IMPROVEMENT OF JUTE THROUGH MANIPULATION WITH USEFUL GENE(S)

Ph. D. THESIS

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By

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DECLARATION

I hereby declare that this thesis entitled "Improvement of jute through manipulation with useful gene(s)" has been composed by me and all the work presented herein is my own. I further declare that this work has not been submitted anywhere for any academic degree.

Date: 16.05.2011

(Md. Shahidul Islam)

CERTIFICATE

This is to certify that the research work embodying the results reported in this thesis has been carried out under my guidance and direct supervision in the experimental field of Bangladesh Jute Research Institute, Dhaka and Molecular Biology laboratory, Department of Biochemistry and Molecular Biology, University of Dhaka. It is further certified that the work presented here is original and suitable for submission for the award of Ph. D. degree.

Professor Haseena Khan, Ph. D.
Department of Biochemistry and Molecular Biology
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Dedicated to

Abu Ashfaqur Sajib

Muhammad Shafiul Azam

As a token of gratitude for their continued support in my molecular biology endeavour...

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Author

Abstract

The study focused on developing an efficient genetic transformation system for jute and identification of useful genes from the repository of different jute species in the gene bank at the Bangladesh Jute Research Institute for transformation into farmer popular cultivar. This study therefore consisted of (a) development of an efficient transformation system for *Corchorus olitorius* L. (b) identification and characterization of gene(s) resistant to the jute pathogens from different jute species as a source of useful genes for genetic modification of jute and (c) development of a salt tolerant jute species using an *E.coli* gene.

Jute is the principal cash crop of Bangladesh. But this crop is now cultivated in marginal lands due to the tremendous pressure for the cultivation of food and vegetables crop in order to feed the fast growing people of this country. Therefore, jute faces many biotic and abiotic stresses in the inhospitable terrain where it is now being cultivated. Moreover, improvement of jute is hampered by classical breeding methods due to a narrow genetic base and a strong barrier to inter-specific crosses. Genetic transformation provides a promising methodology for introducing new genes that encode desirable traits to a wide range of crop plants. Success in genetic transformation has been achieved in many of the important crop species, such as soybean, cotton, rice, corn etc. There have been limited studies on transformation of jute due to the recalcitrance of jute varieties to tissue culture especially for *Corchorus olitorius*.

In this study, transgenic jute plants have been generated by a tissue culture independent *Agrobacterium tumefaciens* mediated transformation procedure. Heritable transmission of the transgene to progeny from genetically modified plants was confirmed by various molecular analyses such as GUS gene expression by histochemical analysis, selection on kanamycin containing medium, reverse transcription polymerase chain reaction (RT-PCR), Polymerase chain reaction (PCR) and Southern hybridization. Efficiency of transformation was determined by selection on medium containing kanamycin.

The gene bank at the Bangladesh Jute Research Institute is a rich repository of genes for many desirable traits. Two of the most widely cultivated species of jute, *Corchorus capsularis* and *C. olitorius*, suffer severely from stem rot disease caused by *Macrophomina phaseolina*. Therefore in order to identify useful genes from the jute gene bank for the purpose of using them in genetic transformation, a genetic approach, called differential display was employed to identify differentially expressed genes, under both infected and un-infected conditions, between fungus sensitive and resistant jute varieties. One of the genes identified through this method encode a xyloglucan endotransglycosylase/hydrolase (XTH) - an enzyme involved in cell wall elongation and restructuring. Two xyloglucan endotransglycosylase/ hydrolase (XTH) genes designated *Co*XTH1 (from *Corchorus olitorious*, sensitive to fungus infection) and *Ct*XTH1 (from *Corchorus trilocularis*, resistant to fungus infection) were identified from each of the two species that showed opposite expression patterns after fungal infection. By exploiting two independent molecular biology approaches i.e. northern blot analysis and semi-quantitative RT-PCR expression in response to fungus infection was found to increase with time for *Co*XTH whereas it decreased for *Ct*XTH.

Salt tolerant jute plant is an urgent need to cope with the changing environment. In order to improve salt tolerance in jute, *kat*E gene from *Escherichia coli* was introduced into jute cultivar O-72, a popular local cultivar in Bangladesh. The *in planta* method developed in our lab was used for transformation. Molecular analyses of transgenic plants clearly indicate successful integration of *katE* in jute genome. These plants were shown to be more tolerant to salt stress than the non-transgenic plants.

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IMPROVEMENT OF JUTE THROUGH MANIPULATION WITH USEFUL GENE(S)

General Introduction

1.1 Jute

Jute is a natural bast fibre crop occupying second place only to cotton in economic importance (Singh, 1976). It is grown primarily in the Indian sub-continent, especially in eastern India and Bangladesh. Combinedly, this region traditionally supplied the majority of the world's demand for raw jute. Its cultivation is confined to this region due to the climatic conditions that are particularly suitable for growing this crop. India and Bangladesh rank as the top two countries in terms of jute production (Islam *et al.*, 2005). Jute grows under wide variation of climatic conditions of the tropic and subtropics. Apart from Bangladesh and India the crop is now also grown in Myanmar, Nepal, China, Taiwan, Thailand, Vietnam, Cambodia, Brazil and some other countries. However, Bangladesh, India, China, Myanmar, Nepal and Thailand are the major producers accounting for over 98% of the global output (FAO, 2008).

Jute (*Corchorus* spp. 2n= 14) plants are tall, usually annual herbs reaching a height of 2-4 m, unbranched or with only a few side branches and mostly self-pollinating crop. The leaves are alternate, simple, elliptic-lanceolate, apically acute or acuminate, 5-15 cm long, with an acuminate tip and a finely serrated or lobed margin. Flowers of both the species have yellow colour, are small in size (2-3 cm diameter) and occur in condensed cymes. Flowers occur in the axel of leaves, and composed of 4-5 sepals and petals, 5 to numerous stamens. Sepals are about 3 mm long, oblong, apiculate. Ovary is locular and 2-6 in number. Seeds are small and numerous. Fibres are derived from the secondary phloem fibres from the bark of the stem (Kundu, 1951).

The genus *Corchorus* comprises 50-60 species (Puresglove, 1968). The members of this genus show extreme variation, but all are apparently highly fibrous. Among these, only two species are cultivated commercially for fibre production namely, *Corchorus capsularis* L. and *C. olitorius* L. These two species are distinct in their growth habitat, branching habit, and characteristics relating to leaf, flower, fruit, seed, bast fibre, and photosensitivity (Basu *et al.*, 2004), and do not cross fertilize with each other (Patel

and Dutta, 1960). They also differ in origin. Indo-Burma region is the centre of origin for *Corchorus capsularis* L. while Africa the centre of origin for *C. olitorius* L. (Kundu, 1951; Mahapatra *et al.*, 1988).

1.2 Jute and Bangladesh

Bangladesh is an agro-based economy where agriculture accounts for 20.60% of country's GDP (Bangladesh Economic Review, 2010) and employs 48.1% of her labor force (BBS, 2009; Labour Force Survey, 2005-06). Jute is the principal cash crop of Bangladesh. The contribution of jute is very significant in the field of agriculture, industry, employment generation, rural economy as well as export earning of the country. Jute industry is the second largest industrial employer in the country. It directly and indirectly provides livelihoods to more than 35 million people including farmers, businessmen, workers, laborers and self employed artisans and weavers of the country (BBS, 2009).

Jute is an agricultural commodity that singly earns foreign exchange equivalent to 38.69 billion taka annually to our national economy, which is the second largest source of foreign exchange (BBS, 2009). It is used extensively in the manufacture of different types of packaging materials for various agricultural and industrial products. Now-a-days novotex, blanket, fabrics, shopping bags, knitwear, nursery sheet and pot, microcrystal cellulose for pharmaceutical products, geo-textile, photostable dye etc. are being made from jute (Islam, 1997). Therefore, the importance of jute to the national welfare of Bangladesh cannot be underestimated.

Despite its high socio-economic importance for Bangladesh, globally jute is now confronted with a number of problems. The global demand for traditional products of jute is declining day by day due to invasion of synthetic fibre. On the other hand, the global food demand is increasing sharply; as a result jute is being pushed to the marginal lands where it faces various limitations resulting in low yield of jute. In addition to this, farmers are facing problem in retting green jute for fibre extraction, due to lack of retting water during the time when jute is harvested.

Recently natural fibres have attracted the attention of scientists and technologists because of the advantages that these fibres provide over conventional reinforcement materials, and the development of natural fibre composites has been a subject of interest for the past few years (Jha, 2009). These natural fibres are low-cost fibre with

low density and high specific properties. These are bio-degradable and non-abrasive, unlike other reinforcing fibres. Also, they are readily available and their specific properties are comparable to those of other fibres used for reinforcements (Danladi *et al.*, 2009). The worldwide awareness on environment and health is likely to provide new opportunities on jute (Anonymous, 2010b). Jute fibre which can be used in many different areas, supplementing and/or replacing synthetics, has been receiving increasing attention from the industry. Their interests focus not only on the traditional uses of jute, but also on the production of other value-added products such as, pulp and paper, geotextiles, composites and home textiles etc. Jute has again proved the old saying true, "Traditional and natural commodities are never old fashioned and never out-of-date". This golden fibre of the past will hopefully regain its golden image again in the near future.

Bangladesh has the largest gene bank of jute and allied fibre (JAF) crops. At present the gene bank of Bangladesh Jute Research Institute has a collection of a total of 5935 accessions. A total of 53 species of fibre producing plants are available here of which, 15 species belong to *Corchorus*, 22 to *Hibiscus* and 16 to other allied fibres of other genera. For *Corchorus*, 3344 accessions are of indigenous collection and 737 accessions have been collected through International Jute Study Group (IJSG) formerly International Jute Organization (IJO). For *Hibiscus* species, 585 accessions were collected locally and 925 were from IJO. Among the allied fibre genera, 343 accessions were collected from IJO while one from local source. About 3000 germplasms have been characterized based on morphological and physiological characters (Khatun, 2007).

1.3 The aim of this work

During the last few decades, the productivity of jute in Bangladesh improved only marginally from 1691 kg/ha to 1800 kg/ha (BBS, 2009). This is largely because of inadequate or limited number of recommended varieties, in terms of meeting the requirements of wide agro-ecological conditions. Most of the available elite cultivars are essentially the products of pure line selection from a few common accessions. They have narrow genetic bases and are susceptible to various biotic and abiotic stresses such as insects, diseases, drought, water logging, low temperature etc (Kar *et al.*, 2009). The two cultivated species of jute namely, *Corchorus capsularis* L. and *C. olitorius* L. are mostly self-pollinating and contain very limited genetic variability with

respect to (i) adaptability to different agronomic environments, (ii) fibre quality, (iii) fibre yield, and (iv) susceptibility to diseases and pests (La Farge *et al.*, 1997). *Corchorus capsularis* is comparatively more resistant to flood and drought but slightly more susceptible to diseases and pests. It provides white commercial fibre which is slightly weak. *C. olitorius* is relatively tolerant to diseases and pests and produces the stronger tossa commercial fibre. Therefore, a combination of useful characters of these two species in a single genotype is highly desirable. But, unfortunately, these two species do not cross successfully with each other, possibly because of the presence of a strong sexual incompatibility barrier between them (Patel and Datta, 1960; Swaminathan *et al.*, 1961).

Addressing these challenges through traditional plant breeding program has limitations due to lack of genetic diversity among cultivated jute varieties and sexual incompatibilities between the cultivars of the two jute species, as mentioned above. In addition, jute has limited information on fibre yielding traits. All these factors combined with the increasing demand of new types of jute to meet various agro-industrial needs, identification and characterization of fibre yield associated genes is critical for genetic manipulation to improve fibre traits using molecular approaches. Jute faces both biotic and abiotic stresses in the inhospitable terrain where it is now being grown. Therefore, for developing superior jute varieties with traits such as biotic and abiotic stress tolerance, genetic modification with foreign gene(s) may provide a suitable alternative.

The analyses presented in this work focused on three objectives-

1. Establishment of an easy and efficient transformation protocol for jute.
2. Identification and characterization of important genes in the jute germplasm for future use in transforming farmer popular jute cultivars with the same.
3. Using the established transformation protocol for the development of salt tolerant jute plant by introducing in the jute genome a bacterial gene known to confer salt tolerance.

CHAPTER TWO

ESTABLISHMENT OF AN EASY AND EFFICIENT TRANSFORMATION PROTOCOL FOR JUTE

2.1 Introduction

More than ten percent of the available land on the planet today is arable and permanent cropland. By using this land, to produce more food for ever growing population, is a big challenge for agriculture in the 21st century. Biotechnology can contribute to meet the future food and fibre needs. It enables the addition of new or modified genetic traits to existing varieties, circumventing the sexual process and, thus, enables an acceleration of the whole breeding process. It is a potential tool for food security, human health care, environmental pollution management and alternative energy sources, which are the key socio-economic challenges in the 21st century (Wong, 2007).

2.1.1 Genetic engineering of plants

Genetic engineering of plants enables the addition of new or modified genetic traits to existing varieties. Commercialized transgenic plants to date carry traits like disease resistance, insect resistance, herbicide resistance, etc. Eventually transgenic technology may be used for increasing photosynthetic efficiency, nitrogen fixing ability and production of crops for efficient food processing and molecular farming (Kung, 1993; Suzuki *et al.*, 2000; Daniell, 2001; Aharon *et al.*, 2003). A study carried on the impact of genetically engineered crops on farm sustainability in the United States revealed that genetic engineering (GE) of crops can be effective in reducing pest problems with economic and environmental benefits to farmers (Ervin *et al.*, 2010). GE crops could help address food insecurity through development of plants with improved nutritional qualities or resilience to a changing climate (Anonymous, 2010a). In 2006, an estimated 136 million acres of U.S. cropland was used to grow GM crops. Some 89% of soybeans and 61% of corn crops are now genetically engineered (Fox, 2009). Canola is also genetically engineered, and vegetable oils (canola and corn) along with soy protein and lecithin, are used widely in a variety of

prepared foods for people and their pets. Genetically engineered sugar beet will soon be planted widely as a source of sugar for the food industry. We even have GM wheat now and it will soon be cultivated widely (Zeller *et al.*, 2010; Razzaq *et al.*, 2011).

Genetic engineering requires three elements: the gene to be transferred, a host cell into which the gene is inserted, and a vector to bring about the transfer. Various methods have been developed to achieve this and many plant species have been successfully transformed (Slater *et al.*, 2003).

2.1.2 *Agrobacterium* mediated transformation

Agrobacterium-mediated gene transfer is the method of choice when aiming for stable transformation in dicotyledonous species (Hewezi *et al.*, 2002), although an increasing numbers of monocotyledonous plants are also being transformed (Hiei *et al.*, 1994). *Agrobacterium tumefaciens* mediated gene transfer is achieved by modified vectors based on its Ti plasmid. The T-DNA sequence on Ti plasmid is naturally transmitted and stably integrated into plant genome (Barton *et al.*, 1983; Herrera-Estrella *et al.*, 1983).

2.1.3 Direct gene transfer

Direct gene transfer or vector less gene transfer involves the transfer of DNA into protoplasts, cells, tissues or whole plants by using different chemical or physical methods (Huang *et al.*, 1993).

2.1.3.1 Chemical methods

These methods rely on using different membrane-active agents such as polyethylene glycol (PEG), polyvinyl alcohol and dimethyl sulfoxide (DMSO), which destabilize membranes by creating momentary pores allowing uptake of DNA from the surrounding solution (David, 2001).

2.1.3.2 Physical methods

Various physical methods are used in plant transformation and the most important methods are summarized in next page.

2.1.3.3 Electroporation

Electroporation is the process where cells are mixed with a DNA construct and briefly exposed to pulses of high electrical voltage which creates transient pores in membrane thereby allowing foreign DNA to enter the host cell (Prescott *et al.*,, 1999). Although, transgenic rice and soybean have been produced by electroporation, success rates are low and the technique is not very reproducible (Wheeler *et al.*, 1991; Hagio, 2009).

2.1.3.4 Particle bombardment

Particle bombardment is a popular method for direct gene delivery into cells, tissues and organs. This technique uses pressurized helium to accelerate sub-cellular size microprojectiles of tungsten or gold coated with DNA (or other biological material) into cells over range of velocities necessary to optimally transform many different cell types (Bhatnagar *et al.*, 2002). Since the late 1980's particle bombardment has become an efficient tool for the study of gene expression and production of stably transformed tissues and whole transgenic plants from different species (Taylor and Fauquet, 2002; Cheng *et al.*, 2010; Guenoune-Gelbart *et al.*, 2010). Biolistics has the advantage of being applicable to plant cells in suspension or to intact or sliced plant tissues. In addition, particle bombardment is a unique gene delivery approach of particular utility to monocotyledonous species. Using biolistics, transgenic corn and soybean plants have been produced which contain heritable copies of the inserted gene (Wheeler *et al.*, 1991). Although, this methodology is very useful for molecular genetic studies (Birch, 1997), it often suffers from the problem associated with direct DNA transfer method, possibly leading to gene fragmentation and silencing. Silencing results from the interactions and integration of multiple copies of the transgene oriented in opposite directions (Bhat and Srinivasan *et al.*, 2002).

2.1.3.5 Microinjection

Microinjection is the process of directly injecting foreign DNA into the cell nucleus using ultra-fine needle (Prescott *et al.*, 1999). The essence of the method is a heat–induced expansion of a liquid metal alloy (galinstan, an alloy of gallium, indium and tin) in combination with silicon oil within the pipette to force probes from the

capillary tip). This technique is effective with plant protoplasts and tissues but the most obvious drawback of microinjection is damage of the cell inflicted by the penetrating glass pipette because of cellular pressure loss which is often accompanied by a drastic change in the cellular ultra-structure followed by cell death. The technique is laborious, technically difficult and limited to the number of cells actually injected. However, microinjection has produced a few transgenic plants (Prüfer, 2003). Recently, Okuda *et al.*, 2009 reported that they developed a laser-assisted thermal-expansion microinjector to efficient microinjection into plant cells with an intact cell wall and high turgor pressure.

2.1.4 *Agrobacterium*- mediated transformation of plants

Through the introduction and expression of diverse genes in tobacco more than twenty-five years ago, the concept of using *Agrobacterium tumefaciens* as a vector to create transgenic plants was established (Horsch *et al.*, 1985). Today, many agronomically and horticulturally important species are routinely transformed using this bacterium and the list of species that is susceptible to *Agrobacterium*-mediated transformation seems to grow daily and successes include most major economic crops, vegetables, ornamental, medicinal, fruit, tree and pasture plants (Gelvin, 2003; Paz *et al.*, 2006; Song *et al.*, 2007; Subramanyam *et al.*, 2011).There are numerous examples like that of Park *et al.*, (1996) who described the transfer of gene coding for glufosinate-ammonium (PPT) resistance into japonica rice by using *Agrobacterium*. Cheng *et al.*, (1998) who used *Agrobacterium*, which harbored the *cryIA(b)* and *cryIA(c)* genes to introduce stem borer resistance into a japonica rice. Japonica rice cultivar has also been engineered quite a while ago to express the *Bt* gene for insect resistance (Fujimoto *et al.*, 1993). Several rice cultivars have been engineered to express the *bar* gene for herbicide resistance (Christou *et al.*, 1991). Many other successes have been achieved with rice (Park *et al.*, 1996). In most cases, the GUS gene or antibiotic selectable markers were used. Park *et al.*, (1996) introduced the genes containing herbicide (*pat*) and insect resistance (*Bt*, lepidoptera-specific protein) into a tropical japonica rice using *Agrobacterium*-mediated transformation. *Agrobacterium* mediated transformation has been achieved with other important plants such as wheat (Jones *et al.*, 2005), soybean (Opabode, 2006), kenaf (Kojima *et al.*, 2004), buckwheat (Kojima

et al., 2000; Bratić *et al.*, 2007) cotton (Keshamma *et al.*, 2008), pigeon pea (Rao *et al.*, 2008), tomato (Yasmeen *et al.*, 2009), Wheat (Razzaq *et al.*, 2011) etc.

2.1.5 Jute and Genetic Engineering

Whole plant regeneration following tissue culture and transformation has not been very successful with jute as it is comparatively more recalcitrant in response to regeneration and transformation than other crops (Sarker *et al.*, 2007). Though there are a number of reports on *in vitro* regeneration of jute (Islam *et al.*, 1982; Rahman *et al.*, 1985; Seraj *et al.*, 1992; Khatun *et al.*, 1993; Hossain *et al.*, 1994; Saha *et al.*, 1999), the regeneration protocols described in different reports need to be improved in view of their reproducibility (Sarker *et al.*, 2007).

As jute has a narrow genetic base and inter-species crosses do not give fruitful outcomes, introduction of useful genes into jute varieties necessitates the development of easy and efficient transformation protocols. There is only one established transformation protocol for jute (Ghosh *et al.*, 2002) involving *Corchorus capsularis*. However, this study involved costly particle bombardment technique and was subjected to tissue culture. Though regeneration following tissue culture is hard to achieve with *Corchorus capsularis*, it is near to impossible, if not completely impossible, with *C. olitorius*.

Therefore, the aim of this study was to establish a tissue culture independent *Agrobacterium tumefaciens* mediated transformation protocol for *C. olitorius*.

2.2 REVIEW OF LITERATURE

Conventional plant breeding, combined with improved agricultural practices and modern technologies, have contributed to dramatic crop improvements over the past 50 years, and will continue to provide benefits in future (Ali and Batra, 2009). But there are limitations to conventional plant breeding technology, either due to the limited gene pool or the restricted range of organisms between which genes can be transferred due to species barriers (Kumar and Bhatt, 2006). Plant biotechnology continues the trend of improving crops with more precise methods, permitting the transfer of a single gene with a known function into existing crop varieties (Sharma *et al.*, 2005). New technologies, such as DNA recombinant biotechnologies, if properly focused, offer a responsible way to enhance agricultural productivity for now and the future (Izquierdo, 2000).

2.2.1 Genetic transformation of plants

Despite significant advances over the past decade for genetic transformation of plants, the development of efficient transformation methods can take many years of painstaking research. The major components for the development of transgenic plants are: (1) the development of reliable tissue culture regeneration systems; (2) preparation of gene constructs and insertion into suitable vectors; (3) efficient transformation techniques for the introduction of genes into the crop plants; (4) selection of transgenic plants; (5) molecular and genetic characterization of transgenic plants; (6) transfer of genes to elite cultivars by conventional breeding methods if required; and (7) evaluation of transgenic plants for their effectiveness without being an environmental biohazard (Birch, 1997).

