xa21 expression was completely missing in this line. A similar transgene inactivation of the Xa21 expression in transgenic IR72 plants was reported by Tu et al. (1998). Variation in transgene expression is usually observed in the transgenic plants. These phenotypic variations in transgenic plants might be the result of position effect (integration site), dosage effect (transgene copy number), insertion mutagenesis or gene silencing (Fladung, 1999; Maqbool and Christou; Matzke et al., 2000; James et al., 2002; Kathuria et al., 2007). Zhai et al. (2004) observed no obvious
position or dosage effects of \textit{Xa21} gene in the transgenic rice lines. However changes in
gene expression or silencing of the transgene \textit{Xa21} have been reported by some other

Generation of selectable maker-free transgenic plants is an important advancement for
commercial development of the genetically modified (GM) crops (Yang \textit{et al.}, 2009).
Use of antibiotic and herbicide resistance genes in the transgenic crops is a major public
concern especially with reference to food and environmental biosafety (Daniell, 1999).
Therefore, an important area of research during the recent years has been the
development of marker free transgenic plants (Miki and McHugh, 2004). In this study a
co-transformation strategy was used where gene of interest (\textit{Xa21}) and the selectable
marker (\textit{hpt}) genes were on separate plasmids and these were mixed just before coating
on gold micro-particles. Segregation of \textit{Xa21} and \textit{hpt} genes in the T$_2$ generation of the
transgenic lines was observed. Thirty-two plants were analysed by PCR for the
presence/absence of \textit{Xa21} and \textit{hpt} genes in the T$_2$ generation, out of which twenty-seven
plants contained both \textit{hpt} and \textit{Xa21} genes, three plants contained only the \textit{hpt} gene while
two plants contained only \textit{Xa21} gene. These results confirmed that two plants were
marker free containing only the bacterial blight resistance (\textit{Xa21}) gene. These results are
in agreement with the findings of Zhao \textit{et al.} (2007). They co-transformed minimal gene
cassettes having selectable marker (\textit{bar}) and non-selected cecropin-B into rice (\textit{O. sativa}
L.) by particle bombardment. By adopting the hereditary segregation strategy, they were
able to eliminate the selectable marker (\textit{bar} gene) in R$_1$ generation having only the
\textit{cecropin-B} gene without any superfluous DNA. Similarly, Matthews \textit{et al.} (2001)
reported elimination of marker gene from transgenic barley developed through
\textit{Agrobacterium} method having twin T-DNAs in the standard vector. They observed that
co-insertion took place in two-thirds of transformants from which one quarter segregated
in the next generation to yield marker free transgenic plants.

This study resulted in the development of simple and reproducible protocols for the
genetic improvement of Basmati rice through transgenic approach. The bacterial blight
resistant rice developed during the course of this study can further be exploited to
incorporate other (\textit{Xa/xa}) genes for developing broad spectrum resistance against
bacterial blight. Further more this study has paved the way for the improvement of Basmati rice for incorporation of genes for insect, diseases and abiotic stress resistance and for other quality traits like nutritional improvement etc.
REFERENCES