2.2.2 *Agrobacterium* mediated indirect transformation of plants

Agrobacterium tumefaciens is a soil bacterium that has been implicated in gall formation at the wound sites of many dicotyledonous plants (Matthysse and McMahan, 2001). This oncogenic bacterium has been modified for use as a biological vector for plant transformation. For more than two decades, scientists have used *Agrobacterium*-mediated genetic transformation to generate transgenic plants (Lee

and Gelvin, 2008) and has continued to be the most commonly-used method for DNA introduction into plants (Mello-Farias and Chaves, 2008). *Agrobacterium tumefaciens* has the ability to transfer a portion of its DNA, known as T-DNA, to the genome of the plant (Sheng and Citovsky, 1996). Modified *Agrobacterium* strains have been developed that do not induce tumor formation, and these strains can carry genes of interest for the purpose of plant transformation (Citovsky *et al.*, 1988). Since the genes required for the transfer process are not within the T-DNA, any gene of interest can be placed between the 25 base pair sequences that flank the T-DNA (Lee and Gelvin, 2008). This feature makes *Agrobacterium* an excellent candidate for manipulating plant genomes (Rossi *et al.*, 1996). Today, many agronomically and horticulturally important species are routinely transformed using this bacterium, and the list of species that is susceptible to *Agrobacterium*-mediated transformation seems to grow daily (Gelvin, 2003).

Agrobacterium-mediated transformation has strong advantages, such as stable integration into genomic DNA and simple segregation pattern by low copy number, making it preferred by breeders more than direct transformation methods (Hiei and Komari, 1994). The use of *Agrobacterium* is less labor intensive, does not require sophisticated equipments, and is more cost effective.

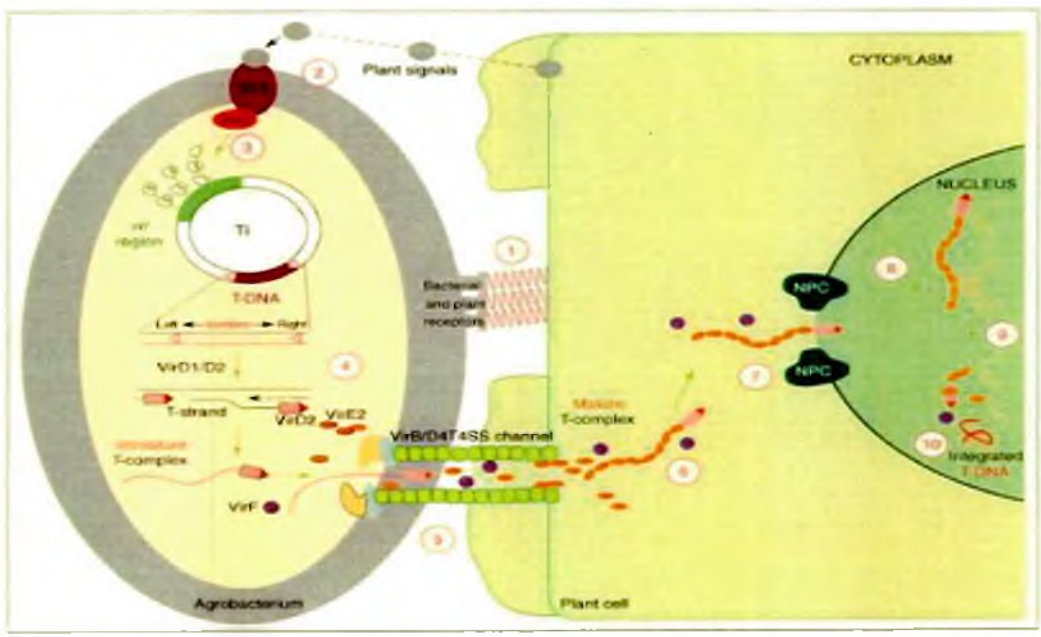
2.2.3 Molecular basis of *Agrobacterium* mediated transformation

The molecular basis of this transformation revolves around the activities of a large (~200 kb) tumor-inducing (Ti) plasmid resident in virulent strains. The transferred DNA (T-DNA) is transferred into the plant cells, integrated into the nuclear DNA, and expressed (McCullen and Binns, 2006). Virtually any DNA cloned into the T-DNA can be transferred into plant cells. Using such modified strains and appropriate selectable markers, the host range over which DNA can be transferred now includes species ranging from other bacteria, fungi, virtually all classes of plants, and even to some mammalian cells (McCullen and Binns, 2006).

The intense mechanistic analysis of *Agrobacterium*-mediated transformation has established *Agrobacterium* as an important model system, at the forefront in understanding how pathogens recognize hosts and deliver macromolecules into

target cells, resulting in disease. From these studies a basic model for the cellular transformation of plants by *A. tumefaciens* has been developed (McCullen and Binns, 2006). The mechanism of T-DNA transfer process is shown in Figure 2.2.1.

The transformation process comprises 10 major steps and begins with (1) recognition and attachment of the *Agrobacterium* to the host cells and (2) sensing of specific plant signals by the *Agrobacterium* VirA/VirG two component signal-transduction system. Following (3) activation of the *vir* gene region, (4) a mobile copy of the T-DNA is generated by the VirD1/D2 protein complex and (5) delivered as a VirD2–DNA complex (immature T-complex), together with several other *vir* proteins, into the host cell cytoplasm. Following (6) the association of VirE2 with the T-strand, the mature T-complex forms, travels through the host-cell cytoplasm and (7) is actively imported into the host-cell nucleus. (8) Once inside the nucleus, the T-DNA is recruited to the point of integration, (9) stripped of its escorting proteins and (10) integrated into the host genome (Tzfira and Citovsky, 2006).



Courtesy: Current Opinion in Biotechnology, 2006, 17:147-154

Figure 2.2.1: A model for the *Agrobacterium*-mediated genetic transformation.

2.2.4 Vectors for *Agrobacterium* mediated transformation of plants

For dicot plants, *Agrobacterium tumefasciens*-mediated transfer of T DNA from the Ti plasmid produces high transformation frequencies, perhaps with less DNA rearrangement and silencing than direct DNA transfer (Gallie, 1998). Initially it was difficult to transform monocots using *Agrobacterium*, but eventually this constraint was overcome (Cheng *et al.*, 2004).

Most of the progress achieved to date in establishing protocols for the transformation of new host species has relied on a relatively small number of binary vectors and genetically modified Ti-helper plasmids (Tzfira and Citovsky, 2006). They are extensively modified so that most of the features of a natural Ti plasmid are removed; only the left - and right border sequences are present to ensure transfer of the T-DNA region between them. Virulence genes required for transfer of the T-DNA are often located on a separate plasmid in the bacterium (Kojima *et al.*, 2000).

The borders discussed above define the start and end of the T-DNA. The T-DNA used in engineering plants is usually 5 to 10 kb, and can be upto 50 kb in size, with the capacity to encode 2 to 20 genes (Comai, 1993). Most vectors used for the genetic transformation of plants carry 'marker' genes that allow the recognition of transformed cells, by either selection or screening. These genes are dominant, usually of microbial origin, and placed under the control of strong and constitutive, eukaryotic promoters, often of viral origin (Birch, 1997).

2.2.5 Plant Binary vector pBI121

The widely used expression vector pBI121 (accession number AF485783) for plant transformation has 14758 bps. The T-DNA region (6193 bp) contains the right border, expression cassettes for a neomycin phosphotransferase II (npt II) selection marker and β-glucuronidase (GUS) reporter gene, and the left border. The β-glucuronidase gene is driven by CaMV promoter and NOS (nopaline synthase) terminator (Jefferson, 1987). The neomycin phosphotransferase (npt II) gene is driven by NOS promoter terminator conferring kanamycin resistance for bacterial selection (Figure 2.2.2). The non-T-DNA region (8565 bp) was constructed according to the Bin 19 vector (Chen *et al.*, 2003). Although the vector pBI121 is no longer available from the

manufacturer (Clontech Laboratories, USA), many researchers still use it (Chen *et al.*, 2003).

Agrobacterium-mediated transformation using pBI121 as a donor for the kanamycin resistance was developed from several plants including peanut (Rohini and Rao, 2001), shallot (Zheng *et al.*, 2001), spruce (Le *et al.*, 2001), buckwheat (Kojima *et al.*, 2000), and even in a bacterium *Azotobacter* (Chen *et al.*, 2003). Thus, the binary vector pBI121 is still a valuable vector for plant transformation (Chen *et al.*, 2003).

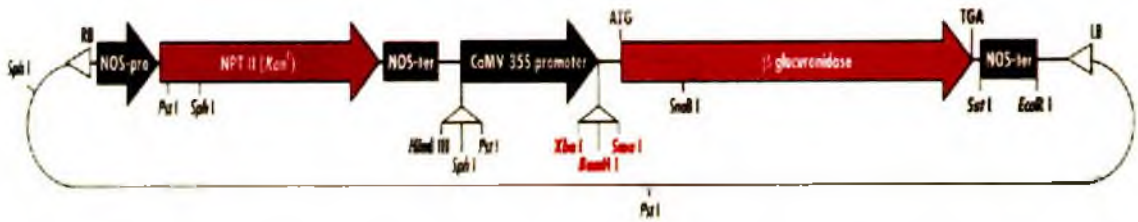


Figure 2.2.2: Structure of binary vector pBI121 (www.btc.nchu.edu.tw/myweb3/SP3-2/SP3-2_img_4.jpg).

2.2.6 *In planta* transformation

Transformation protocols requiring extensive periods of tissue culture, have several drawbacks, including time (typically many months), space, labour and, importantly, the tendency to induce somaclonal variation. Tissue culture based techniques are also needed to be conducted under contamination-sensitive aseptic conditions and a series of selection procedures are needed to be designed for establishing authentic transformants (Seo *et al.*, 2008). There is, therefore, considerable interest in developing transformation methods that obviate the need for tissue culture. A number of laboratories have pursued plant transformation methods that avoid tissue culture or regeneration. In many cases these methods have targeted meristems or other tissues that will ultimately give rise to gametes (Chee and Slighton, 1995; Birch, 1997).

For non-tissue culture approaches, *Agrobacterium* or tungsten particles have been used in a number of species to transform cells in or around the apical meristems that are subsequently allowed to grow into plants and produce seeds (Chee and Slighton,

1995). Injection of naked DNA into ovaries has also been reported to produce transformed progeny (Zhou *et al.*, 1983). Electroporation-mediated gene transfer into intact meristems *in planta* and a variety of pollen transformation procedures have also been reported. However, most of these methods have been difficult to reproduce and have not gained widespread acceptance (Bent, 2000).

One of the most promising and easy method, that was developed using Arabidopsis as the target plant, was the floral dip (Bent, 2006). This was a simple method in which plants with young flowers were dipped into a culture of *Agrobacterium* which also contain surfactant. The plant was subsequently allowed to set seed, whereupon a number of seeds produced were transgeneic. This, or similar techniques have been applied to other plant species, including alfalfa (Weeks *et al.*, 2008) buckwheat (Kojima *et al.*, 2000) and rice (Supartana *et al.*, 2005). For example, in rice, seeds (*Oryza sativa* L. var. Koshihikari) were soaked in water for 2 days. Thereafter, the embryo containing an apical meristem was inoculated with *Agrobacterium tumefaciens* by piercing a site of the husk overlying the embryonic apical meristem with a needle that had been dipped in an *A. tumefaciens* inoculum. The inoculated seeds were then grown to maturation (T_0 plants) and allowed to pollinate naturally to set seeds (T_1 plants) in pots under nonsterile conditions. Although the methods have not been widely reproduced or adapted, *Agrobacterium*-mediated shoot apex transformation and related methods that minimize tissue culture have been reported for a number of other plant species. Multiple plant species have been reported to be successfully transformed using *Agrobacterium* mediated *in planta* approaches such as, kenaf (Kojima *et al.*, 2004), buckwheat (Bratić *et al.*, 2007), cotton (Keshamma *et al.*, 2008), pigeon pea (Rao *et al.*, 2008), tomato (Yasmeen *et al.*, 2009) etc.

The benefits of *in planta* methods are clear: transformation without tissue culture can provide a high throughput method that requires minimal labor, expense, and expertise. Rates of unintended mutagenesis are reduced. More importantly, simplified transformation protocols facilitate positional cloning, insertional mutagenesis, and other transformation-intensive procedures, thus reducing the effort required to test any given DNA construct within plants (Bent, 2000).

2.2.7 Selectable markers

Plant transformation is in many cases a very low frequency event. The use of selection gene has been vital to the development of strategies for the production of transgenic plants (Haldrap *et al.*, 2001). However, selection of the transformed plant cells has traditionally been accomplished by the introduction of an antibiotic or herbicide resistant gene, enabling transformed cells to survive on medium containing inhibiting compound (Haldrap *et al.*, 1998).

The commonly used selectable marker genes include the neomycin phosphotransferase-II gene (nptII) that confers resistance to aminoglycoside antibiotics such as kanamycin and its analogues paramomycin and geneticin (Bevan *et al.*, 1983) and hygromycin phosphotransferase gene, which confers resistance to hygromycin (Van Den Elzen *et al.*, 1985; Weeks *et al.*, 2000), have been successfully used as selectable markers in transformation of a variety of crop plants. The bar gene which encodes for bialaphos confers resistance to the herbicide BASTA and phosphinothricin has also been used in transformation of monocots and dicots (Thompson *et al.*, 1987; Strauch *et al.*, 1988; Murakami *et al.*, 1986).

Kanamycin, which has played a prominent role in the development of plant transformation technologies, is produced by the soil actinomycete *Streptomyces kanamyceticus* as a trisaccharide composed of a deoxystreptamine and two glucosamines (Miki and McHugh, 2004). This antibiotic inhibits protein synthesis in bacteria by binding to the ribosomal subunits and similarly inhibits protein synthesis in eukaryote plastids and mitochondria (Pestka, 1975). The aminoglycoside antibiotics, kanamycin, is very toxic to plant, animal and fungal cells (Miki and McHugh, 2004).

2.2.8 Marker genes

The use of visual marker for selection increases transformation efficiency by reducing the time and the number of materials to be handled at the screening step. This has been demonstrated for rice, barley and woody fruit plants (Stewart, 2001; Ghorbel *et al., 1999;* Vain *et al., 2000).* At present, only a small number of reporter genes are in widespread use in plant transformation vectors, these being β-glucuronidase (*uidA* or *gus*, E.C. 3.2.1.31), green fluorescent protein (gfp), luciferase

genes (*lux* and *luc*) and, to a lesser degree (although it is widely used in animal systems), the chloramphenicol acetyl transferase gene (*cat*) (Slater *et al.*, 2003). Reporter genes have played an important role in developing and optimizing transformation protocol for plant species (Sreeramanan *et al.*, 2005).

β-glucuronidase is perhaps the most widely used reporter gene in plant transformation vectors (Jefferson, 1987). Its widespread acceptance is due to its many advantages over the previously existing marker genes. β-glucuronidase can be assayed extremely sensitively using quick, easy and non-radioactive methods. It can be used to obtain both quantitative (i.e. the level of gene expression) and qualitative (i.e. localization of gene expression) data (Miki and McHugh, 2004). GUS activity is found widely in microorganisms, vertebrates and invertebrates but there is no or very little background activity (in reproductive tissues) in plants. The GUS enzyme is very stable within plants and is non-toxic when expressed at high levels. The major drawback with the use of GUS as a reporter is that the assays are destructive to the plant cells (Miki and McHugh, 2004).

Green fluorescent protein (gfp) isolated from jelly fish, *Aequorea victoria* encodes a small barrel-shaped protein surrounding a fluorescent chromophore, which immediately emits green fluorescent light in the blue to ultraviolet range (Jaiwal *et al.*, 2002). Detection is possible at any times in the living cells without cell destruction and without the addition of any cofactor or external substances. In addition, the gfp gene product does not adversely affect cell growth, regeneration and fertility of transformed plants (Jaiwal *et al.*, 2002).

2.2.9 Study of *Gus* gene expression

GUS gene expression is often studied for detecting transformed plants particularly when detection by selectable marker is ambiguous. For assaying the transfer of T-DNA to the transformed kenaf plants, transient GUS expression study is performed several days after the infection using the histochemical GUS assay (Kojima *et al.*, 2004). GUS expression frequency is calculated as the percentage of the number of explants that show GUS expression out of the number of explants tested for GUS expression from each treatment. To confirm T-DNA delivery, genomic DNA is extracted from putative transgenic plants and analyzed by the polymerase chain

reaction. Amplified products are visualized by electrophoresis on agarose gel stained with ethidium bromide (Herath *et al.*, 2005).

2.2.10 Transformation and Jute

Jute, the principal cash crop of both India and Bangladesh, has special significance, as the livelihood of millions of small and marginal farming families depend on its production and utilization (Pathak, 1991). Unfortunately, no significant progress in the genetic improvement of jute has been achieved. As mentioned earlier the major problems encountered in the development of new cultivars are the lack of genetic variability and the existence of a strong sexual incompatibility barrier between two cultivated species, *Corchorus olitorius* L. and *C. capsularis* L. (Patel and Dutta, 1960; Swaminathan *et al.*, 1961).

Since selection has limited scope in bringing about improvement in both species, ionizing radiation and chemical mutagens have been used in the past to produce desirable varieties. However, the use of ionizing radiation did not prove very fruitful. For instance, during the last 30 years or so, only one variety, *C. capsularis* (Atom Pat), has been released by the Bangladesh Institute of Nuclear Agriculture through the use of ionizing radiation (Rahman, 2007).

In vitro regeneration has been quite difficult with the species of *Corchorus* through tissue culture techniques. Regeneration has only been reported from meristematic tissue but not from totally dedifferentiated tissue, like callus. Rahman *et al.*, 1985 showed that callus initiated from both apical meristems and cotyledons of var. D-154 of *C. capsularis* when cultured on BAP and tyrosine fortified MS media formed shoots. Ahmed *et al.*, (1989) reported the regeneration experiments using NDGA, an antioxidant. Regeneration from hypocotyls-derived callus has been reported by Seraj *et al.*, (1992). Regeneration from cotyledon derived callus is reported by Khatun *et al.*, (1992). They used growth regulators BAP and IAA with MS medium in order to get multiple shoots from cotyledon derived callus. However, in the case of both hypocotyls and cotyledons the regeneration percentage was low and sporadic. Saha *et al.*, (1999) described that regeneration from jute was only reproducibly obtained from meristematic regions of seedlings or calli containing pre-existing shoot primordial.

Hossain *et al.*, (1998) observed that transgenic *gus* gene could be delivered into the cut surface of the meristematic zone of both plumules and cotyledonary bases by *Agrobacterium* as was evident by GUS assay. However, they found no mature transgenic plants. Islam *et al.*, (1999) showed the stable transformation of jute (*Corchorus capsularis* L.var CVL-1) calli and high efficiency of marker gene insertion in explants. Transformed calli were easily obtained from cotyledonary-petiole explants with efficiencies of 20-60% depending on the type of vector used. However, regenerated plantlets failed to grow in selection medium for longer than two months, probably due to the chimeric nature of transformed plantlets.

Ghosh *et al.*, (2002) developed a biolistic particle delivery system for stable jute transformation of *Corchorus capsularis* var. JRC 321. They used apical, meristematic region of a germinating seedling as target for transformation. They observed the transmission of the trait to T_1 generation. However, this technique was expensive and involved laborious tissue culture steps.

2.2.11 Improvement of jute by biotechnological approach

Jute suffers from a number of diseases of which 10 are known to be seed-borne (Fakir, 2001). Among the seed borne diseases, only leaf mosaic is caused by virus and the rest are caused by fungi. Fazli and Ahmed (1959) made a comprehensive list of pathogenic fungi which include *Macrophomina phaseolina, Botryodiplodia theobromae, Colletotrichum corchori, Glomerella cingulata* and *Fusarium* (semi-pathogenic). Among these, *M. phaseolina* causes stem-rot at any stage from seedling to fruiting and *C. corchori* causes seedling blight and both lead to deterioration of yield of fibre and quality of seed. Stem-rot alone causes huge loss of fibre (Ahmed, 1968). It also causes loss of fibre quality (Ahmed, 1968; Biswas *et al.*, 1980). Anthracnose is another important disease of jute.

In Bangladesh, loss due to seed-borne diseases of jute varies from 8% to 20% depending on the severity of diseases from year to year (Ahmed *et al.*, 1985). Genes, encoding disease and insect resistance, inserted into jute genome may be effective in avoiding the loss but this will require development of an efficient transformation and regeneration protocol for jute.

2.3 Material and Methods

2.3.1 *Agrobacterium tumefaciens* strain and binary vector

In the present study *Agrobacterium tumefaciens* strain LBA4404 (Hoekema *et al.*, 1983) carrying the binary vector pBI121 (Bevan, 1984) was used for introducing foreign gene(s) in jute. The redundant vector, pBI121 contains a nopaline synthase promoter sequence in front of a neomycin phosphotransferase II (NPTII) gene which confers resistance to kanamycin and a GUS (β-glucuronidase) gene driven by CaMV 35S promoter.

2.3.2 Culture of *Agrobacterium tumefaciens* strain LBA4404

Agrobacterium strain LBA4404 was grown on Yeast Mannitol (YM) solid medium containing kanamycin as the selective agent. Single colonies from these plates were later used to inoculate YM broth medium. Bacterial concentration was determined by spectrophotometer (SPECORD ® 50) using the software WinASPECT® at wavelength 600 nm. Bacteria from these cultures were used for infection of shoot apical meristematic region of young jute plants.

2.3.3 Culture in Yeast-Mannitol solid medium

2.3.3.1 Materials

Mannitol	Yeast extract	NaCl
$MgSO_4.7H_2O$	K_2HPO_4	Agar

2.3.3.2 Method

A typical medium for *Agrobacterium* is YMB. Yeast-Mannitol media with Kanamycin was used for culturing *A. tumefaciens* strain LBA4404 containing binary vector pBI121.

- Components of YMB medium were mixed in the following amounts in autoclaved conical flasks:

Mannitol	1.0% (w/v)
Yeast extract	0.04% (w/v)

NaCl	0.01% (w/v)
$MgSO_4.7H_20$	0.02% (w/v)
K_2HPO_4	0.05% (w/v)

- Volume was made upto the required mark (for example, 100 ml in a measuring cylinder if 100 ml of medium is to be made).
- The pH of the media was adjusted to 7.0-7.2 (The initial pH of the medium was above 7.2. Acid was slowly added to the medium as alkali can not be added to raise the pH if it falls bellow 7.0 due to inadvertent addition of excess volume of acid).
- Agar (1.5% w/v) was added to the medium and autoclaved.
- Once autoclaving of the medium was complete and cooled to a level that the temperature could be tolerated by bare hand, kanamycin was added and mixed by swirling. The medium was spread on Petri dishes in a volume of approximately 20 ml per plate.
- Upon solidification, the plates were streaked with *Agrobacterium* from a glycerol stock or from a previously prepared culture.
- The streaked plates, sealed with parafilm, were incubated in an incubator at 28^0C in dark for three days.

2.3.4 Culture in Yeast Mannitol Broth

2.3.4.1 Materials

Mannitol	Yeast extract	NaCl
$MgSO_4.7H_2O$	K_2HPO_4	

2.3.4.2 Method

YMB media with Kanamycin was used for culturing *A. tumefaciens* strain LBA4404 containing binary vector pBI121.

- Components of YMB medium were mixed in the following amounts in autoclaved conical flasks:

Mannitol	1.0% (w/v)

Yeast extract	0.04% (w/v)
NaCl	0.01% (w/v)
$MgSO_4.7H_20$	0.02% (w/v)
K_2HPO_4	0.05% (w/v)

- Volume was made upto the mark (for example, 100 ml in a measuring cylinder if 100 ml of medium is to be made).
- The pH of the media was adjusted to 7.0-7.2% (The initial pH of the medium was above 7.2. Acid was added slowly to the medium as alkali cannot be added to raise the pH, if pH falls below 7.0 for inadvertent addition of huge volume of acid).
- Once autoclaved the medium was cooled, kanamycin (100 µl) was added and mixed by swirling. The medium was poured approximately to a volume of one-third of the volume into 10 ml screw cap tubes.
- Screw cap tubes containing medium were inoculated with single colony from a previous culture on solid medium. Appropriate controls were used to measure the growth and concentration of bacteria in the medium.
- The tubes were incubated overnight in a rotary shaker at 28°C in dark at 200 rpm.

2.3.5 Determination of growth of *Agrobacterium*

After overnight incubation, the extent of growth of *Agrobacterium* in the liquid culture medium was determined by measuring the optical density (O. D.) with a spectrophotometer (SPECORD ® 50) using the software WinASPECT®. O.D. was measured in 500 µl glass cuvettes at a wave-length of 600 nm.

2.3.6 Preparation of glycerol stock of *Agrobacterium*

2.3.6.1 Materials

Glycerol	$MgSO_4$	Tris

2.3.6.2 Method

For preparing 50 ml of Glycerol stock solution the following procedure was followed:

- 1.2323g of $MgSO_4$ and 1.5 ml of 1M tris (pH= 8.0) was added to 32.5 ml of 99.9% glycerol.
- The volume was adjusted to 50 ml using distilled H_2O.
- The solution was autoclaved and then stored at - 4°C if necessary.

For preparing stock culture of *Agrobacterium,* 1 ml of liquid culture of bacteria was mixed with 2 ml of glycerol stock solution. Bacterial glycerol stock was stored at – 80°C.

2.3.7 Plant Growth and Inoculation

2.3.7.1 Plant material

BJRI Tossa Pat-4 (O-72), a popular tossa (*Corchorus olitorius* L.) jute variety developed at Bangladesh Jute Research Institute (BJRI), Dhaka was used in this study. Seeds were sown on pots in soil (5-7 seeds each). Plants were grown inside the glass house with 12 hours photoperiod at 30°C.

2.3.7.2 Experimental Design

Experiments were conducted using five replicates. For each replication procedure, at least 15 plants were infected with *Agrobacterium*. To determine the most suitable OD of Agrobacterium for effective transformation, five different levels/concentrations were tested - 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0.

2.3.7.3 Transformation procedure

Early young plants (25-30 days old) were infected according to the following steps -

- For infection, *A. tumefaciens* strain LBA4404 was grown in liquid medium without kanamycin. As kanamycin was not present in the medium, appropriate controls were there to ensure that the growth pertained solely to *Agrobacterium*.

- The meristematic regions at the shoot tip of the plants were injured by a needle an hour before infection.
- Bacterial suspension in medium was directly used for infecting the plants. Cotton buds soaked in the bacterial suspension were used for the inoculation of the injured regions.
- Upon infection, the plants were placed in dark at 28±2°C for three days.
- After this period, plants were allowed to grow under normal conditions.
- As these plants matured and set fruit, seeds were collected and planted for obtaining the next generation plants.

2.3.8 Selection of putative transformed plant

After the collection of seeds from T_0 plant, transformed plants were selected according to the following steps:

2.3.8.1 Sterilization of seeds

Both transformed and non-transformed (control) plants were surface sterilized by the following procedure -

- The seeds were washed in 99% ethanol in sterile conical flask for 2-3 min.
- Seeds were then washed with 0.1% $HgCl_2$ solution containing 2-3 drops of Tween 20 and incubated in a shaker for 20 min.
- The seeds were washed 5 times with autoclaved ddH_2O.

2.3.8.2 *In vitro* seedling growth under selection medium and transfer to soil

To select transformed plants, sterilized seeds were aseptically placed in kanamycin selection medium and then grown for 10-15 days in control environment at 25°C under 14h light. Once the growth was sufficient to sustain further development in an open environment, seedlings were transferred to the soil in the garden.

2.3.8.2.1 Preparation of culture medium

2.3.8.2.1.1 Materials

Murashige and Skoog (MS) medium (Table 2.1) was used for *in vitro culture* of jute.

Table 2.1: Components and their concentrations in MS medium.

MS medium							
Macro-nutrients	Final conc. (mg/l; mM)	Stock solution (20×); g			Working solution (1×); ml		
		1000ml	500ml	100ml	500ml	200ml	100ml
NH_4NO_3	1650/20.6	33	16.5	3.3	25 ml	10 ml	5 ml
$CaCl_2.2H_2O$	440/3.0	8.8	4.4	0.88			
$MgSO_4.7H_2O$	370/1.5	7.4	3.7	0.74			
KNO_3	1900/18.8	38	19	3.8			
KH_2PO_4	170/1.3	3.4	1.7	0.34			
Micro-nutrients	Final conc. (mg/l; µM)	Stock solution (400×); g			Working solution (1×); ml		
		1000ml	500ml	100ml	500ml	200ml	100ml
H_3BO_3	6.2/100	2.48	1.24	0.124	1.25 ml	0.5 ml	0.25 ml
$CoCl_2.6H_2O$	0.025/.01	0.01	0.005	0.001			
$CuSO_4.5H_2O$	0.025/0.1	0.01	0.005	0.001			
$MnSO_4.H_2O$	16.9/100	6.76	3.38	0.676			
KI	0.83/5	0.332	0.166	0.0332			
$Na_2MoO_4.2H_2O$	0.25/1	0.1	0.05	0.01			
$ZnSO_4.7H_2O$	8.6/30	3.44	1.72	0.344			
Iron source							
Na_2EDTA	37.3/100	14.92	7.46	1.492	1.25 ml	0.5 ml	0.25 ml
$FeSO_4.7H_2O$	27.8/100	11.12	5.56	1.112			
Organics	Final conc. (mg/l; µM)	Stock solution (200×); g			Working solution (1×)		
		1000ml	500ml	100ml	500ml	200ml	100ml
Thiamine HCl	0.5/	0.1	0.05	0.01	2.5 ml	1.0 ml	0.5 ml
Myo-inositol	100/550	20.0	10.0	2.0			
Glycine	2.0/26.6	0.4	0.2	0.04			
Nicotinic acid	0.5/4.1	0.1	0.05	0.01			
Pyridoxine HCl	0.5/2.4	0.1	0.05	0.01			

Others

- Myoinositol 100 mg/l
- Sucrose 40 gm/l
- PF-68 0.1% (w/v)
- PVP 250 mg/l

Antibiotic stock

- Kanamycin 50 mg/ml

2.3.8.2.1.2 Method

- The basal medium was based on Murashige and Skoog (MS) medium. Appropriate quantities of macro-nutrients, micro-nutrients, organics, myo-inositol, sucrose, PF-68 and PVP were mixed together and the volume was adjusted to the required level by adding ddH_2O.
- pH of the medium was adjusted to 5.8.
- Phytagel was added to the medium at 0.4% (w/v) as a solidifying agent.
- The medium was autoclaved at 121°C for 20 min.
- Once the autoclaved medium cooled, thiamin and kanamycin were added and mixed by swirling. The medium was poured approximately to a volume of 100 ml in 250 ml conical flasks and allowed to solidify.

2.3.9 Estimation of transformation efficiency

Transformed plants were identified as kanamycin resistant from seedlings that produced green leaves and well established roots on the kanamycin selection medium. Transformation efficiency was determined by the following formula:

$$\text{Efficiency (\%)} = \frac{\text{Number of germinated seedlings and subsequent growth}}{\text{Total numbers of seeds were placed in selection medium}} \times 100$$

2.3.10 Assay for β-Glucuronidase (GUS) Activity

Transgenic cells were confirmed by histochemical GUS assay as described by Jefferson (1987).

2.3.10.1 Materials

a) 0.5M MES buffer (pH 5.6)

2.67 gm of Morpholinoethane sulphonic acid (MES) was taken in 20 ml ddH_2O. pH was adjusted to 5.6 with 5M KOH (freshly prepared) and volume was made to 25 ml by ddH_2O, filter sterilized and stored at room temperature.

b) Fixation solution (pH 5.6)

Fixation solution was prepared by mixing 750 µl of formaldehyde, 2ml of 0.5M MES solution and 5.46g of mannitol. The volume was made to 100ml with ddH_2O. The solution was stored at 4°C.

c) 50 mM Phosphate buffer (pH 7.0)

Stock solutions were prepared as follows: Solution A (pH< 7): 50 mM solution of $NaH_2PO_4.2H_2O$ was prepared by adding 0.39g of $NaH_2PO_4.2H_2O$ in 50 ml of ddH_2O.

Solution B (pH> 7): 50 mM solution of $Na_2HPO_4.2H_2O$ was prepared by adding 0.45g of $Na_2HPO_4.2H_2O$ in 50 ml of ddH_2O.

Solution A (39 ml) was mixed with solution B (59 ml) and the pH was adjusted to 7.0 by addition of either solution A or B as required. The solution was filter sterilized and stored at 4°C.

d) Histochemical reagent

X-gluc (5-bromo-4chloro-3-indoyl β-D-glucuronide)

For the preparation of 10 ml of X-gluc 10 mg of X-gluc was dissolved in 100 µl of dimethyl formamide and the final volume was made to 10ml with 50 mM phosphate buffer (pH 7.0). X-gluc is photo-sensitive, so, this solution was stored at -20°C in an eppendorf tube wrapped with aluminum foil.

2.3.10.2 Methods

- The plant tissues (such as leaves) or seedlings to be tested for GUS activity were first washed in 70% ethanol for 30 seconds followed by ddH_2O for three times.
- The plant parts were placed in fixation solution and were vacuum infiltrated briefly (for about 20 min).
- The tissues were washed three times with phosphate buffer (pH 7.0).
- Adequate volume of X-gluc (~500µl - 700 µl) was added to each eppendorf tube so that the tissues got immersed into the solution. These were incubated at 37°C for at least 72 hours.
- Samples that showed blue colour were washed with 70% ethanol solution and kept at least 24 hours at 4°C and examined under a microscope for GUS gene expression.

2.3.11 Isolation of transgenic plant DNA using CTAB

DNA was isolated from both transgenic and non-transgenic jute plants following a procedure modified from Doyle and Doyle (1990) using nonionic detergent Cetyl Trimethyl Ammonium Bromide (CTAB).

2.3.11.1 Materials

CTAB extraction buffer (100 ml)

Materials

CTAB	2 g
1M Tris- HCl (pH= 8.0)	10 ml
0.5M EDTA	4 ml
5.0M NaCl	28 ml
2-Mercaptoethanol	2 ml

Preparation of CTAB extraction buffer

- 2g of CTAB was taken into ~ 40ml of ddH_2O and kept in a water bath at 60°C for 3-4 hours until CTAB was dissolved.

- 10 ml of 1M Tris- HCl (pH 8.0), 4 ml of 0.5M EDTA and 28 ml of 5.0M NaCl was added to the dissolved CTAB solution.
- The volume was made 100 ml by adding ddH_2O.
- 2 ml of 2-mMercaptoethanol was added to the mixture just before use.
- The buffer was stored at room temperature.

CTAB NaCl buffer (100 ml)

Materials

- CTAB 1.0 g
- NaCl 4.1 g

Preparation

- 1g of CTAB and 4.1g of NaCl were dissolved in ~ 50ml of ddH_2O
- The mixture was kept in a water bath at 60°C for 3-4 hours until CTAB was dissolved.
- The volume was made to 100 ml by adding ddH_2O.
- The buffer was stored at room temperature.

CTAB precipitation buffer (100ml)

Materials

- CTAB 1 g
- 1M Tris-HCl 5 ml
- 0.5M EDTA 2 ml

Preparation

- 1g of CTAB was taken into ~ 50 ml of ddH_2O and dissolved by heating in a water bath at 60°C for 3-4 hours.
- 5 ml of 1M Tris-HCl and 2 ml of 0.5M EDTA was added to the solution.
- The volume was made to 100 ml by adding ddH_2O.
- The buffer was stored at room temperature

High salt TE buffer

Materials

- 1M Tris- HCl (pH 8.0) 1 ml
- 0.5M EDTA 20 ml
- 5.0M NaCl 20 ml

Preparation

- All the reagents were mixed in ddH_2O and the volume was made 100 ml.

The buffer was stored at room temperature.

Other materials for DNA extraction

- 80% ethanol
- Mortar and pestle
- Organic solvent-resistant screw cap test tubes

23.11.2 Procedure for extracting DNA

- 5 ml of extraction buffer was taken into each screw cap tube.
- 125 µl of 2-mercatoethanol was added to it.
- The tubes were warmed in a water bath at 65°C for 30 min.
- After 30 min, the samples were ground with mortar and pestle using liquid nitrogen.
- The homogenates were added to warmed extraction buffer in screw cap tubes.
- These mixtures were incubated again at 65°C for 60 min.
- 6 ml of $CHCl_3$: IAA (24:1) was added in each tube and shaken.
- The weights of each tube were measured and balanced using $CHCl_3$: IAA.
- The mixtures were centrifuged at 4000 rpm for 20 min.
- Supernatants were collected.

- Equal volume of $CHCl_3$: IAA (~ 6 ml) was added.
- These were centrifuged again at 4000 rpm for 20 min.
- Supernatants were collected and CTAB-NaCl solution was added to each tube at a volume of $1/10^{th}$ of the collected supernatant.
- Weight of each tube was measured and balanced with an equal volume of $CHCl_3$: IAA.
- These were centrifuged at 400 rpm for 20 min.
- Supernatants were collected and mixed with an equal volume of CTAB-precipitation buffer.
- If DNA could be seen, these mixtures were centrifuged as in the next step. However, in case when DNA could not be seen, the mixtures were incubated at 65°C for 30 min.
- These were centrifuged at 4000 rpm for 20 min.
- The pellets were collected.
- Pellets were dissolved in high-salt buffer (3-5 ml, depending on the quantity of the pellet) by mild shaking, if necessary.
- RNase solution was added (for 1 ml of solution 1µl of RNase (100 mg/ml) solution was added)
- These mixtures were kept at 37°C for 30 min inside an incubator.
- Equal volume of $CHCl_3$: IAA was added to these mixtures.
- These were centrifuged at 4000 rpm for 10 min.
- Supernatants were collected and transferred (~ 750 µl in each) to eppendorf tubes.
- 0.6 volume Iso-propanol (2-propanol) was added.
- The mixtures were kept at -30°C for 2 h (if there was no precipitate, the mixture was kept at -80°C for ~2 h).

- These were centrifuged at 12000 rpm for 20 min and the supernatants were collected.
- 500 µl of 80% ethanol was added to the pellets and washed twice.
- These were centrifuged at 12000 rpm for 5 min.
- Pellets were dried in a vacuum drier for 10 min.
- 50 µl of PCR TE buffer (depending on the amount of pellet) was added to the pellets.
- These were stored at -20°C.

2.3.12 Isolation of plasmid DNA

The primary objective of the isolation process was to achieve the maximum yield of plasmid DNA devoid of protein. Plasmid was isolated according to the following protocol which was modified from Sambrook *et al.* (1989).

2.3.12.1 Materials

1. Solution I
   - Glucose 50mM
   - Tris.HCl (pH 8.0) 25mM
   - EDTA (pH8.0) 10mM
2. Solution II (Freshly prepared)
   - SDS 1% (w/v)
   - NaOH 0.2N
3. Solution III
   - K-acetate 3M
   - Glacial acetic acid 11.5% (v/v)
4. Phenol:Chloroform:Isoamylalcohol 25:24:1 (v/v)
5. Isopropanol

6. RNase solution 10μg/ml

7. TE buffer

   - Tris.HCl (pH 8.0) 10mM
   - EDTA (pH 8.0) 1mM

All the solutions except solution II were autoclaved (110°C for 15 min) and stored at 4°C.

2.3.12.2 Procedure

- Bacterial colony/cells were grown overnight in YMB (liquid) medium at 28°C with vigorous shaking (kanamycin in YMB media was at a concentration of 100μg/ml).
- 10ml of culture was transferred to each screw cap tube and centrifuged at 4000rpm for 10 min.
- Supernatant was discarded and pellet was dried completely.
- 200μl of solution I (ice-cold) was added and mixed.
- The suspension was transferred to an eppendorf tube and 400μl of solution II was added to each tube after 30 seconds, mixed the contents by inverting the tubes rapidly five times and kept on ice for exactly 5 min.
- 300μl of solution III (ice-cold) was added and mixed until a clot formed and then incubated on ice for 30 min.
- The mixture was centrifuged at 14000rpm for 10 min at 4°C.
- Supernatant was transferred into a fresh eppendorf tube and extracted twice with equal volume of phenol: chloroform: IAA (25:24:1) by centrifugation at *14000rpm for 10 min at* 4°C.
- Aqueous phase was transferred into fresh eppendorf tubes.
- DNA was precipitated with equal volume of isopropanol (ice-cold) at room temperature for 2-4 min and centrifuged for 10 min at 14000rpm at 4°C.
- Supernatant was discarded and pellet was washed with 70% ice-cold ethanol.

- (If no precipitate was observed, then 500µl of absolute (99%) ethanol (ice-cold) and 10µl of 3M Na-acetate was added).
- Centrifugation was done two times at 14000 rpm for 10 min at 4°C.
- Pellet was dried completely and suspended in 30-50µl of TE buffer.
- 0.2µl of RNase at a concentration of 10µg/ml was added and incubated at 37°C for 2 min.

2.3.13 Polymerase chain reaction (PCR)

2.3.13.1 Primer design

The success with transformation was confirmed by PCR for GUS gene sequence. The free software Primer3 (http://frodo.wi.mit.edu/) was used for designing primers. Parameters for GUS primers were set to provide high stringency during PCR. The primers obtained using this software was cross-checked by software, GeneFisher2 (http://bibiserv.techfak.uni-bielefeld.de/genefisher2/). The primers were also investigated for their homology to other sequences by BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3.13.2 Components of PCR

The Following components were required for the polymerase chain reaction.

1. 10X PCR reaction buffer, which contained
    - 500mM KCl
    - 100mM Tris.HCl (pH 8.3)
    - 0.1% gelatin
2. $MgCl_2$ (25mM) in water
3. Sodium salt of deoxyadenosine-5′-triphosphate (dATP)

    (10mM aqueous solution, pH 7.35)
4. Sodium salt of deoxy-thymidine -5′- triphosphate (dTTP)

    (10mM aqueous solution, pH 7.35)

5. Sodium salt of deoxyguanosine-5′-triphosphate (dGTP)

   (10mM aqueous solution, pH 7.35)

6. Sodium salt of deoxycytidine-5′-triphosphate (dCTP)

   (10mM aqueous solution, pH 7.35)

7. *Taq* DNA polymerase

8. 1 Kb+ ladder.

9. DMSO (20%)

10. DNA template, the sample DNAs was collected from jute plants.

11. Primers

Primers	Direction	Sequence	Bps
Gus	Forward	TTGTCAAGACCGACCTGTCC	23
Gus	Reverse	GCGATACCGTAAAGCACGAG	24

12. Autoclaved ultra-pure water

13. TE buffer for the dilution of the template DNA

    i. 10mM TrisHCl (pH 8.0)

    ii. 0.1mM EDTA (pH 8.0)

2.3.13.3 Preparation of dNTPs mixtures

100µl of dATP, dGTP, dCTP and dTTP (their concentrations being 10mM each) were mixed in fresh, autoclaved eppendorf tubes and the final volume was made 1000µl by adding 600µl of nuclease free water and dispensed as aliquots in tubes and stored at –20°C. The concentration of each of the nucleotide in the above mixture was 1.0mM.

2.3.13.4 Dilution of the DNA template

Since the isolated DNA was highly concentrated and unsuitable for using in PCR, it was diluted with TE buffer before use. Thus, the working concentration of the template DNA was made 50ng/µl.

2.3.13.5 Dilution of primer

Primers were diluted with TE buffer before use and the final concentration of the primers were 60ng/µl.

2.3.13.6 Preparation of the Master Mix

Each PCR reaction (25 µl) mixture consisted of 1X PCR reaction buffer, 2.5 mM $MgCl_2$, 200µM dNTPs, 0.5µM of each oligonucleotide primer, 1.25 unit Taq DNA polymerase and approximately 50ng of template DNA. Volume was made 25µl by adding required amounts of sterilized ultra-pure water. Taq DNA polymerase was added just before the start of the reaction. Finally, the tubes were subjected to momentary spin and transferred to a thermocycler (Applied Biosystem) for the amplification reaction. The oligonucleotide primers were also tested by amplifying the GUS gene by using 10ng of plasmid DNA (pBI121) as positive control.

2.3.13.7 Thermal Cycling Profile Used in PCR

The thermal cycling profiles that were programmed to amplify the gene by Polymerase Chain Reaction (PCR) for 35 cycles were as follows:

Steps	Temperature	Time	No. of Cycles
Initial denaturation	94°C	5 min	1 (First)
Denaturation	94°C	1.0 min	35
Annealing	60°C	1.0 min	35
Elongation	72°C	1.0 min	35
Final elongation	72°C	5.0 min	1 (last)
Hold temperature	4°C	Infinite period	-

2.3.13.8 Visualizing the PCR Product

4 µl of DNA dye was added to the PCR amplified DNA. After a momentary spin the PCR products were loaded in wells of 1.5% agarose gel and electrophoresed at 75 volts. Then the gel was stained in ethidium bromide (0.5µg/ml) and de-stained in

water, followed by visualization under UV transilluminator (MicroDoc, Cleaver Scientific Ltd). Photographs were taken using a Sony G-10 camera.

Composition of DNA dye

i.	Xylene cyanol	0.25%
ii.	Bromophenol blue	0.25%
iii.	Glycerol	30%

2.3.14 Southern Blot Analysis

Putative transgenic plants were analysed at the molecular level for the presence of the GUS transgene by Southern blot (Sambrook and Russell, 2001). The steps of Southern blot followed are as follows:

- Probe preparation and labeling
- Restriction digestion of genomic DNA

  Transfer to digested DNA to nylon membrane
- Pre-hybridization and hybridization
- Washing
- Detection

2.3.14.1 Probe preparation and Labeling

DNA samples from plasmid were PCR amplified using programs described above (2.3.14) and this PCR product was used as a probe in the hybridisation reaction. Probe was labelled with digoxigenin-dUTP following manufacturer's protocol (DIG DNA labeling Kit, Roche).

15 µl of purified PCR product was denatured by heating at 95^{0}C for 10 min and then quickly chilled on ice. Then 2uL hexanucleotide mix, 2uL dNTP mix and 1uL Klenow enzyme were added to it. Finally, the tubes were subjected to momentary spin and incubated overnight at 37°C. The reaction was stopped by heating at 65°C for 10 min.

2.3.14.2 Restriction Digestion of Genomic DNA

Genomic DNA from both transgenic and non-transgenic jute plants was digested with restriction enzyme *EcoRI* (Invitrogen, USA). The plasmid DNA (pBI121) was also digested using the same enzyme and used as the positive control.

Materials

EcoRI (10U/μL)	Genomic DNA
10x restriction buffer	3M sodium acetate
Nuclease free water	100% ethanol

Methods

- Each restriction digestion reaction (100 μl), contained 15μg of genomic DNA, 1X restriction buffer, 75 units of restriction enzyme *EcoR1*. The volume was made up to 100μl by adding required amount of nuclease free water.
- DNA was allowed to stand at 4°C for several hours after dilution and addition of 10 x restriction enzyme buffer followed by a gentle stirring of the DNA solution from time to time. This was done to ensure a homogeneous dispersion of genomic DNA.
- After addition of half the amount of restriction enzyme, the solution was stirred gently for 2-3 min at 4°C before warming to 37°C in a water bath.
- After digestion for 30 min, a second aliquot of restriction enzyme was added and stirred.
- The restriction digestion reaction mixture was kept overnight at 37°C in a water bath with gentle shaking.
- After overnight incubation 0.1 volume of 3 M sodium acetate and 2.5 volumes of ice-cold 100% ethanol was added to the restriction digestion reaction mixture and mixed well. Then the mixture was kept at -30°C for 2 hours.
- The mixture was centrifuged at 10000 rpm at 4°C for 20 min. After discarding supernatant the pellet was dried and finally dissolved in 15 μl of TE buffer.

2.3.14.3 Transfer of digested DNA to Nylon membrane

- Digested DNA was loaded in 0.7% agarose gel and electrophoresed at 40 volt until the dye reached at the edge of the gel.
- DNA was denatured by soaking the gel in an alkaline transfer buffer (1 M NaCl and 0.4 N NaOH) for 15 min at room temperature with constant and gentle agitation.
- A fresh scalpel was used to cut a piece of positively-charged nylon membrane ~1 mm larger than the gel in each dimension. Then the membrane was soaked in distilled water until it was completely wet. This was followed by soaking the membrane in alkaline transfer buffer for 5 min.
- DNA was blotted to positively-charged nylon membrane by capillary action with alkaline transfer buffer. The buffer tank was filled with alkaline transfer buffer until the level of liquid reached almost to the top of the support.
- The transfer platform was made and covered with a sheet of What-man™ 3 MM filter paper, saturated with blotting buffer. When the blotting paper on the top of the support was thoroughly wet, all air bubbles were carefully removed with a glass rod.
- The gel was removed from the denaturation step (after treatment with alkaline transfer buffer) and was placed on the support in the transfer system.
- Then the gel was placed upside down on the support by avoiding trapping of air bubbles beneath it.
- The Nylon membrane was placed on the top of the gel, again avoiding trapping air bubbles beneath the membrane.
- Three sheets of Whatman™ 3 MM filter paper were cut to size of the membrane and wetted with blotting buffer and placed on the top of nylon membrane.
- A stack of absorbent paper towels was placed on top of the 3 MM paper. A glass plate was placed on the top of the paper towels and pressed with a 0.5 kg weight on it.

- The gel was surrounded with parafilm to prevent the paper towels from coming in contact with the wet paper below the gel. Evaporation was also prevented from the transfer solution by sealing with parafilm on either end of the tray.
- The paper towels were replaced as they became wet. It was necessary to prevent the entire stack of towels from becoming wet with buffer. Upward capillary transfer of DNA was performed at room temperature for 18 hours. This capillary transfer drew the buffer up by capillary action through the gel to the membrane, which then bound to ssDNA.
- After transfer of the DNA, the nylon membrane was marked with a pencil to identify both the orientation of the gel and to which side of the membrane the DNA was bound to.
- The membrane was soaked in neutralization buffer (0.5 M Tris-Cl, pH 7.5 and 1.5 M NaCl) for 15 min and followed by 2X SSC for few min and then allowed to air dry.
- DNA was fixed by UV crosslink for 3 min followed by baking in an oven for 30 min.

2.3.14.4 Pre-hybridization and Hybridization

2.3.14.4.1 Pre-hybridization

A pre-hybridization step is required to prevent non-specific binding during hybridization with probe.

Materials

- Maleic acid buffer (0.1 M maleic acid and 0.15 M NaCl; pH 7.5)
- 10x blocking solution

  4 gm blocking powder (Cat. No. 11096176001, Roche Applied Science) was mixed with maleic acid buffer. The volume of the solution was made up to 40.0 ml and autoclaved. The solution looked turbid.
- 20% SDS

- 20X SSC (3 M NaCl and 0.3 M sodium citrate)
- N-laurylsarcosine sodium salt

Prehybridization solution

0.02 % SDS

5x SSC

1x blocker (DIG DNA labeling Kit, Roche)

0.1 % N-laurylsarcosine sodium salt

Procedure

In the hybridization bottle, 10.0 ml of pre-hybridization solution was poured and the membrane was placed inside. The hybridization bottle was then kept in a hybridization chamber at 65°C in a rotary motion.

2.3.14.4.2 Hybridization

Preparation of probe for hybridization

Denatured probe was added to the pre-hybridization solution to facilitate the binding to the complementary sequence fixed on the membrane. For this purpose the probe was kept in 98°C heating block for 12 min. It was immediately transferred and maintained on ice until use.

Calculation of hybridization temperature

The following equation was used to calculate the optimum hybridization Temperature:

$Tm = 49.82 + 0.41\ (\%\ G+C) - 600/L$

Where,

(% G + C) = % of G and C residues in probe sequence

L = length of sequence in base pairs

Procedure

30.0 µL of labeled probe was added to the hybridization bottle in the pre-hybridization solution and temperature was set to 65°C. This hybridization process was carried out for 20 hours.

2.3.14.5 Washing

Finally, the unbound probe was washed from the membrane with a salt-detergent mix.

Materials

- Stringency solution I (2X SSC and 0.1% SDS)
- Stringency solution II (0.5X SSC and 0.1% SDS)

Procedure

- The membrane was washed with stringency solution I twice, each time for 5 min at room temperature.
- Then the membrane was washed with stringency solution II, twice, each time for 15 min at 65°C.

2.3.14.6 Detection

Materials

- Washing buffer (0.1 M maleic acid, 0.15 M NaCl; pH 7.5 and 0.3% Tween 20)
- Antibody solution (1X blocking solution-15.0 ml and Antibody 3.0 µl)
- Detection buffer (0.1 M Tris-Cl and 0.1 M NaCl, pH 9.5)
- Color substrate solution (detection buffer 9.8 ml and colour substrate 0.2 ml)

Procedure

- The membrane was rinsed with washing buffer for 2 min at room temperature with gentle shaking.
- The membrane was incubated in 80.0 ml of 1X blocking solution for 30 min at room temperature with gentle shaking.

- The membrane was then washed with 15 ml antibody solution for 30 min at room temperature.
- This was followed by two washes with washing buffer for 15 min at room temperature.
- The membrane was then equilibrated with detection buffer for 3 min.
- The membrane was incubated with freshly prepared color substrate solution in the dark without shaking.
- As the bands appeared on the membrane, the reaction was stopped by washing the membrane for 5 min with sterile distilled water or with TE buffer.

2.3.15 Isolation of Total RNA

To study the expression of GUS gene in transformed jute plants, total RNA was isolated from both the transformed and non-transformed seedlings (4 day old) according to the modified procedure from Chomczynski and Sacchi (1987). Details of the protocol are described in section 3.3.5.

2.3.16 Reverse-Transcription polymerase chain reaction (RT-PCR)

2.3.16.1 First Strand Synthesis

First strand cDNA was synthesized from isolated total RNA by using Gus reverse primer following the manufacturer's protocol for SuperScript TM III Reverse Transcriptase (Invitrogen, USA). A detailed procedure is described in section 3.3.10.

2.3.16.2 PCR using first strand cDNA

PCR amplification of the cDNA was carried out for 35 cycles using Gus forward and Gus reverse primers following the aforementioned PCR conditions (section 2.3.14).

2.3.17 Precaution to ensure aseptic condition

Aseptic conditions were maintained by using laminar airflow cabinet. To avoid contamination, all kinds of glassware, water, forceps, knife handle, conical flasks etc. were autoclaved at 121°C for 20 min.

2.4 Results

2.4.1 Culture of the *Agrobacterium tumefaciens* strain LBA4404

Growth rate of *Agrobacterium tumefaciens* strain LBA4404 harbouring the binary vector pBI121 was slower in both Yeast Mannitol broth and solid medium. It took at least two days for visible colonies to appear on the solid medium and about 15 hours for sufficient growth (with Optical density > 0.6 at 600 nm wavelength) in liquid medium.

Presence of adequate air during the growth of *A. tumefaciens* in liquid medium was ensured. At least two-third volume of air in a 10 ml screw cap tube was necessary for sufficient growth of *Agrobacterium*. Inadequate air caused clumping of bacterial cells and ceased growth. Rotation of shaker at 200 rpm or more allowed better growth of *Agrobacterium* cells.

2.4.2 Optimal optical density

To determine the most suitable optical density (OD) for *Agrobacterium* suspension used in this experiment, eight different concentrations (0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0) were tested. The optimal OD was determined by using GUS assay. This was done by observing the number of plants that exhibited sporadic blue colored spots on the leaves (newly developed leaves above the infection site) for that particular OD measurement. The percentage of plants showing GUS activity at this stage is given in Figure 2.4.1. The highest number of GUS expressing plants was observed at OD 0.6-0.8 and it decreased with an increase in optical density.

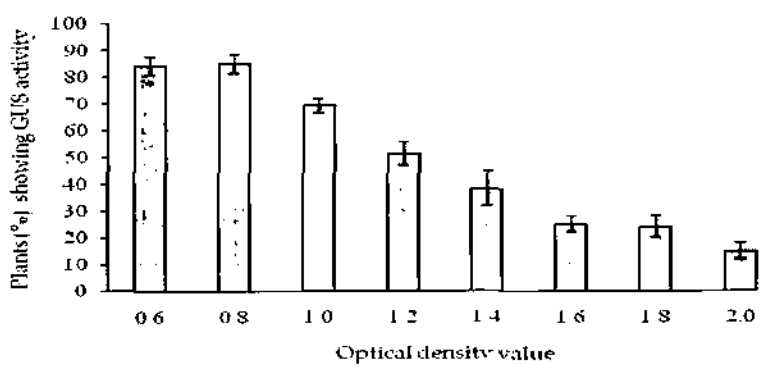


Figure 2.4.1: Optimal optical density of LBA4404 (pBI121) *Agrobacterium* engineered strains as measured by the percentage of plants showing GUS activity in T_0 generation. Vertical bars represent standard error of the means. Optical density (OD) was measured at 600nm.

2.4.3 Indirect transformation of jute shoot tips

Five sets of inoculation experiments were conducted on jute shoot tips with *Agrobacterium tumefaciens* strain LBA4404. A total of ~120 plants were infected with *Agrobacterium* suspension in each experiment. All the wounded plants (T_0) survived and showed normal growth upon infection with *A. tumefaciens* (Figure 2.4.2a). However, at the injection sites the stems became little thicker than the surrounding regions (Figure 2.4.2b) which may be due to deposition of wound responsive molecules. Some of the plants also showed branching at the infection site. These plants were allowed to grow under normal condition subsequent seed development.

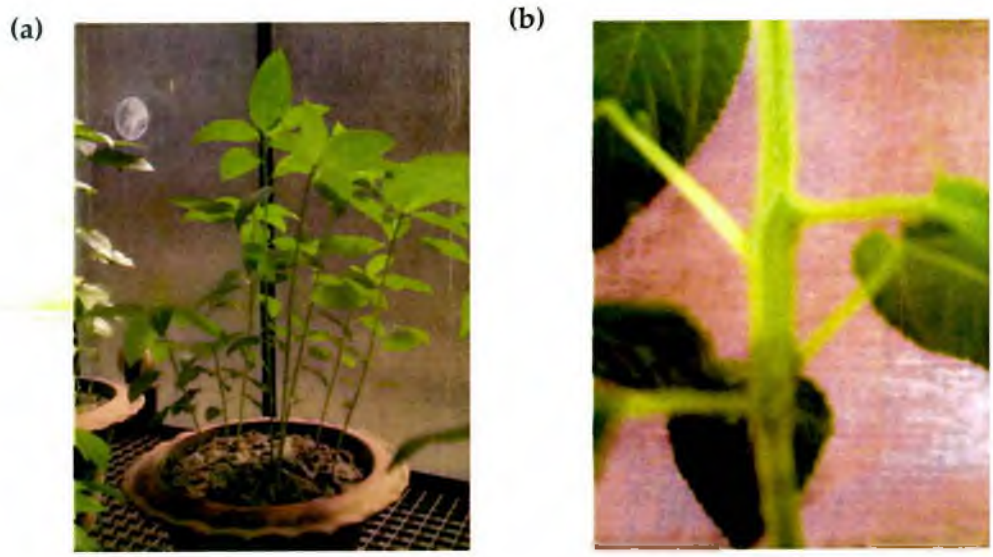


Figure 2.4.2: (a) Jute plants after two weeks of infection with *A. tumefaciens* at the shoot tips. (b) Increased thickness at the site of wounding after two weeks of infection with *A. tumefaciens*.

2.4.4 Integration and expression of the transgene in T_0 plants

Infection of the already differentiated somatic tissues with *Agrobacterium* may result in gene integration. However, the feasibility of gene integration in some tissues by *in planta* transformation protocol can be ascertained based on the GUS histochemical assay.

Newly developed leaves above the infection sites were collected after three weeks of infection. Assaying for GUS expression using leaves showed sporadic GUS activity (Figure 2.4.3a) whereas GUS activity was not seen in the non-transformed controls.

The leaves were examined under light microscope (Olympus CH20BIMF200) and photographed using a microscope compatible digital camera (Nikon E995). Intensity of blue colour (due to GUS activity) was in some cases comparable, to the positive transgenic control tobacco plants (Figure 2.4.3b). Plants with positive GUS expression were chosen for further analysis in the subsequent T_1 generation.

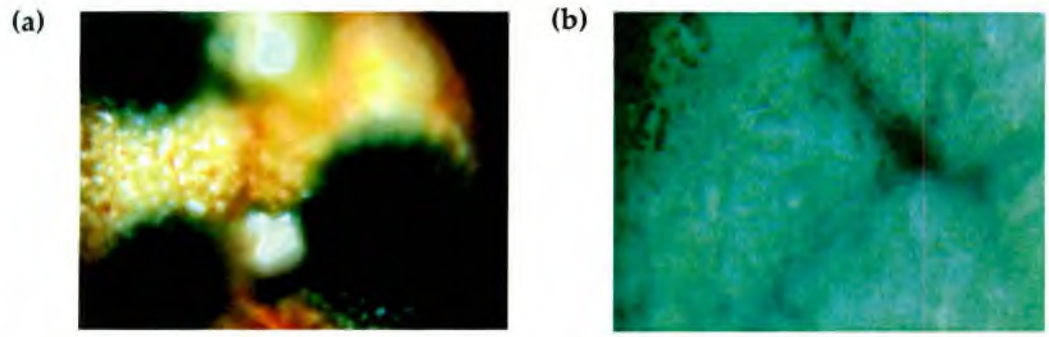


Figure 2.4.3: (a) Sporadic GUS activity observed under microscope (400x) in the new jute leaves above the infection site after three weeks infection. (b) GUS activity observed under microscope (400x) in the leaves of transgenic Tobacco plants observed under microscope.

2.4.5 Selection of T_1 transgenic plants

Transformants were expected to be resistant to kanamycin. T_1 jute seeds were grown on MS medium containing kanamycin (0.1 mg/ml) as a selective agent for transgenic plants. For comparison seeds from non-transgenic control plants from the same species were grown on the same medium.

Figure 2.4.4: Selection of transformants (T_1 generation) on kanamycin selection medium. Conical flasks 1, 3 and 4 contain transformed seeds and 2 contain non-transformed control seeds.

After 10 days, all non-transgenic control seedlings despite germinationbut failed to sustain growth. Whereas transformed seeds were germinated and continued to grow photo-autotrophically (Figure 2.4.4). These plants were transferred to soil for normal growth and seed development for next generation.

2.4.6 Efficiency of the transformation protocol

Efficiency of transformation process was determined following selection on kanamycin containing medium. Only putative transformants grew well on the selective medium containing kanamycin. Non-transformed seeds were germinated initially but bleached rapidly and died. Efficiency varied between 13.11% and 48% for different transformation events with an average of 27.23 ± 7.01 (Table 2.4.1). However, for both transgenic and non-transgenic plants similar growth rate were observed in non-selection medium attesting to the viability of the seeds tested.

Table 2.4.1: Transformation efficiency based on selection on medium containing kanamycin

Replication	No. of seeds that germinated and had subsequent growth	No. of seeds that failed to germinate	Efficiency (%)
R_1	35	154	18.55
R_2	8	53	13.11
R_3	19	96	16.52
R_4	48	52	48.00
R_5	32	48	40.00
Mean ± SE			27.01 ± 7.01

2.4.7 Integration and expression of the transgene in T_1 plants

Stable T_1 transformants were confirmed by two lines of evidences –

a) GUS assay

b) PCR analysis

2.4.7.1 GUS assay

Leaves from T_1 generation plants were tested for GUS activity. The entire leaves showed GUS activity (Figure 2.4.5b) in comparison to the sporadic activity in the

leaves of T_0 plants (Figure 2.4.3a). A portion of a leaf is shown in the figure as the whole leaf could not be photographed under microscope. Figure 2.4.5a shows a portion of a leaf from non-transformed plant of the same species which was used as a negative control in the experiment.

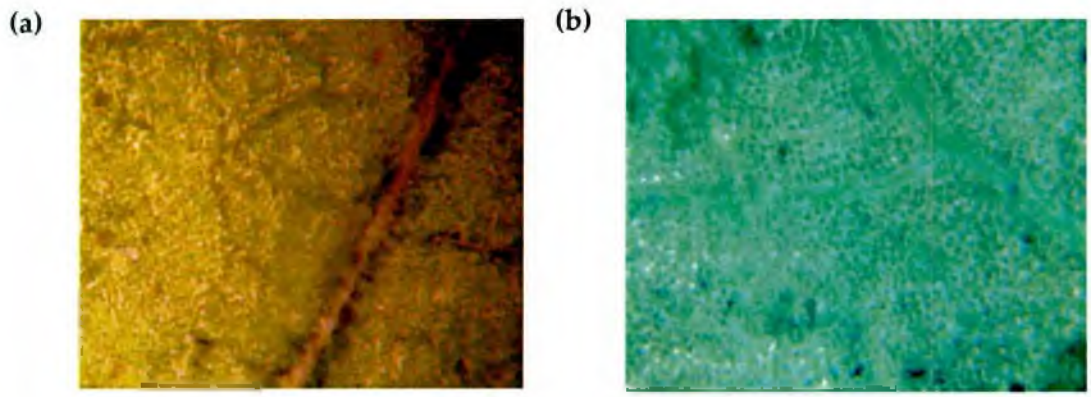


Figure 2.4.5: (a) No GUS activity observed under microscope (400x) in leaves of non-transgenic jute plants. (b) Expression of GUS activity observed under microscope (400x) in whole leaves of T_1 transgenic jute plants.

2.4.7.2 PCR analysis

DNA was isolated from both transgenic plants (T_1) and non-transgenic plants. PCR amplification of GUS gene sequence from transgenic plants using GUS specific primers were performed. Specific size band (511 bps) was amplified only from transgenic plants. But no band was amplified from non-transformed negative control plants of same species (Figure 2.4.6). These progeny plants were phenotypically normal and set flowers and seeds in a normal fashion.

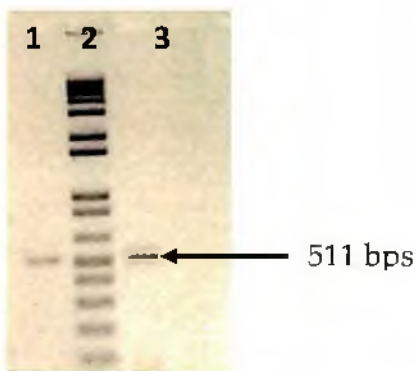


Figure 2.4.6: Gel electrophoresis of the amplified GUS gene sequence. Lane 1: Transformed jute; lane 2: 1 kb+ ladder; Lane 3: pBI121 plasmid containing GUS gene, used as positive control; Lane 4: Non-transformed control plant.

2.4.8 Stability of the transgene in T_2 plants

Seeds from selected plants (T_1) were sown in pots in the greenhouse for further analysis. Presence of transgene in T_2 generation was confirmed by three lines of evidences –

a) At the genomic DNA level (PCR and Southern blotting)

b) At the transcript level and

c) At the phenotypic expression

2.4.8.1 At the genomic DNA level

DNA was isolated from both transgenic seedlings (T_2) and non-transgenic seedlings. PCR was performed with primers specific for the GUS gene. The expected size of the amplified (511 bps) was detected upon gel electrophoresis from transgenic seedlings (T_2) (Figure 2.4.7). No band was amplified from DNA of non-transformed jute plants of the same species. Same size amplified product was detected from *Agrobacterium* plasmid DNA which was used as a positive control.

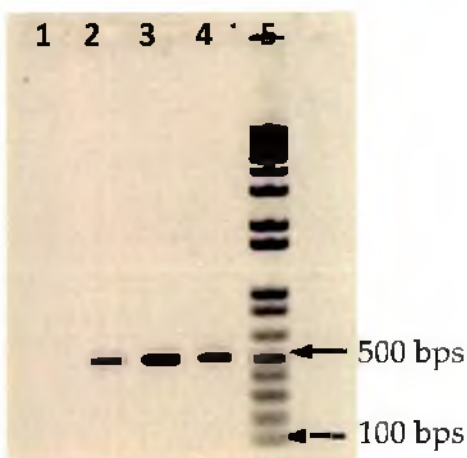


Figure 2.4.7: Gel electrophoresis of the amplified GUS gene sequence. Lane 1: non-transformed jute; lane 2: pBI121 plasmid containing GUS gene, used as positive control; lane 3 & 4: T_2 generation seedlings from transgenic plants; lane 5: 1 kb+ ladder.

Further confirmation of the integration and inheritance of the transgene in the progeny plants was obtained by Southern blot analysis. Results of Southern blot

analysis of T_2 generation plants for the presence of GUS gene are shown in Figure 2.4.8. As mentioned in Section 2.3.14, 511 bps GUS gene specific PCR product from plasmid pBI121 was used as probe. DNA from non-transformed jute plants was used as a negative control, and PCR amplified DNA from plasmid pBI121 was used as a positive control.

Southern hybridization with GUS specific probe gave specific signals for EcoRI digested genomic DNA from different transformed (T_2) plants, pBI121 and GUS specific PCR product from transformed (T_2) jute plants, whereas non-transformed plants did not give any signal (Figure 2.4.8).

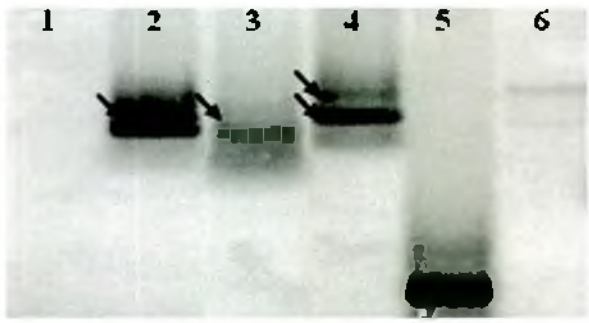


Figure 2.4.8: Southern hybridization of transformants (T_2 generation) by LBA4404 strain harboring pBI121 binary vector. Lanes 2-4 different transformants; Lane 1 non-transformed jute plants and Lane 5-6 used as positive control. Lane 5 contain PCR product and Lane 6 contain intact plasmid (pBI121) containing GUS gene.

2.4.8.2 At the Transcript level

Total RNA from T_2 seedlings was isolated and Reverse Transcription PCR (RT-PCR) was performed with GUS specific primers. Specific band for GUS gene was observed only in transformed seedlings, but not in non-transformed seedlings. To confirm that this amplification did not occur due to DNA contamination of the RNA samples, simple PCR was performed using the RNA with GUS specific primers. However, no sequence was amplified, even from the RNA sample of transgenic seedlings (Figure 2.4.9).

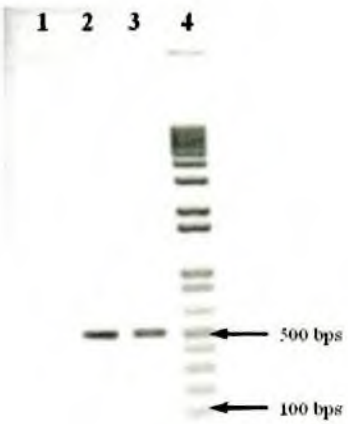


Figure 2.4.9: Gel electrophoresis of the RT-PCR product specific for GUS gene sequence. Lane 1: non-transformed jute; lane 2: and 3: T_2 generation seedlings from transgenic plants. Lane 4: 1 kb+ ladder.

2.4.8.3 At the phenotypic expression

Seeds from the second generation transgenic plants were germinated and seedlings (T_2) were tested for GUS gene activity (Figure 2.4.10). All transgenic T_2 seedlings expressed GUS gene activity (Figure 2.4.10b, c, d) whereas no expression was observed in non-transgenic jute plants(Figure 2.4.10a). The transgenic seedlings had similar expression pattern of GUS activity, with more intense blue deposition in leaves and roots and a lighter blue colour in the stem region (Figure 2.4.10b, c, d).

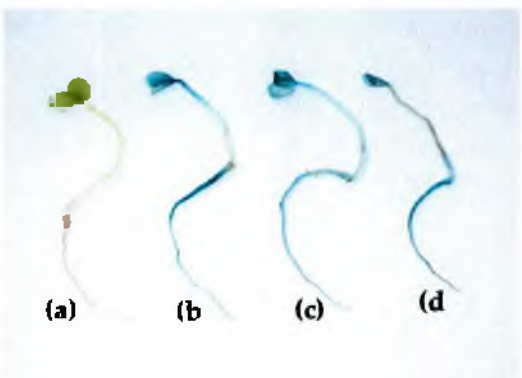


Figure 2.4.10: Expression of GUS activity in T_2 seedlings. (a) The first seedling in the left is non-transgenic control plant. (b, c, d) The other three are transgenic seedlings.

All the above three lines of evidences unambiguously confirmed the presence and inheritance of transgene up to T_2 generation.

2.5 Discussion

The genetic transformation of crop plants has become an essential tool for crop improvement and a vital part of the development of functional genomics resources (Bartlett *et al.*, 2008). The principal goal of this study was to establish an easy and efficient transformation protocol for jute (*Corchorus olitorius* L.). It was noted that whole plant regeneration following tissue culture and transformation was not successful with jute due to its recalcitrance in tissue culture. Therefore, under this study tissue culture independent technique was used for establishing a transformation protocol for jute.

Primary growth of a plant occurs from the activities of apical meristems (Figure 2.5.1) and subsequent division and differentiation of the derivative cells into the tissues and organs of the plant. The principal behind the current study was that if some of the dividing cells in the meristematic region could be transformed by *Agrobacterium*, then this transgene(s) would be transferred to subsequent cells, and if some of these cells later differentiate to form floral buds, seeds generated from these buds would inherit the transgene(s) to next generation.

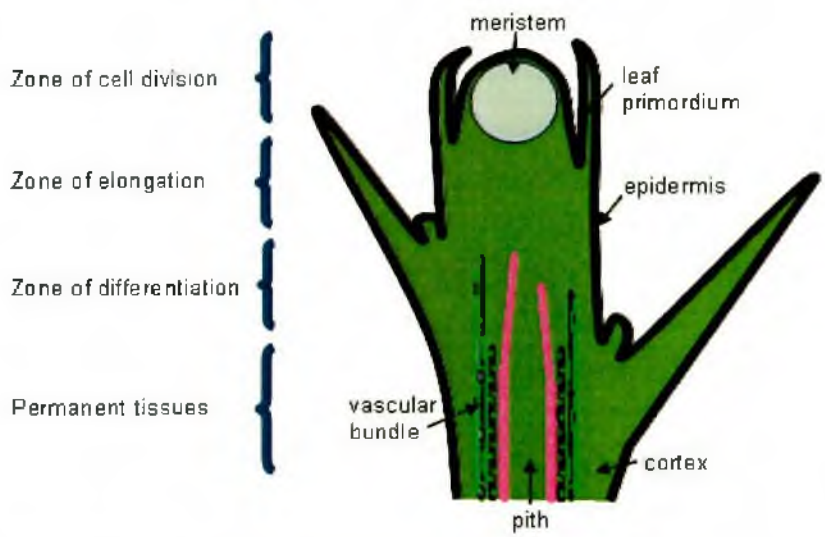


Figure 2.5.1: Various tissues and zones of a plant shoot tip.

In this transformation experiment with *C. olitorius*, the apical meristematic regions of young jute plants were infected with *Agrobacterium tumefaciens* harbouring pBI121. Some of the seeds collected from these plants showed stable transformation and

inheritance of the GUS gene. Most of the seeds (T_2) obtained from T_1 generation transgenic plants were also transgenic.

As infection was carried out on young plants, not all the cells could be transformed, even in the meristematic regions. Therefore, the whole new set of cells above the infection site was not supposed to carry the transgene(s). This theoretical consideration was in harmony with the observation of sporadic activity β-glucuronidase (GUS) activity seen in T_0 generation. However, as traits are transmitted to next generation through germ cells, the whole plants were supposed to show GUS activity. In fact, GUS gene activity in next generation (T_1) plants was evident in whole leaves. Similar whole plant expression of GUS activity was seen in T_2 generation (Figure 2.4.10).

GUS expression in T_0 generation correlated negatively with optical density of *Agrobacterium* suspension. The highest number of GUS expressing plants was observed at OD 0.6-0.8. Using *Agrobacterium* at OD of 0.6-0.8 was coincidental with the fact that most *Agrobacterium* strains would be experiencing the early log (exponential) phase during which active cell division occurs at 0.4-0.8 OD (Janssen and Gardner, 1989). All OD readings were measured at an absorbance of 600 nm.

Five different lines of evidence attest to the success of the transformation process: (i) histochemical GUS assay was performed in order to assess GUS activity in transgenic plants, (ii) confirming the presence of GUS gene in the transgenic plants, PCR with GUS gene specific primers was performed, (iii) ascertaining the presence of GUS transcript in the GUS positive plants, GUS gene specific RT-PCR was conducted, (iv) GUS specific Southern hybridization, and (v) selection of transgenic plants were also selected on kanamycin containing medium.

There is a disadvantage in using pBI121 as the reporter and the selectable marker genes, GUS and *npt II*, were taken from *E. coli*. It would have been easier to determine false positive results, if the vector sequence contained marker genes from more than one organism. However, pBI121 is a popular binary vector and has recently been used by researchers for several plants including peanut (Chen *et al.*, 2010; Rohini and Rao, 2001; Sharma and Anjaiah, 2000), shallot (Zheng *et al.*, 2001), spruce (Le *et al.*, 2001), buckwheat (Kojima *et al.*, 2000), poppy (Park and Facchini,

2000) and pine (Tang, 2001). pBI121 has also been used for *in planta* transformation of cactus (Razzaq *et al.*, 2011; Jung *et al.*, 2009; Seol *et al.*, 2008).

β-Glucuronidase (GUS) activity is found widely in microorganisms, vertebrates and invertebrates but there is very little background activity, if any, in plants. So, the blue colour deposition in transgenic plant leaves and seedlings was possibly due to the activity of introduced GUS gene. Though, false positive GUS activity could show up due to *Escherichia coli* contamination, there was a high confidence to rule this possibility out as all such assays were performed inside laminar flow hood under sterile conditions, all the tissues were disinfected before assay, a huge number of negative controls were used and none of these negative controls showed any colour which would be definitive of GUS activity. Some of the GUS positive leaves showed diffusion of blue colour from leaves in the solution during GUS assay, although the positive control plants (transgenic tobacco) had relatively little diffusion of the same in the solution. In an experiment in this lab (data not shown), jute leaves were found to release more carbohydrates and proteins into solution than tobacco leaves which might account for the greater loss of the blue colour.

To ascertain the expression of GUS genes in transgenic T_2 seedlings, total RNA was isolated and RT-PCR was performed using primers specific for GUS transcripts. Expected size fragment was visible upon gel electrophoresis. To exclude the chance of amplification from contaminating DNA in the RNA sample, PCR was performed directly with the same sample of RNA without reverse transcription. However, no amplification of sequence was visible. None of the non-transgenic plants of the same species, used as negative control, gave amplified products in RT-PCR using the same primers.

To further confirm the transformation event, PCR was performed with DNA isolated from both T_1 and T_2 plants using GUS specific primers. To increase the specificity of PCR amplification long primers (22 bps) with high annealing temperatures (60°C) were designed. Primers were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/) and crosschecked by another (GeneFisher2: http://bibiserv.techfak.uni-bielefeld.de/genefisher2/). The primers were also searched for homology to other sequences using BLAST to avoid getting multiple spurious

bands during PCR amplification. Using these primers the amplified products from transgenic plants gave a single and expected size band of 511 bps, identical to the positive control (pBI121 plasmid containing GUS gene). DNA from non-transgenic plants of the same species did not produce any band (Figure 2.4.7). This strengthened the fact that the plants that tested positive for transformation in the previous two assays were true transformations.

Southern hybridization with GUS specific probe confirmed further the success of the transformation process (Figure 2.4.8). None of the transformed plants showed any phenotypic change except for thickening at the infection sites in T_0 plants. This might possibly be the result of plant defense responses. Plants produce signal molecules that induce resistance responses due to wounding or pathogen attack. Induced defense responses include reinforcement of cell wall synthesis among others (Mello-Farias and Chaves, 2008).

Selection on kanamycin containing medium also provided proof for transformation as only seeds from transformed plants germinated and showed growth while no germination was observed with seeds of non-transgenic plants. But seeds of non-transgenic plants had normal growth in non-selection medium. This eliminated doubts about the vigour of non-transgenic seeds.

Efficiency of transformation process at an average of 27.23 ± 7.01 was much lower than those obtained with other *in planta* transformation studies obtained with *Notocactus scopa* cv. Soonjung (Seol *et al.*, 2008), *Triticum aestivum* L. (Supartana *et al.*, 2006), *Hibiscus sabdariffa* (Gassama-Dia *et al.*, 2004) and *Oryza sativa* L. (Supartana *et al.*, 2005), which had maximum transformation efficiencies of upto 100%, 75%, 68% and 43%, respectively. However, the efficiency obtained in the current study is sufficiently high for practical purposes.

The simple and tissue culture independent transformation protocol for *C. olitorius* described here opens up new possibilities for the improvement of jute varieties through the introduction of new traits through *Agrobacterium*-mediated transgene expression.

CHAPTER THREE

IDENTIFICATION OF IMPORTANT GENES FROM DIFFERENT JUTE SPECIES UNDER STRESS CONDITION

3.1 Introduction

Plants live in a complex environment where many biotic and abiotic factors limit or promote productivity (Boyer, 1982; US DOE, 2008). Plants frequently face challenges from pathogens including bacteria, fungi, and viruses as well as from herbivores (Mahajan and Tuteja, 2005). Plants also face challenges from abiotic stress conditions such as extreme temperatures, salt; drought, flooding, heat, oxidative stress and heavy metal toxicity (Mahajan and Tuteja, 2005). All these stress factors pose challenges for plants, preventing them from reaching their full genetic potential and limit crop productivity worldwide (Tuteja *et al.*, 2009). One of the challenges confronting scientists worldwide is the development of new and sustainable ways of protecting crops from pests and diseases. Biotic stress caused by plant pathogens, insect pests and weeds, accounts for some 30-40% of crop loss worldwide (Tennant *et al.*, 2010). Annual loss for plant pathogens alone is estimated to be 12% (Cook, 2006).

Every year jute is infested by the pathogens and embrace loss to the tune of 10-20% (Mahapatra and Saha, 2008). There are as many as 14 diseases of jute caused by 6 fungal pathogens. The major diseases, which reduce the yield of jute, as well as fibre quality are stem rot, root rot, collar rot and seedling blight. All these 4 diseases are caused by *Macrophomina phaseolina* (Mahapatra and Saha, 2008). Average loss of yield due to this pathogen is around 10 percent. (Rao, 1980; Ghose, 1983; Mandal, 1990). Late infection causes transmission of the diseases through contaminated seeds. Infected seed is a more important source of primary inoculum than infection through soil (Richardson, 1990).

The organism is also known to cause root and stem diseases of many economic crops, notably charcoal rot of soybean, corn, sorghum and root rot of cotton. It infects the root and lower stem of over 500 plant species and has a wide geographic distribution (Wyllie, 1988; Das *et al.*, 2008).

Pathogen attack initiates a cascade of signal transduction pathways so that a systemic or induced resistance begins in tissues remote to the initial infection (Öktem *et al.*, 2008). Analysis of transcriptional changes that occur during systemic defense responses in *Arabidopsis* plants elicited by fungal pathogen *Arabidopsis brassicicola* revealed the presence of 25 up-regulated and 10 down-regulated genes in distal tissues of the infected plants (Schenk *et al.*, 2003). Recent findings suggest that individual pathogens induce different defense responses in the host and this may reflect a differential response to individual virulence mechanisms employed by pathogens (Veronese *et al.*, 2003). Defense reactions can also change in function with the host's age and developmental stage (Neale *et al.*, 1990; Gaudet *et al.*, 2003). Based on this, plants can potentially generate a myriad of individualized defense responses (Hahlbrock *et al.*, 2003). Identifying the common and most efficacious plant defense response(s) effective against individual pathogens or groups of pathogens will provide a framework for genetically engineering new disease resistant varieties.

Identification of differentially expressed genes in various cells or under different conditions is one of the main areas of molecular biology. Patterns of gene expression can be compared using differential hybridization screening (Tedder *et al.*, 1988), representational difference analysis (Lisitsyn *et al.*, 1993), serial analysis of gene expression (Velculescu *et al.*, 1995), differential display (Liang and Pardee, 1992), subtractive library construction (Hedrick *et al.*, 1984) and DNA microarray (Schena *et al.*, 1995) etc.

To identify genes that are differentially expressed during fungus infection, we have used the technique of differential display and employed differential screening techniques. Differential display (DD) is a genetic approach that allows the analysis of differentially expressed genes in eukaryotes (Liang and Pardee, 1992; Wan *et al.*, 1996). The method is based on comparing the RT-PCR products of mRNA isolated from two tissue sources in which differences in gene expression are expected. This technique has been used successfully to identify developmentally, environmentally, or hormonally regulated genes in protozoa, plants, animals, and humans (Abrahamsen *et al.*, 1995; Joseph *et al.*, 1994; Sharma and Davis, 1995; Utans *et al.*,

1994; Van der Knaap and Kende, 1995.). In this procedure, two or more sets of differentially expressed mRNAs to be compared are used as templates to generate the corresponding cDNAs. Subsequently, the cDNA fragments are amplified by means of PCR using a combination of an mRNA-specific oligo(dT) "anchor" primer together with an arbitrary primer in the presence of radioactively labeled deoxynucleoside triphosphates (dNTPs). After separation by denaturing polyacrylamide gel electrophoresis, differentially amplified cDNAs are identified by autoradiography. The differentially expressed bands can readily be cloned and used as probes in Northern or Southern (DNA) blots and can also be used to isolate genes from cDNA or genomic libraries.

For introducing resistance to fungus in widely cultivated jute varieties, it turns out that a wild species of jute could be the best source organism of the gene(s) of interest. Wild jute species *C. trilocularis* shows resistance to *Macrophomina phaseolina* while *C. olitorius* var. O-72, a cultivated variety, is susceptible to the same fungus (Biswas *et al.,* 1968). The goal of this study was to identify gene(s) differentially expressed between these two species under both stress and non-stress conditions and at different time intervals upon infection. The results of these approaches led to the identification of a gene that shows specific expression patterns during fungus infection.

3.2 REVIEW OF LITERATURE

Differential gene expression is generally reflected by the different mRNA species expressed in a given cell at any time point. Changes in relative mRNA levels may also have important implications in the development of pathological processes (Wang and Feuerstein, 1999). Therefore, it is essential to identify and characterize the genes that are differentially expressed under diseased conditions in order to understand the molecular nature of diseased and normal state (Wang *et al.*, 1996).

3.2.1 Methods of gene identification

A large number of techniques have been used in the last decade to study gene expression for the improvement of plants (Wilkinson *et al.*, 1995). The most widely used methods for gene identification and isolation often rely on examining differential gene expression in experiments under different growth conditions. Patterns of gene expression can be compared using differential hybridization screening (Tedder *et al.*, 1988), representational difference analysis (RDA) (Lisitsyn *et al.*, 1993), serial analysis of gene expression (SAGE) (Velculescu *et al.*, 1995), differential display (DD) (Liang and Pardee, 1992), and subtractive library construction (Hedrick *et al.*, 1984). Such techniques are tedious, labor intensive, and time consuming. Some require a large amount of mRNA (cDNA) or are biased for high abundance mRNA. However among these techniques the differential display has advantage over other techniques as it does not require large amount of RNA for analysis (Willekens *et al.*, 1995).

3.2.2 Application of differential display for identification and isolation of differentially expressed genes

Compared to the subtractive hybridization method, Differential Display Reverse-Transcriptase PCR (DDRT-PCR) is simpler, quicker and more sensitive. However, false-positive results can generate a large number of spurious sequences that do not represent differentially expressed genes. A number of technical modifications have been introduced to reduce the problem of false positives and to increase the

reproducibility of the technique (Liang and Pardee, 1995; Huang *et al.*, 1996; Jones *et al.*, 1997; Averboukh *et al.*, 1996). Modifications that allowed the display of longer cDNAs have also been reported (Averboukh *et al.*, 1996). Therefore, with properly designed primers and controls, DDRT-PCR could produce results that truly reflect gene expression patterns of different tissues.

A number of researchers have isolated and characterized various plant genes involved in physiological events, stress responses, signal transduction and secondary metabolism using differential display. Some of the isolated genes encode transcription factors, membrane proteins and rare enzymes that were previously difficult to purify (Yamazaki and Saito, 2002). These results suggest that differential display is a powerful tool used to investigate rare genes involved in a plant's life cycle without using information from proteins.

3.2.3 Isolation of genes involved in biotic stress response

Application of differential display to identify biotic stress response genes in plants is summarized in Table 3.2.1. A number of defense genes which are expressed in response to attacks by pathogens (Truesdell and Dickman, 1997; Collinge and Boller, 2001; Yi and Hwang, 1998; Seehaus and Tenhaken, 1998; Jagoueix-Eveillard *et al.*, 2001; Mazeyrat *et al.*, 1998; Timmusk and Wagner, 1999; Chen and Chen, 2000; Vercauteren *et al.*, 2001; Hermsmeier *et al.*, 2000; Mahalingam *et al.*, 1999) and herbivores (Schittko *et al.*, 2001; Hermsmeier *et al.*, 2001) have been obtained so far. In plant-microbe interactions such as symbiosis, induced genes have been isolated and characterized (Neumann and Werner, 2000; Szczyglowski *et al.*, 1997; Goormachtig *et al.*, 1995; Niebel *et al.*, 1998; Heidstra *et al.*, 1997; Martin-Laurent *et al.*, 1997; Lapopin *et al.*, 1999). Information about the functions of these genes are expected to provide agriculturally valuable leads.

Table 3.2.1. Application of differential display technique to identify biotic stress response genes in plants.

Stress treatments	Plant species	Obtained gene or encoded protein	References
Colletotrichum trifolii	*Medicago sativa*	defense-related protein, tree pollen allergen homologue	Truesdell and Dickman (1997)
Phytophthora infestans	*Solanum tuberosum*	putative peroxidase, NAC domain protein, pathogen-induced genes	Collinge and Boller (2001)
Phytophthora sojae	*Glycine max*	*basic peroxidase*	*Yi and Hwang* (1998)
Pseudomonus syringae		chalcone isomerase, ubiquitin, signaling molecule, G-6-P dehydrogenase, leucin-rich protein	Seehaus and Tenhaken (1998)
mollicutes	*Catharanthus roseus*	genes involved in photosynthesis, sugar transport, stress response, phytosterol synthesis	Jagoueix-Eveillard *et al.* (2001)
Plasmopara halstedii	*Helianthus annuus*	auxin-induced gene homologue	Mazeyrat *et al.* (1998)
Paenibacillus polymyxa	*A. thaliana*	drought-stress-responsive defense genes	
TMV	*N. tabacum* (resistant/susceptible lines)	WRKY transcription factor	Chen and Chen (2000)
Meloidogyne incognita (nematode)	*A. thaliana*	trypsine inhibitor, peroxidase, mitochondrial uncoupling protein, endomembrane protein, 20S proteasome *a*-subunit, diaminopimelate decarboxylase	Vercauteren *et al.* (2001)
Heterodera schanttii (cyst nematode)	*A. thaliana*	infection-responsive genes	Hermsmeier *et al.* (2000)
Heterodera glycines (cyst nematode)	*G. max* (resistant/susceptible plants)	Polygalacturonase	Mahalingam *et al.* (1999)
Herbivore attack Symbiosis	*Nicotiana attenuate*	insect-responsive genes	Schittko *et al.* (2001); Hermsmeier *et al.* (2001)
Sinorhizobium	*Medicago sativa* (xenobiotic treated)	copper transporter homologue, 60S ribosomal protein	Neumann and Werner (2000)
Rhizobium lori	*Lotus japonicas*	nodule specific protein phosphatase, peptide transporter, nodule-specific P450	Szczyglowski *et al.* (1997)
Azorhizobium cauiinodans	*Sesbania rostrata*	hydroxyprolin-rich cell wall protein, chitinase, chalcone reductase	Goormachtig *et al.* (1995)
Nod factor treatment	*Medicago truncatula*	Annexin	Niebel *et al.* (1998)
	Vicia sativa	Leghemoglobin	Heidstra *et al.* (1997)
Glomus mosseae	*Pisum sativum* (defective mutant)	proline-rich protein	Martin-Laurent *et al.* (1997); Lapopin *et al.* (1999)

3.2.4 Methodology of differential display

The method of mRNA differential display consists of two basic steps -

1. The mRNAs within the total RNA population are used as the templates for first strand cDNA synthesis by reverse transcription. The current methodology makes use of three "anchored" oligo-dT primers that target the poly-adenylation (poly-A) site of eukaryotic mRNA.
2. PCR amplification of cDNA fragments using arbitrary (upstream) primers and anchored (downstream) primers.

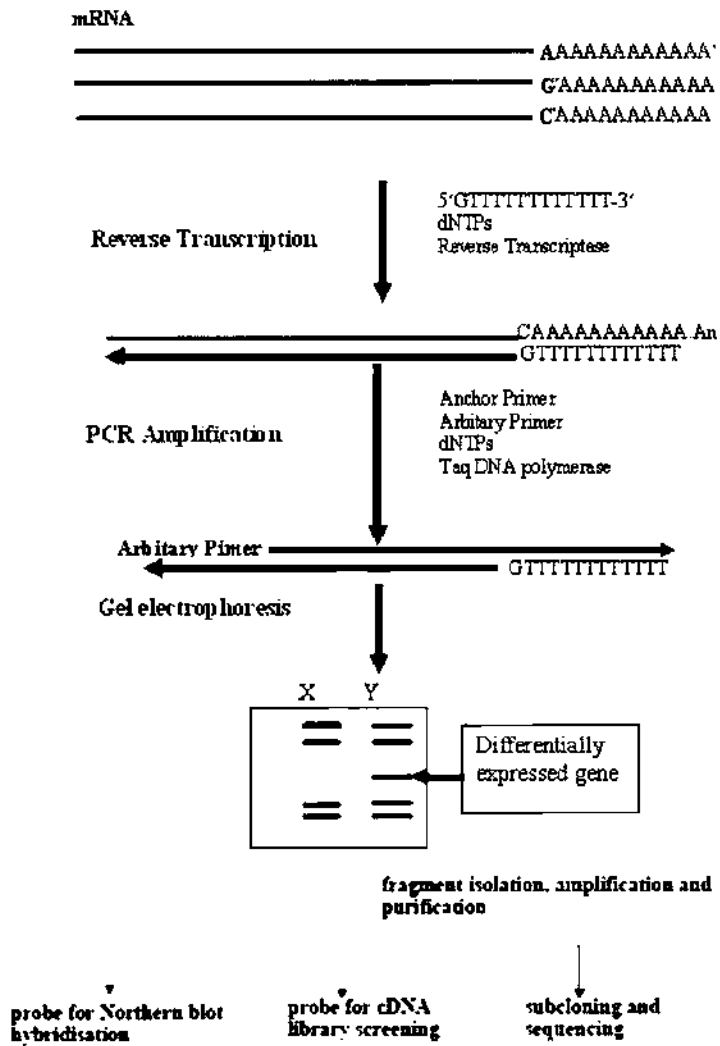


Figure 3.2.1: Schematic representation of mRNA differential display. One-base anchored oligo-dT primers are used in combination with arbitrary primer to reverse transcribe and amplify the mRNA.

Typically, reverse transcription (RT) reaction is divided into several subgroups, each using a different anchored primer. Because a large number of mRNA species are present in a cell, the divisions or subgroups for the RT allows a portion of the mRNA species to be displayed, which will increase the resolution of cDNA species after amplification (Wang *et al.*, 1996). The amplified cDNAs are separated following gel electrophoresis. A band of interest, such as one produced by one kind of cell versus another, can be excised from the gel, its cDNA is re-amplified by PCR, cloned and then sequenced. Its sequence can be directly used as a probe for cDNA library screening or northern blot hybridization, and ultimately be studied for function (Liang and Pardee, 1995) (Figure 3.2.1).

Polyacrylamide gels are the most commonly used method for identifying differentially expressed band because they offer the best band resolution and allow for easy and efficient recovery of genes (Liang *et al.*, 2007). Upon completion of the gene expression profiles by gel electrophoresis, cDNA bands of interest are excised from the gel and reamplified with the same primer combination as the original DD-PCR and under the same reaction conditions. Characterization of each potential gene includes sequencing of the cDNAs of interest from DD, with the results giving an indication of whether the cDNA is a known or unknown sequence after searching the GenBank database (Liang *et al.*, 2007).

3.2.5 Limitations of mRNA differential display

While the differential display technique has significant advantages, several difficulties may arise when the technique is used. The major concerns are the incidence of false positives, poor reproducibility, redundant gene identification and the labor intensive nature of this procedure for large scale screening (http:// profs.sci.univr.it /~delledon /Insegnamenti /Dispense% 20Genomi% 20-%202010.pdf; Pillutla *et al.*, 2002). Poor separation can mask differentially expressed genes of low abundance under the intense signals generated by highly expressed genes. Under these conditions false positives and redundancy pose a great problem. In addition, the cDNA fragments isolated by this method are often small and frequently located in the 3'-untranslated region. Therefore, in order to identify the differentially

expressed gene, one may need to screen a cDNA library to isolate the full length cDNA clone. Moreover, in order to observe the differentially expressed genes in the mRNA population, at least 20-25 (arbitrary primer) upstream primers (it can be more for much better discrimination) in combination with downstream anchored primers should be used (Wang *et al.*, 1996).

3.2.6 Primers for differential display

Anchor primers

The original method of differential display used a two base-anchored oligo-dT primer in combination with an arbitrary decamer to amplify the 3'-termini of mRNAs (Liang and Pardee, 1995). The initial choice of using two-base anchored oligo-(dT) primers instead of one-base anchored primers was due to historical rather than scientific reasons (Liang, 1998). Later, longer one-base anchored primers were shown to be much more efficient for differential display in subdividing the mRNA populations into three groups. One-base anchored primers have significant advantages over two-base anchored primers in that the former reduce the redundancy of priming, eliminate the high background smearing problem for two-base anchored primers ending with the T, and reduce the number of reverse transcription reactions from 12 to 3 per RNA sample (Liang, 1998).

The use of oligo-dT primers for a simplified RT step for cDNA synthesis, creates a serious problem during PCR by the generation of "smeared" PCR products as a result of non-anchored priming by the carried over primer (Liang and Pardee, 1995).

Arbitrary primers

The optimal length of arbitrary primers is determined by statistical consideration that each primer will recognize 50-100 mRNA species. To do so, these primers must hybridize 6-to 8-mers (Liang and Pardee, 1992). In practice, however, primers shorter than 9 bases failed to be used for PCR amplification (Williams *et al.*, 1991). Initially, arbitrary decamers (10-mers) were employed for differential display in combination with two-base anchored primers. Although the method worked well in displaying the expected number of mRNAs per primer under optimal conditions, weak signals,

fewer bands, and poorer reproducibility were encountered arising possibly from performing the experiments under suboptimal conditions due to the variation of the RNA samples (Liang *et al.*, 1994).

Although primers of 20 bases or longer have been used to display the mRNA in an attempt to increase the "reproducibility" of the cDNA pattern, longer primers are used to amplify specific genes and shorter primers are used to non-specifically amplify multiple genes. The longer primers under degenerate conditions hybridize in a non-predictable way, making it difficult to design rationally a representative set of primers (Liang, 1998).

3.2.7 Band separation and retrieval

The original method of differential display employed a denaturing polyacrylamide gel commonly used for DNA sequencing in order to obtain high resolution of a large number (50-100) of amplified cDNAs. On the other hand, non-denaturing polyacrylamide gel was reported to simplify the band complexity in the lower half of the gel (Bauer *et al.*, 1993). Recently, separation of PCR products on an agarose gel has been used for differential display (Kim *et al.*, 2004). DDRT-PCRs can be displayed in 1.6% agarose gels and stained with ethidium bromide. An approach such as this has the advantages of speed and ease, without having to use isotopes, allowing a few genes to be quickly obtained for analysis. However, given the low resolution of an agarose gel and low sensitivity of ethidium bromide staining, systematic identification of most, and especially rare, mRNAs is difficult with this method (Liang and Pardee, 1995).

3.2.8 Re-amplification, confirmation and cloning of differentially expressed genes

Considering the rate of false positives and redundancy, differential display seems much less attractive than thought originally. The downstream verification process is not only labor intensive, it also requires significant amount of RNA, which compromises one of the major advantages of the method. Many different approaches have been proposed to meet the necessity of large-scale screening of candidate cDNA fragments with low amounts of RNA, such as Reverse Northern blots with Southern blotted, slot or dot-blotted clones, eventually in combination with the use of

amplified RNA as a probe. Another important factor that adds to the post-display effort is intrinsically linked to the design of the technique. Because the cDNA fragments obtained from differential display are short (typically 100–500 bps) and correspond to the 3'-end of the gene that represents mainly the 3'- untranslated region, they usually do not contain a large portion of the coding region (Xu, 1999). Unless a model system is studied for which a significant amount of sequence information is available in public databases, labor-intensive full-length cDNA screening is needed before significant sequence homology, informative for gene classification and prediction of function, can be obtained (Lievens *et al.*, 2001).

Cloning of an amplified cDNA is often necessary for its sequence analysis and northern blot for unambiguously confirming its differential expression (Liang *et al.*, 1993). Several vectors are available for cloning cDNAs from differential display: TA cloning (Invitrogen), pCR-TRAP cloning (GenHunter) and One-way Cloning (Stratagene) systems are the most widely used (Liang and Pardee, 1995).

3.2.9 Fungal pathogenicity to plants

Approximately among the 100,000 known species of fungi, less than 10% are able to colonize plants and cause disease (Knogge, 1996). This is because plants have evolved sophisticated active defense mechanisms against potential pathogens (Van den Burg *et al.*, 2006). This defense mechanism can be the result of a number of distinct mechanisms working either together or separately. On the host side, these mechanisms include constitutive structural barriers (e.g. leaf cuticle) and chemical defenses (e.g. certain antifungal seed proteins and secondary metabolites) as well as inducible defense responses such as the so-called pathogenesis-related (PR) proteins, antibiotics known as phytoalexins, localized cell death (the hypersensitive response - HR) and structural alterations - e.g. lignification of the cell wall (Collinge and Slusarenko, 1987; Collinge *et al.*, 1993; Collinge *et al.*, 1994).

Some pathogenic fungi colonize aerial parts, others infect below the ground, while even more specialized fungi only infect specific organs in the plant. This organ specificity is well known but poorly understood (Schäfer, 1994). Many fungi enter the host through stomata or wounds and then may penetrate through cell walls once

they are established inside the host. Other fungi enter the host by penetrating directly without producing special infection structures. Some, like the tomato pathogen *Cladosporium fulvum* grow only inter-cellularly (Rivas and Thomas, 2005). Plant fungus interactions are generally described as susceptible if the fungus produces characteristic symptoms and reproduces or resistant if the plant can counter the infection to restrict symptom development and pathogen reproduction. Understanding mechanisms of fungal pathogenicity and the genes that control them may allow the use of recombinant DNA technology to modify the plant's defense system to induce resistance to attack (Jia *et al.*, 2010).

3.2.10 Molecular mechanism of plant defense

Many plants defend themselves against fungi and other microbial pathogens by inducing both localized and systemic resistant responses. The defense responses include suicide of the attacked host cell - a process that is called hypersensitive response (HR) (Morel and Dangl, 1997), phytoalexin formation (Bailey and Mansfield 1982), the production of other antimicrobial secondary metabolites and pathogenesis related proteins (PR-proteins) (Linthorst, 1991; Van Loon, 1985), many of which exert antimicrobial properties, and cross linking of cell wall proteins (Bradley *et al.*, 1992). The effect of the defense responses often determines whether plants are susceptible to infection by pathogens or not.

Plants respond to foreign microbes by identifying them as pathogens and this recognition is governed by the interaction of a resistant (R) plant gene product and a corresponding pathogen-derived avirulence (Avr) gene product. Subsequent to the recognition, signal transduction pathways are engaged in host cells. Both generation of reactive oxygen species (ROS) and changes in ion fluxes are observed during the early phases of many plant pathogen interactions (Baker and Orlandi, 1995; Atkinson and Baker, 1989). The consequences upon infection can be listed as the local accumulation of pathogenesis related (PR) proteins, the deposition of callose and lignin in the cell wall near the infection site and the rapid death of plant cells distinctively at the site of infection (Ergen *et al.*, 2007). Homologs of R genes from a number of different plant species have been isolated and characterized by PCR based

degenerate primers (Xue, 1998). The proteins encoded by R genes carry motifs that are found in receptor and signal transduction proteins, and can further be classified into four groups. The majority of these proteins contain a template nucleotide-binding site/leucine-rich repeat (NBS/LRR) structure resembling intracellular receptors. One other group of this family of proteins contains a common LRR receptor and a membrane anchor. The other two groups carry a serine/ threonine kinase signature with or without an obvious LRR domain. These different individual domains assemble to function as pathogen recognition and signal transduction features of R proteins to initiate the defense mechanism at the beginning of signaling pathways (Richter, 2000). Until now, several components of signaling cascades have been identified; however, the pathogen-associated molecular patterns are still largely unknown in plants (Cohn *et al.*, 2001). The understanding of the signaling pathways and pathogen-associated molecular patterns are critical for finding ways to develop disease resistance in plants for various types of pathogens (Ergen *et al.*, 2007). mRNA differential display is a powerful method for isolating different cDNAs that are specifically induced or repressed against the same triggering factor (Neslihan *et al.*, 2007).

3.2.11 Fungal diseases of Jute

Both the species of jute, *Corchorus capsularis* and *C. olitorius*, suffer from a number of diseases. Of these the most important is *stem rot* caused by *Macrophomina phasiolina* (Roy *et al.*, 2008). This fungus is seed borne. The pathogen may cause infection and damage at all stages of growth of the plants, right from seedling emergence to maturation. Other major seed borne fungal diseases caused by *Botryodiplodia theobromae* and *Colletotrichum corchori* are also frequently transmitted through jute seeds. In a study on the frequency of occurrence of pathogenic fungi in jute seeds, the predominant fungal pathogens found in order of prevalence were *M. phaseolina, C. corchori, B. theobromae, Fusarium semitectum, F. moniliforme, Curvularia lunata* and *Corynespora cassiicola*, respectively, with *M. phaseolina* constituting 31.59% of the total seed borne fungal infections (Islam, 2007).

3.2.12 Taxonomy of *Macrophomina phaseolina*

According to Wheeler (1975), taxonomic description of *Macrophomina phaseolina* is given below:

Division	Eumycota
Sub Division	Deuteromycotina
Class	Coelomycetes
Order	Sphaeropsidales
Family	Sphaeropsidaceae
Genus	*Macrophomina*
Species	*phaseolina*

M. phaseolina is a soil-borne plant pathogenic fungus. The pycnidial and sclerotial stage are primarily responsible for the disease and the perfect stage is very rarely seen (Ghosh, 1957; Mandal, 1990). The pycnidiospores are ellipsoid to ovoid. During the sclerotial formation, 50–200 individual hyphal cells aggregate to give multicellular bodies named as microsclerotia. The microsclerotia are black and are variable in size (50–150 μm).

3.2.13 Host range and distribution

M. phaseolina has a wide host range and is responsible for causing losses on more than 500 cultivated and wild plant species (Khan, 2007). Major cultivated hosts include: *Arachis hypogaea* (peanut), *Beta vulgarius, Brassica oleracea* (Cabbage), *Capsicum annuum* (pepper), *Cicer arietinum* (chick pea), *Citrus spp. Corchorus* sp., *Cucumis* spp., *Fargaria* sp., *Glycine Max* (soybean), *Gossypium* sp., *Helianthus annuus* (sunflower), *Ipomoea batatas* (sweet potato), *Medicago sativa* (alfalfa), *Phaseolus* spp., *Pinus* spp., *Prunus* spp., *Sesamum indicum* (sesame), *Solanum tuberosum* (potato), *Sorghum bicolor* (sorghum), *Vigna unguiculata* (bean), and *Zea mays* (corn).

3.2.14 Pathogenesis

Pectinolytic and cellulolytic enzymes play a significant role in the pathogenesis of seedling blight of jute caused by *M. phaseolina*. Both these enzymes have been extracted from infected and surrounding regions (Ali *et al.*, 2005).

3.2.15 Pathogenic variation

The fungus isolated from different parts of various hosts showed wide range of variation in their morphological shapes and sizes. Based on the size, *M. phaseolina* is classified into four groups (A, B, C and D). Isolates from jute collected from different jute growing areas in the Indian sub-continent belonged to group C. However within this group there is morphological and pathological variation and further classification into four sub-groups has been made depending on their morphology and pathology (Mandal *et al.*, 1998). Eight physiological races of this fungus have been identified at BJRI (Ahmed and Ahmed, 1969). From the cultural characteristics, however, all the eight isolates differed from each other.

3.2.16 Disease cycle and epidemiology

M. phaseolina survives as microsclerotia in the soil and on infected plant debris. The microsclerotia serve as the primary source of inoculums and have been found to persist within the soil up to three years (Dhinga and Sinclai, 1977). The microsclerotia are black, have spherical to oblong structures that are produced in the host tissue and released into the soil as the infected plant decays. These multi-celled structures allow the persistence of the fungus under adverse conditions such as low soil nutrient levels and temperature above 30°C. Microsclerotial survival is greatly reduced in wet soils; surviving no more than 7 to 8 weeks and mycelium no more than 7 days. Seeds may also carry the fungus inside the seed coat. Infected seed do not germinate or produce seedlings that die soon after emergence (Bowers and Russin, 1999).

Germination of the microsclerotia occurs throughout the growing season when temperatures are between 28°C and 35°C. Microsclerotia germinate on the root surface, germ tubes form appresoria that penetrate the host epidermal cell walls by mechanical pressure and enzymatic digestion or through natural openings (Bowers and Russin, 1999). The hyphae first grow intercellularly in the cortex and then intracellularly through the xylem, colonizing the vascular tissue. Once in the vascular tissue *M. phaseolina* spreads through the taproot and lower stem of the plant producing microsclerotia that plug the vessels (Wyllie, 1998). The rate of infection

increases with higher soil temperatures and low soil moisture which further enhances disease severity (Wyllie, 1988).

Hot, dry weather promotes infection and development of charcoal rot disease. *M. phaseolina* can grow and produce large amounts of microsclerotia under relatively low water potentials allowing this disease to be recognized as favoring drought (Olaya and Abawi, 1996). The mechanical plugging of the xylem vessels by microsclerotia together with toxin production, enzymatic action and mechanical pressure during penetration lead to disease development (Wyllie,1998). The population of *M. phaseolina* in soil increases when susceptible hosts are cropped in successive years and can be redistributed by tillage practices (Wyllie, 1988).

3.2.17 Plant cell wall and Endoxyloglucan transferase

Plant cell walls provide a physical barrier between pathogens and the internal content of the cells. In this respect, several biochemical and physiological events occur in plant cells in response to pathogen recognition (Miedes and Lorences, 2007). The primary immune response is initiated after recognition of microbial-associated molecular patterns (MAMPs) by cell surface receptors and subsequent activation of regulatory proteins, protein kinases, and transcription factors (Bittel and Robatzek, 2007). These signaling cascades usually lead to up-regulation of defense-related genes. Plant innate immunity can also include the hypersensitive response (HR), which results in tissue necrosis and localized cell death at the site of infection, as well as reinforcement of cell walls surrounding the infected region by deposition of lignin and related wall-bound phenolics (Eulgem, 2005; Garcia-Brugger *et al.*, 2006).

Studies over the last few decades suggest that land plants have evolved a fundamentally similar primary wall structure: layers of cellulose microfibrils enmeshed in matrices of structurally varied hemicellulosic and pectic polysaccharides, with additional quantitatively minor components such as structural proteins (Carpita and Gibeaut, 1993). While compositional analysis can confirm the presence of different classes of polysaccharides and proteins, it is much more difficult to establish how they are associated with each other in a mature wall. However, there is evidence that one of the key interactions in the primary wall of

dicotyledons is formed between cellulose and the hemicellulose xyloglucan, which together typically comprise about two thirds of the dry wall mass. Xyloglucan binds non-covalently to cellulose coating and cross-linking adjacent cellulose microfibrils (Rose *et al.*, 2002) and the resulting extensive xyloglucan cellulose network is thought to act as the major tension bearing structure in the primary wall (Hayashi, 1989). Xyloglucan metabolizing enzymes therefore represent potentially important agents for controlling wall strength and extensibility, for example, through the modification of load-bearing xyloglucan (McDougall and Fry, 1989).

Xyloglucans are one of the classes of molecules that cross link cellulose microfibrils in the cell wall, and consequently xyloglucan endotransglucosylase/hydrolases (XTHs) have been postulated to play an important role in cell expansion and rearrangement (Fry *et al.*, 1992). XTHs are encoded by large gene families, the best described to date is the Arabidopsis XTH family with 33 members (Yokoyama and Nishitani, 2001).

3.2.18 Endoxyloglucan transferase as a key enzyme for molecular grafting

Endoxyloglucan transferase (EXGT) is responsible for rearrangement of the cellulose/xyloglucan framework in the cell wall (Yokoyama and Nishitani, 2001). The enzyme catalyzes transfer of a large segment of one xyloglucan molecule and thereby mediates molecular grafting between the polysaccharide molecules, a molecular process essential for rearrangement of the cell wall architecture (Nishitani and Tominaga, 1991; Nishitani, 1998; Okazawa *et al.*, 1993). Many homologues of this enzyme have been found and characterized (Fry *et al.*, 1992; Farkas *et al.*, 1992; Talbott and Ray, 1992). Together, these homologues constitute a large multi-gene family designated as xyloglucan-related proteins (XRP) (Xu *et al.*, 1996). EXGT mediates interchange of cross links in the cell wall matrix and functions as a key enzyme responsible for the regulation of cell wall reconstruction, an essential process in cell growth and development in plants (Nishitani and Tominaga, 1991).

3.2.19 Mode of action of endoxyloglucan transferase

Growth processes in plants involve structural changes in the molecular architecture of the cell wall that are necessary to allow cell expansion (Romo *et al.*, 2005). One of the candidates susceptible to these modifications is the xyloglucan (XG) chain. It has been demonstrated that during cell wall expansion changes occur in the turnover of this polymer (Thompson and Fry, 2000). Xyloglucan endotransglucosylase/ hydrolases (XTHs) are enzymes that cut XG chains and transfer the fragment with the new reducing end either to another XG (called xyloglucan endotransglucosylase, or XET activity) or to water (called xyloglucan endohydrolase or XEH activity). XTH has been proposed to catalyze a type of wall-loosening that leads to cell wall extension both by cutting and restructuring the existing wall-bound XG, and in some cases by rejoining with newly secreted XG chains (Romo *et al.*, 2005).

3.2.20 Structure of endo-xyloglucan transferase

Johansson *et al.*, (2004) analyzed the crystal structure of native PttXET (*Populus tremula x tremuloides* XET). The enzyme was found to have an overall structure typical of glycoside hydrolase family 16, with the addition of a C-terminal linker that crosses the convex surface and forms a short, additional ß-strand on the concave side of the molecule in the region of the acceptor binding site (Figure 3.2.2).

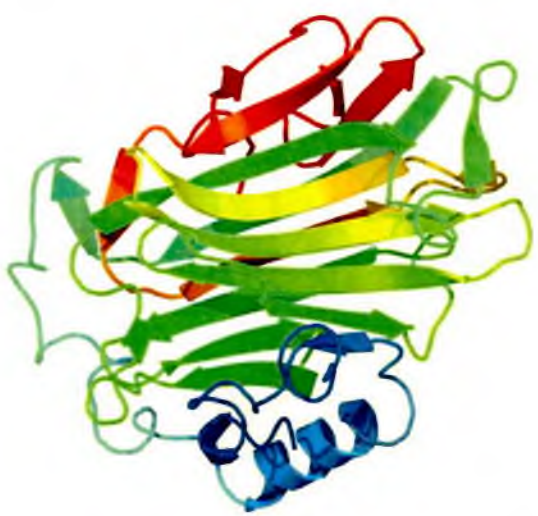


Figure 3.2.2: 3D structure of *Ptt*XET (*P. tremula x tremuloides* XET); Johansson *et al.*, (2004).

3.2.21 Endoxyloglucan transferase and fungus infection

Among cell-wall-modifying enzymes, a putative cellulose synthase and a glucanase have been found to be upregulated during fungus infections but an endoxyloglucan transferase was downregulated (Azaiez *et al.*, 2009).

Filgueira *et al.*, (1999) worked on the role of xyloglucan and its oligosaccharides (XGOs) during the invasion of *Fusarium* on carnation plants. They observed an inverse relation between the xyloglucan concentration in the resistant and susceptible plants, i.e., the resistant varieties had less xyloglucan on their walls than the susceptible ones on carnation. Also, an increase in XTH1 mRNA accumulation and XET activity during the defense reaction associated with the incompatible tomato-cuscuta interaction has been reported (Albert *et al.*, 2004). In another study, Kortekamp (2008) found that PR-2 (β-1– 3 glucanase) expression was associated with responses to *Pseudoperenospora cubensis* infection in the resistant grape rootstock 'Gloire de Montpellier' (*Vitis riparia*) compared to the susceptible cultivar 'Riesling' (*Vitis vinifera*).

3.3 Materials and methods

3.3.1 Plant materials

Macrophomina phaseolina is a plant pathogen which infects many plant species including the cultivated species of jute. In this study, a *M. phaseolina* susceptible jute species, *Corchorus olitorius* L. (variety BJRI tossa pat-4; O-72) and a resistant wild jute species *Corchorus trilocularis* L. (Biswas *et al.*, 1968) were used. Seeds of both species were collected from the Genetic Resources and Seed Division of Bangladesh Jute Research Institute, Dhaka, Bangladesh.

3.3.2 *Macrophomina phaseolina* strain and culture

The *Macrophomina phaseolina* strain *ms-6* isolated from stem rot specimen of jute was used in this experiment. Strain *ms-6* is the most virulent strain of *M. phaseolina* (Islam *et al.*, 2003). This strain was collected from the Plant Pathology Department of Bangladesh Jute Research Institute, Dhaka.

Macrophomina phaseolina strain *ms-6* was grown on Potato-Dextrose-Agar (PDA) media. PDA media provides a good nutrient source for *M. phaseolina* culture. Fungi from these cultures were used for infecting jute seedlings.

Materials

For 1000 ml of PDA media the following reagents were mixed in the given amount:

Potato	250 g
Dextrose	20 g
Agar	10 g

Methods

Potato starch and dextrose support luxuriant growth of fungi. Lowering the pH of the medium to approximately 3.5 with sterile tartaric acid inhibits bacterial growth.

- 250g potato was cut into small pieces and boiled with water until the volume was reduced to half of the starting volume.
- Supernatant was collected and residual potato pieces were discarded.

- 20g dextrose was suspended in supernatant, mixed thoroughly and the volume was made up to 1000 ml.
- The pH of the media was adjusted to 3.5-4.0.
- Agar (1.0% w/v) was added to the medium and autoclaved.
- The solution was cooled to 45-50°C and poured onto sterile Petri dishes or in 20 ml test tubes. Media was allowed to solidify for a minimum of 30 min.
- Upon solidification, the plates or tubes were inoculated with fresh culture of *M. phaseolina.*
- Upon infection the inoculated media were incubated at room temperature for 3 days. For long term storage, these test tubes were preserved at 4°C.

3.3.3 Preparation of *M. phaseolina* inoculum for jute seedlings

Fungus inoculum was prepared for artificial infection of jute seedlings according to the procedure described by Bhowal *et al.*, 2006. *Macrophomina phaseolina* was grown on potato dextrose agar media at room temperature for 5 days. Then mycelia, including sclerotia, were separated from the culture and 1% fungal suspension was prepared in sterile distilled water. This suspension was used as infection inoculum.

3.3.4 Inoculation of jute seedlings with *M. phaseolina*

- Seeds from both the susceptible and the resistant plants were germinated on Petri dishes simply in presence of water and in the absence of light for three days without any growth medium.
- After three days, three sets of plants from both species were sprayed with fungal suspension in dH_2O. One set of seeds from both species were kept uninfected and were collected at the same time with the first collection of infected seedlings.
- One set of infected seedlings from each species were collected at intervals of 15, 24 and 36 hours, respectively, from the time of spraying with fungal suspension, washed several times with dH_2O and stored at -80^0C.

- Fungal infection on the seedlings was checked by visual observation of the symptom i.e., brown coloration of the seedlings.
- Total RNA was isolated from these seedlings for differential display experiment.

3.3.5 Isolation of total RNA

Total RNA was extracted from both the susceptible and resistant plant species at 15, 24 and 36 hours after infection with fungal suspension according to the modified procedure from Chomczynski and Sacchi (1987). Total RNA was also isolated from both uninfected susceptible and resistant seedlings.

Materials

Reagents

- GNTC Buffer

  4M Guanidine thiocyanate

  25mM Sodium citrate

  0.5% Sodium lauryl sarcosinate pH 7.0

  filter- sterilized and stored at 4°C

  8 μl of β-mercaptoethanol was added to every ml of GNTC buffer before use)

- 2M Sodium acetate, pH 4.0
- 3M Sodium acetate, pH 5.2
- DEPC-phenol (RNase-free phenol equilibrated in DEPC- water)
- Chloroform: Iso-amyl alcohol (49:1)
- Isopropanol
- 75% DEPC ethanol

Method

Day One:

- 0.2g of seedlings was ground to powder in liquid nitrogen using mortar and pestle. After homogenizing the tissue 2 ml of GNTC buffer was added. This was followed by the addition of following reagents. The mixture was vortexed between each addition:
    a) 0.1 vol. of 2M Sodium Acetate, pH 4.0
    b) 1 vol. of DEPC-phenol.
    c) 0.3 vol. of Chloroform: iso-amyl alcohol.
- The mixture was incubated on ice for 20 min.
- The mixture was centrifuged at 10,000 rpm for 25-30 min at 4°C.
- Supernatant was collected and 1vol. of isopropanol (2-propanol) and 3M Sodium Acetate (pH-5.2) was added to each tube.
- The mixture was incubated overnight at -20°C.

Day Two:

- Sample was centrifuged at 10,000 rpm for 25 min at 4°C.
- Supernatant was discarded very carefully and 1 ml 75% ethanol was added.
- Again sample was centrifuged at 10,000 rpm for 5 min at 4°C.
- Pellet was collected and air dried.
- Pellet was dissolved in 100 µl of nuclease free water by tapping.
- ¼ vol. 10M $LiCl_2$ was added and mixed by tapping.
- Eppendorf containing the sample was dipped in Liq. Nitrogen for 2min.
- Then the sample was allowed to thaw.
- Sample was centrifuged at 10,000 rpm for 25 min at 4°C.

- The pellet was washed with 75% ethanol.
- After air-drying the pellet was dissolved in 30 µl of DEPC treated water.

3.3.6 Precautions for isolating total RNA

The precautions listed below were strictly maintained to avoid contamination with RNases and subsequent damage of RNA.

- Clean gloves were used.
- Triple autoclaved filter tips and other RNase-free consumables were used.
- Nuclease free pipette barrel was used.
- Non-disposable items were treated with RNase *AWAY*™ to remove RNase contamination.
- DEPC treated doubly autoclaved water was used for all sorts of solution preparations and dilutions. Section 3.3.7 describes the protocol.
- RNA was isolated under a laminar flow hood. The air conditioner was switched off before the start of the work to prevent in-flow of air into the laminar air flow hood.
- To inactivate cellular RNAses, GNTC buffer was added rapidly after the seedlings had been ground.

3.3.7 Preparation of 0.1% DEPC treated water

0.1% (v/v) solution of Diethyl Pyrocarbonate (DEPC) in distilled water was prepared, shaken vigorously, and allowed to stand overnight at room temperature. The solution was autoclaved twice for removing DEPC activity completely.

3.3.8 Quality of RNA

Quality of purified RNA was determined after electrophoresis in 1.3% agarose gel.

Materials

- Ultra pure agarose

- RNA gel loading dye
- MOPS buffer
- Gel electrophoresis kit

Preparation of 10X MOPS Buffer

- The following reagents were mixed in the given amounts to make 1000ml of 10X MOPS Buffer -

3-Morpholinopropane sulfonic acid (MOPS)	41.86g
Na-Acetate	4.1g
0.5 M EDTA (pH 8.0)	20.0 ml

- The pH was adjusted to 7.0 using 1N NaOH prepared with DEPC treated water.
- This volume was made up to 1000ml with DEPC treated water.

Preparation of 1.3% Agarose Gel

Materials

- 1X MOPS (Electrophoresis buffer)
- Agarose
- Formaldehyde
- DEPC-treated water

Method

- To prepare 25 ml of 1.3% gel, 0.325g of agarose powder was mixed with 2.5 ml of 10x MOPS buffer and 21.25 ml of DEPC treated water in a conical flask.
- The mixture was melted in a micro-wave oven at 60°C for 2 min.
- As the mixture cooled to 40-45°C, 1.25 ml formaldehyde was added and mixed by gentle swirling.
- The molten solution was poured on a gel-case to solidify the gel.

Sample preparation and gel electrophoresis

Materials

- Thermal cycler
- RNA-loading buffer
- RNA sample

Composition of RNA loading buffer:

10X MOPS	40 µl
Formamide	200 µl
37% Formaldehyde	70 µl
Glycerol	20 µl
DEPC-treated water	20 µl
0.5M EDTA	0.5 µl
10 mg/ml Ethidium bromide	2.0 µl

Methods

- 1.0 µl of RNA sample was added to 3.0µl of RNA loading dye and the mixtures were denatured in a thermal cycler at 65°C for 20 min.
- Denatured samples were quickly chilled on ice for at least 1 min.
- The gel was assembled in the electrophoresis system and samples were applied into the wells and electrophoresis was carried out at 45V for 4 hours.
- Later, photograph of the gel was taken in a trans-illuminator under UV light.

3.3.9 Quantitation of RNA

0.5 ml quartz cuvette was washed with concentrated HCl: methanol solution (1:1) for 1 hour. The cuvette was rinsed several times in RNase-free water.

1µl of the purified RNA was added to 499 µl of DEPC-treated water in an eppendorf tube. The absorbance was measured at wavelengths of 260nm and 280nm in

SPECORD ® 50 using software WinASPECT®. The ratio between readings of 260nm and 280 nm (OD_{260}/OD_{280}) was also calculated. *Concentration of RNA in the sample* was determined directly using WinASPECT®. Concentration of the RNA present in the sample was also cross-checked using the standard formula:

If the O.D. is 1 then RNA concentration is 40μg/ml

So, if the O.D. is "A" then RNA concentration is 40xA μg/ml.

Dilution factor =500 (in this experiment)

Concentration of RNA = O.D. x 40 x dilution factor, μg/ml

= Ax40x500, μg/ml

= Ax40x500/1000, μg/μl

= Ax20 μg/μl

The RNA concentrations in all the samples were adjusted to give 1 μg of total RNA per μl of solution. RNA concentration was strictly maintained in the experiment.

3.3.10 First Strand Synthesis

For differential display, first strand cDNA was prepared from all eight samples using the following three anchored primers:

$T_{12}A$ 5′-TTT TTT TTT TTT A-3′

$T_{12}C$ 5′-TTT TTT TTT TTT C-3′

$T_{12}G$ 5′-TTT TTT TTT TTT G-3′

Materials

a) Anchored primer

b) 5X RT-Buffer

c) 0.1 M DTT

d) 2.0 μM dNTPs

e) RNaseOUT™

f) SuperScript™ III Reverse Transcriptase

Method

- The following components were added to a nuclease free PCR tube.

Anchored primers (50μM)	1 μl
Total RNA	4.5 μg
2.0 mM dNTPs mixture	4 μi
DEPC treated PCR water	to 13 μl

- The mixture was heated to 65°C for 5 min and then, incubated on ice for at least 1 min.
- The following components were added to the above tubes:

5X RT-buffer	4 μl
0.1 M DTT	1 μl
RNaseOUT™	1 μl
SuperScript™ III RT (200 units/μl)	1 μl

- The mixture was incubated at 25°C for 5 min followed by at 50°C for 60 min.
- The activity of SuperScript™ III RT was inhibited by heating at 70°C for 15 min.
- For removing the RNA complementary to the cDNA, 0.5 μl of *E. coli* RNaseH was added and incubated at 37°C for 20 min.

3.3.11 DDRT-PCR with the differentially expressed mRNA

Taking the different sets of differentially expressed mRNA populations as starting material, DDRT-PCR was performed. The 3′anchored oligo-dT primers (5′-TTTTTTTTTTTTA-3′, 5'-TTTTTTTTTTTTG-3' and 5'-TTTTTTTTTTTTC-3') divided the total RNA population to be studied into three groups. Arbitrary primers 11 (5'-CTGGCGTGTCA-3') were used as 5′primers in different combinations with each of the anchored primers.

Materials

The following components were used for the polymerase chain reaction.

1. 10X PCR Buffer (500mM KCl, 100mM Tris-cl pH 8.3, 0.1% gelatin)
2. 50mM $MgCl_2$
3. 2 mM dNTPs
4. Taq DNA polymerase
5. Anchored primers
6. cDNA prepared from mRNA.
7. Arbitrary primers.
8. Autoclaved ultra-pure water.

Dilution of primer

For dilution of the primers, 20µl of the primer from stock solution (100 µM) was added to 80µl of TE buffer (pH 8.0). So, the final concentration of the primer was 200 ng/µl.

Preparation of the Master Mix

Reaction mixture for PCR using RT- products had the following composition:

10× PCR buffer	2.5 µl
50 mM $MgCl_2$	0.8 µl
10mM dNTPs	0.5 µl
Arbitrary primers	1.5 µl
Anchored primers	1.5 µl
Taq polymerase	0.2 µl
PCR water	15.0 µl
RT product (cDNA)	3.0 µl

Thermal Cycling Profile Used in PCR

RT products were amplified using a thermal cycler (GeneAmp^{R} PCR System 9700, Applied Biosystems). The thermal cycling profile used to amplify the RT products is as given in the table below:

	Temperature	Time	No. of Cycles
Initial denaturation	94ºC	3 min	1 (First)
Denaturation	94ºC	40 sec	40
Annealing	38ºC	50 sec	40
Elongation	72ºC	2.0 min	40
Final elongation	72ºC	5.0 min	1 (last)
Final hold	4.0°C	Infinite period	-

Analysis of amplified products

PCR products were analyzed by separating them electrophoretically according to their size on 10% polyacrylamide gel and visualized by silver staining.

Preparation of Polyacrylamide gel

Materials

a) ddH_2O
b) 1X TBE buffer
c) 40% Acrylamide
d) TEMED
e) 10% APS

Method

26.516 ml of ddH_2O, 3 ml of 1X TBE buffer, 10 ml of 40% acrylamide, 34.0 µl TEMED and 450 µl of 10% APS were mixed in a beaker and poured between the glasses of the gel system. The combs were set between the glasses immediately after the mixture was poured. Once solidified, the gel was placed in an electrophoresis tank.

Electrophoresis of PCR product

Materials

a) Electrophoresis buffer (1X Tris-Glycine buffer)

b) Ladder

c) Loading dye

d) PCR products

Method

- Before sample application, the electrophoresis apparatus and gels were assembled. Then 500 ml of electrophoresis buffer was added such that the top of gel was well covered.
- Samples (10 μl of PCR products + 2 μl of loading buffer) and 1Kb+ ladder (Invitrogen) (3μl) were applied to individual wells.
- Electrophoresis was carried out for 3 hours at 70 volts.
- After electrophoresis, the gel was dissembled and stained.

Silver staining

Silver staining is widely used to detect DNA fragments in polyacrylamide gels with high sensitivity.

Materials

a. Impregnate solution (25% ethanol, 1%HNO_3, 0.2% $AgNO_3$ in ddH_2O)
b. Developer (3% Na_2CO_3, 0.2% HCHO in ddH_2O)
c. Stop solution (10% acetic acid)

Method

- After the gel run was complete, it was fixed by incubating the gel in fixation solution for 20 min. All the steps of silver staining were performed on a slowly rotating shaker (50-60 rpm) at room temperature.
- Then the gel was put into impregnate solution (2-5 min).

- The gel was rinsed with ddH_2O (5-10 min).
- The gel was developed with developer solution (2-5 min).
- When the bands were easily discernable, the developer was discarded and stop solution was added.

3.3.12 DNA recovery from polyacrylamide gel

Materials

a) Elution buffer

i. EDTA pH 8.0 0.5M

ii. Ammonium acetate 1M

b) TE buffer

i. Tris-HCl pH 8.0 10mM

ii. EDTA pH 8.0 1mM

Method

- Differentially expressed band was cut with a sterile scalpel.
- The excised band was put into 2X volume of elution buffer into an eppendorf tube.
- The tube was incubated overnight at 37^0C with gentle shaking.
- Supernatant was recovered and placed in a new eppendorf tube.
- Two volumes of ice-cold 99% ethanol was added and chilled for 1-2 hrs at -20^0C to precipitate the DNA.
- The solution was centrifuged at 14000 rpm for 10 min, supernatant was discarded and the precipitate was washed with 70% ethanol.
- This was followed by centrifugation at 14000 rpm for 10 min.
- Pellet was air-dried and resuspended in minimal amount of ddH_2O.

3.3.13 Re-amplification of isolated band

- The isolated products were re-amplified using their corresponding primers.
- The PCR (25 µl) was carried out with 10X reaction buffer (2.5µl), 10mM dNTPs (0.5µl), 50.0mM MgCl2 (0.8µl), water (17.4µl), *Taq* polymerase (0.2µl), $T_{12}AG$ Anchored dT (0.8µl), Arbitrary primer (0.8µl) and 2µl of template DNA.
- Template DNA was amplified in a thermal cycler programmed as follows: after preheating for 5 min at 94^{0}C; 40 cycles of 40 sec at 94^{0}C (denaturation), 90 sec at 38^{0}C (annealing) and 40 sec at 72^{0}C (extension); and a final extension at 72^{0}C for 10 min followed by cooling to 4^{0}C.
- The amplified products were checked by running in a 1.5% agarose gel followed by ethidium bromide mediated staining.

Agarose gel electrophoresis

Materials

1. Ultra pure agarose,
2. TAE buffer,
3. Gel loading dye and
4. Gel electrophoresis kit.

Preparation of 50X TAE buffer (1 liter)

Tris base	242.0g
Glacial acetic acid	57.1ml
0.5M EDTA (pH 8.0)	100ml

This solution was made up to 1000ml with ddH_2O.

Preparation of 1.5% agarose gel

- To prepare 100ml of 1.5% agarose gel, 1.5g of agarose powder was weighed in a conical flask.

- 2ml of 50X TAE was taken in measuring cylinder and the volume was made up to 100ml with ddH_2O.
- The mixture of 50X TAE and H_20 was poured into the flask containing agarose and then melted in a microwave oven at 100^0C for 2 min.
- The gel was poured in the gel case (in which the comb was assembled for wells) and allowed to solidify. DNA samples were loaded in wells and the electrophoresis was carried out at 80 volts. The DNA in gel was stained with ethidium bromide and detected using ultraviolet trans-illuminator (Kodak EDAS, Japan)

3.3.14 pCR8 GW TOPO TA™ vector mediated cloning

The pCR®8/GW/TOPO® TA Cloning® Kit combines Invitrogen's TOPO® Cloning and Gateway® technologies to facilitate cloning of *Taq* polymerase-amplified PCR products into a plasmid vector with high efficiency. As is the case with other pCR® vectors, clones may be easily sequenced and characterized.

4.3.14.1 Preparation of competent cells

Materials

- $CaCl_2$ solution (60mM $CaCl_2$, 15% glycerol, 10mM PIPES pH 7.0)
- Liquid LB (Luria-Bertani) medium (5g Yeast extract, 10 g Peptone, 10 g NaCl per liter; pH 7.0)
- *E. coli* DH5α strain.

Method

- A single colony of *E. coli* cell was incubated overnight in 5 ml LB medium at 37°C with shaking (200 rpm).
- 1 ml of this culture was taken in a 100 ml LB medium in a 250 ml conical flask. The medium was incubated at 37°C with shaking (250 rpm) until the optical density of this medium at 590 nm reached 0.375.

- The culture was then aliquoted into 2 pre-chilled sterile centrifuge tubes and placed on ice for 5 to 10 min. It was then centrifuged for 10 min at 4000rpm and 4°C.
- Each pellet was resuspended in 10 ml of ice cold $CaCl_2$ solution. It was centrifuged at 4000rpm for 5 min at 4°C.
- The pellets were resuspended again in 10 ml of ice cold $CaCl_2$ solution and kept on ice for 30 min. It was then centrifuged at 4000rpm for 5 min at 4°C.
- Each pellet was then resuspended completely in 2 ml ice cold $CaCl_2$ solution. 250µl of this solution was aliquoted in each sterile pre-chilled microcentrifuge tube and stored at -80°C for later use in transformation.

3.3.14.2 Ligation Reaction

Materials

- PCR product (freshly prepared)
- pCR®8/GW/TOPO® vector
- Salt Solution
- Water

Method

The following ligation reaction was set up (in 200µL thin wall PCR tube) as follows:

• Fresh PCR product	0.5 µL
• Salt solution	0.5 µL
• Water	1.5 µL
• TOPO® vector	0.5 µL

The mixture was then incubated at room temperature for 30 min.

Transformation

Materials

- LB plates with spectinomycin

- High efficiency competent cells (*E. coli* DH5α strain)
- SOC medium (50ml)

Peptone	1.0 g
Yeast extract	0.25 g
1 M NaCl	0.5 ml
1 M KCl	0.125 ml
2 M Mg^{2+} stock	0.5 ml
2 M glucose	0.5 ml

Method

- The ligation reaction was transferred to a sterile 1.5ml microcentrifuge tube and placed on ice.
- A tube of frozen DH5α High Efficiency Competent Cells was removed from –80^{0}C storage and placed on an ice bath until just thawed (about 5 min). The cells were mixed by gently flicking the tube.
- 100µl of cell was transferred carefully into the eppendorf tube containing the ligation product.
- The tube was flicked gently to mix and placed on ice for 20 min.
- The cells were heat-shocked for 90 seconds in a water bath at exactly 42^{0}C.
- Immediately the tube was returned to ice for 2 min.
- 1000µl SOC medium at room temperature was added to the tube containing cells transformed with ligation reactions.
- The sample was incubated for 1.5 hours at 37^{0}C with shaking (~200rpm).
- 100µl of transformation culture was plated onto duplicate LB spectinomycin plates.
- The plates were incubated overnight (16-24 hours) at 37^{0}C.

3.3.14.3 Isolation of plasmid DNA

The primary objective of the isolation process was to achieve the maximum yield of plasmid DNA devoid of protein. Plasmid was isolated according to the following protocol which was modified from Sambrook *et al.* (1989).

The detailed procedure is described in section 2.3.13.

3.3.14.4 PCR of the isolated plasmid

The isolated plasmid with the desired insert was amplified with the M-13 forward and M-13 reverse primers to generate the PCR product which contained the insert of the unknown sequence and which will later serve as the sequencing sample.

3.3.14.5 Sequencing of clone

Selected clone were sent to a molecular service centre in Malaysia (1st Base) for sequencing.

3.3.15 Isolation of DNA from plant

DNA was isolated from both susceptible and resistant jute plant species following a procedure modified from Doyle and Doyle (1990) using nonionic detergent Cetyl Trimethyl Ammonium Bromide (CTAB). The detailed procedure is described in section 2.3.12.

3.3.16 Polymerase chain reaction of genomic DNA

PCR with the plant genomic DNA was performed to identify the sequence of both 3'-end and 5'-end by primer based gene walking.

3.3.16.1 List of primers

Table 3.3.1: Primers used for PCR with plant genomic DNA

Primer name	Type	Sequence	Tm
EXTG F	Gene specific	5'-GTCCAAACAAACGTGTTCGC-3'	60.4
EXTG R	Gene specific	5'-CCTTATGGTTCCATCTAATTTGG-3'	59.2
EXT D2 Rev	Degenerate	5'-CCCAMCKRTAWCKTYKAGCTTC-3'	62.7
EXT 3' end For	Gene specific	5'-GCAGCCGACACTTGTATGGT-3'	62.4
EXT 3' end Rev	Gene specific	5'-GGATTTATCGGTGCAATAAT-3'	56.3

Mixed base nomenclature

R: a/g	M: a/c	W: a/t	H: a/t/c	V: g/a/c
D: g/a/t	Y: c/t	K: g/t	B: g/t/c	N: a/g/t

3.3.16.2 Rapid amplification of cDNA 3'-end (3′-RACE)

Materials

- 10X PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl].
- 25 mM $MgCl_2$.
- 10 mM dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP).
- 0.1 M DTT.
- SuperScript™ II Reverse Transcriptase (RT, 200 units/μl).
- Adapter primer (AP, 10 μM).
- Abridged universal amplification primer (AUAP, 10 μM).
- *E. coli* RNase H (2 units/μl).
- DEPC-treated water.

Table 3.3.2: Primers for 3'-RACE

Primer name	Type	Sequence	Tm
EXT GF	Gene Specific	5'-GTCCAAACAAACGTGTTCGC-3'	60.4
AP	Adapter	5'GGCCACGCGTCGACTAGTACTTTTT TTTTTTTTTTT 3'	68.6
AUAP	Abridged universal amplification primer	5'GCCACGCGTCGACTAGTAC 3'	63.3

Method

First Strand cDNA Synthesis

Total RNA was converted to first strand cDNA following this procedure:

1. Each component was gently mixed and quickly centrifuged before use.
2. 4.5 µg of total RNA and DEPC-treated water was combined in a 0.5-ml microcentrifuge tube. The final volume was 10µl.
3. 1 µl of the 10 µM AP solution was added, mixed gently, and collected by brief centrifugation.
4. The mixture was heated to 70°C for 10 min and chilled on ice for at least 1 min.
5. The contents were collected by brief centrifugation and the following was added-

10X PCR buffer	2 µl
25 mM $MgCl_2$	2 µl
10 mM dNTP mix	1 µl
0.1 M DTT	2 µl
RNase out	1 µl

5. The components were mixed gently followed by brief centrifugation. Mixture was equilibrated to 42°C for 3 min.
6. 1 µl of SuperScript™ II RT was added. The tube was incubated in a heating block at 42°C for 50 min.
7. The reaction was terminated by incubating at 70°C for 15 min.
8. The tube was then chilled on ice. The reaction was collected by brief centrifugation. 1 µl of RNase H was added to the tube, mixed, and incubated for 20 min at 37°C

Amplification of the Target cDNA

Optimal conditions for amplification were dependent on the nature of each particular primer and target sequence used.

10X PCR buffer	5 µl

25 mM $MgCl_2$	3 µl
PCR H_2O	36.5 µl
10 mM dNTP mix	1 µl
EXGT 3' end For (10 µM)	1 µl
AUAP (10 µM)	1 µl
Taq DNA polymerase	0.5 µl

- 2 µl from the cDNA synthesis reaction mixture was added to the tube. The reaction mixture was incubated at 94°C for 3 min.
- 35 cycles of PCR was performed.
- 10 µl of the amplified sample was analyzed, using agarose gel electrophoresis and ethidium bromide staining. Appropriate molecular size standards were used for determining the sizes of the amplified products.

Thermal Cycling Profile Used in 3'RACE

The thermal cycling profile that was used to amplify the gene by Polymerase Chain Reaction (PCR) is as follows:

	Temperature	Time	No. of Cycles
Initial denaturation	95ºC	3min	1 (First)
Denaturation	95ºC	40 sec	35
Annealing	60ºC	30 sec	35
Elongation	72ºC	1 min	35
Final elongation	72ºC	5 min	1 (last)

The amplified product of 3' RACE was gel purified for cycle sequencing.

3.3.16.3 Gel Extraction

For this purpose, QIAGEN MinElute Gel Extraction Kit was used. This protocol was designed to extract and purify DNA of 70 bps to 10 kbps from standard or low-melt agarose gels in TAE or TBE buffer.

Materials

- Agarose gel
- Electrophoresis buffer (1X TAE buffer)
- 1kb plus Ladder
- Loading dye
- PCR products
- Ethidium bromide containing staining solution
- Iso-propanol
- PCR product

Method

- The agarose gel segment containing the DNA fragment was excised with a clean, sharp scalpel.
- The gel slice was weighed in a colourless tube. 3X volumes of Buffer QG (Qiagen™) was added to 1X volume of gel (100 mg ~ 100 µl).
- The gel slice was incubated at 50°C for 10 min (or until the gel slice was completely dissolved). The tube was vortexed every 2–3 min during the incubation to dissolve the gel.
- When gel slice dissolved completely, the color of the mixture was checked and was found to be yellow (similar to Buffer QG without dissolved agarose).
- 1X gel volume of isopropanol was added to the sample and mixed.
- A QIAquick (Qiagen™) spin column was placed on a 2 ml collection tube provided by the manufacturer.
- To bind DNA, the sample was applied to the QIAquick column, and centrifuged for 1 min.
- Flow-through was discarded and QIAquick column was placed back in the same collection tube.
- 0.5 ml of Buffer QG was added to QIAquick column and centrifuged for 1 min.

- To wash, 0.75 ml of Buffer PE was added to QIAquick column and centrifuged for 1 min.
- The flow-through was discarded and the QIAquick column was centrifuged for an additional 1 min at 17,900 × g (or 13,000 × g at 20 sec).
- QIAquick column was placed into a clean 1.5 ml microcentrifuge tube.
- To elute DNA, 20 µl of Buffer EB (10 mM TrisCl, pH 8.5) or water (pH 7.0–8.5) was added to the center of the QIAquick membrane and the column was centrifuged for 1 min.
- The purified DNA was analyzed on a gel, 2X volume of Loading Dye was added to 1X volumes of purified DNA.

3.3.17 Rapid Amplification cDNA end (5' RACE)

Materials

- 10X PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl] .
- 25 mM MgCl2.
- 10 mM dNTP mix.
- 0.1 M DTT.
- SuperScript ®III Reverse Transcriptase(200 units/µl)
- RNase mix.
- 5x tailing buffer (50 mM Tris-HCl (pH 8.4), 125 mM KCl, 7.5 mM MgCl2)
- 2 mM dCTP.
- Terminal deoxynucleotidyl transferase.
- 5'-RACE abridged anchor primer (AAP, 10 µM).
- Abridged universal amplification primer (AUAP, 10 µM).
- DEPC-treated water.
- Control gene-specific primer 1 (GSP1, 1 µM).

- Control nested gene-specific primer (GSP2, 10 μM).
- Control PCR primer, gene-specific primer (GSP3, 10 μM)
- Control DNA (2 x 104 copies/μl; ~0.1 pg/μl).
- Control RNA (50 ng/μl).

DNA Purification System

- S.N.A.P. columns
- Collection tube
- Binding solution (6 M sodium iodide)
- Wash buffer concentrate

All reagents were stored at -20°C except S.N.A.P purification reagent. S.N.A.P purification reagents were stored at 4°C.

Table 3.3.3: Primers for 5′ RACE

Primer name	Type	Sequence	Tm
GSP(EXGT)	Gene Specific	5'-CCT GTCACC CTT TCCAT-3'	57.2
GSP2(EXGT)	Gene Specific	5'-TGCCCACTTCGGTTTCCCAAG A-3'	64.5
GSP3(EXGT)	Gene Specific	5'-CGG GGA CGAGCTTGA TCT TCA T3'	64.5
AAP	Abridged anchor primer	5'GGCCACGCGTCGACTAGTACGGGII GGGIIGGGIIG3'	82.9
AUAP	Abridged universal	5'GGCCACGCGTCGACTAGTAC3'	65

3.3.17.1 First Strand cDNA Synthesis

1. The following solutions were added to a 0.5-ml microcentrifuge tube (or thin-walled PCR tube).

GSP	1 μl (2.5 pmoles)
Sample RNA	2 μl
DEPC-treated water	sufficient for a final volume of 14.5 μl

2. The mixture was incubated for 10 min at 70°C to denature RNA followed by chilling on ice for 1 min.
3. The contents of the tube were collected by brief centrifugation and the following was added in the order given:

10X PCR buffer	2.5
25 mM $MgCl_2$	2.5
10 mM dNTP mix	1.0
0.1 M DTT	2.5
RNase out	1.0
Final volume	9.5

The final volume of step 1 and 2 was 24 µl.

4. The reaction was mixed gently and incubated at 42°C for 1 min.
5. 1 µl of SuperScript™ II RT was added, mixed gently and was incubated for 50 min at 42°C.
6. The reaction was terminated by incubation at 70°C for 15 min.
7. After brief centrifugation the reaction was placed at 37°C.
8. 1 µl of RNase mix was added, mixed gently but thoroughly, and incubated for 30 min at 37°C.
9. The reaction was collected by brief centrifugation and placed on ice.

The procedure was stopped at this point and the reaction was stored at -20°C.

3.3.17.2 S.N.A.P. Column Purification of cDNA

1. The binding solution was equilibrated to room temperature.
2. For each sample to be purified, 100 µl of sterilized, distilled water was equilibrated at 65°C for use in step 9.

3. 120 µl of binding solution (6 M NaI) was added to the first strand reaction.
4. The cDNA/NaI solution was transferred to a S.N.A.P. column and centrifuged at 13,000g for 20 s.
5. The cartridge was removed and the flow through was transferred to a microcentrifuge tube. The solution was saved until recovery of the cDNA was confirmed. The cartridge was inserted back into the tube.
6. 0.4 ml of cold (4°C) 1X wash buffer was added to the spin cartridge and centrifuged at 13,000g for 20 s. The flow through was discarded. This wash step was repeated thrice.
7. The cartridge was washed two times with 400 µl of cold (4°C) 70% ethanol as described in step 6.
8. Sample was washed with 70% ethanol and centrifuged at 13,000 x *g* for 1 min.
9. The spin cartridge was transferred into a fresh sample recovery tube. 50 µl sterilized, distilled, water was added (preheated to 65°C) to the spin cartridge and centrifuged at 13,000g for 20 s.

3.3.17.3 TdT Tailing of cDNA

Variable amounts of purified cDNA from the S.N.A.P. column purification was used in the TdT-tailing reaction.

Procedure:

1. The following components were added to each tube and mixed gently-

DEPC-treated water	6.5
5X tailing buffer	5.0
2 mM dCTP	2.5
S.N.A.P.-purified cDNA sample	10.0
Final volume	24.0

2. The mixture was incubated for 2 to 3 min at 94°C followed by 1 min of chilling on ice. The contents of the tube were collected by brief centrifugation and placed on ice.
3. 1 μl of TdT was added, mixed gently, and incubated for 10 min at 37°C.
4. The tube was incubated for 10 min at 65°C. The contents were briefly centrifuged and placed on ice.

3.3.17.4 PCR of dC-tailed cDNA

Tailed cDNA obtained from the preceding protocol was amplified directly by PCR.

1. The thermal cycler block was equilibrated to 94°C.
2. The following was added to a 0.2 or 0.5-ml thin-wall PCR tube sitting on ice:

Sterilized, distilled water	31.5
10X PCR buffer	5.0
25 mM $MgCl_2$	3.0
10 mM dNTP mix	1.0
Nested GSP2 (10 μM)	2.0
Abridged Anchor Primer (10 μM)	2.0
dC-tailed cDNA	5.0
Final volume	49.5

3. 0.5μl of Taq DNA polymerase (5 units/μl) was added immediately before mixing.
4. The contents of the tube were mixed (Taq DNA polymerase was added immediately before going into the thermal cycler).
5. Tubes were transferred directly from ice to the thermal cycler pre-equilibrated to the initial denaturation temperature (94°C).

6. 35 cycles were performed for PCR.

	Temperature	Time	No. of Cycles
Initial denaturation	94ºC	2 min	1 (First)
Denaturation	94ºC	1 min	35
Annealing	60ºC	40 sec	35
Elongation	72ºC	1 min	35
Final elongation	72ºC	5 min	1 (last)

7. 5-20 µl of 5' RACE products were analyzed by agarose gel electrophoresis.

3.3.17.5 Nested Amplification

Nested polymerase chain reaction is a modification of polymerase chain reaction intended to reduce the contaminations in products due to the amplification of unexpected primer binding sites.

1. 5 µl aliquot of the primary PCR product was diluted in 495 µl TE buffer [10 mM Tris-HCl, (pH 8.0), 1 mM EDTA].
2. The thermal cycler block was equilibrated to 94°C.
3. The following was added to a 0.2 or 0.5-ml thin-wall PCR tube sitting on ice.

Sterilized, distilled water	33.5
10X PCR buffer	5.0
25 mM $MgCl_2$	3.0
10 mM dNTP mix	1.0
Nested GSP3 (10 µM)	1.0
AUAP or UAP (10 µM)	1.0
Diluted PCR product	5.0
Final volume	49.5

4. 0.5 µl of *Taq* DNA polymerase (5 units/µl) was added immediately before mixing.
5. The contents of the tube were mixed (*Taq* DNA polymerase was added just before going into the thermal cycler).
6. Tubes were transferred directly from ice to the thermal cycler pre-equilibrated to the initial denaturation temperature 94°C.
7. 35 cycles were performed for PCR.
8. 5 to 20 µl of the amplified sample was analyzed, using agarose gel electrophoresis.

3.3.18 Southern Blot Analysis

Fifteen-microgram aliquots of *C. trilocularis* and *C. olitorious* gDNA were digested with *Eco*R I and *Bam*H I and transferred to positively charged nylon membrane (Amersham Hybond™-N) according to manufacturer's protocol. A detailed procedure is given in section 2.3.15.

As a probe, a PCR fragment of 728 bp was amplified using primers EXT 5F (5'-TTTGTAATTCAAAACACGACAATGGGT-3') and EXT GR (5'-CCTTATGGTT CCATCTAATTTGG-3').

3.3.19 Semi-quantitative RT-PCR

First strand cDNA was synthesized from the total RNA using the Superscript™ III reverse transcriptase kit according to the manufacture's instruction (Invitrogen, USA). The synthesis was primed with EXT GR (5'-CCTTATGGTT CCATCTAATTTGG-3') and equal amount of RNAs (4.5 µg) isolated from plant samples.

2 µl aliquots of this cDNA were subsequently used as a template for PCR amplification. RT-PCR reactions were performed using a pair of gene specific primers namely, EXT 5F (5'-TTTGTAATTCAAAACACGACAATGGGT-3') and EXT GR (5'-CCTTATGGTTCCATCTAATTTGG-3'). Annealing temperature and the number of cycles were optimized to ensure linearity for the quantification of the

amplification product. The β-actin gene was used as housekeeping control in RT-PCR experiment. To quantify the transcripts of genes, 30 cycles were performed using the following PCR conditions: 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 45 s and final elongation step at 72°C for 10 min. Each quantitative RT-PCR reaction was performed at least twice to confirm results for each cDNA.

3.3.20 Northern Blot analysis

Ten microgram of total RNA from infected (S_{15}, T_{15}, S_{24} and T_{24}) and uninfected (S_0 and T_0) seedlings of both *C. trilocularis* and *C. olitorious* were size separated by electrophoresis and transferred to positively charged nylon membrane (Amersham Hybond™-N).

The steps of northern hybridization are as follows:

- Probe preparation and labeling
- RNA transfer to nylon membrane
- Pre-hybridization and hybridization
- Washing
- Detection

3.3.20.1 Probe preparation and Labeling

For use as probes, a PCR fragment of 496 bp sequences were amplified with EXT 5F (5'-TTTGTAATTCAAAACACG ACAATGGGT-3') and EXT GR (5'-CCTTATGGTTCCATCTAATTTGG-3') from first strand cDNA (previously described in semi-quantitative RT-PCR, Section 4.3.19). Probe was labelled with digoxigenin-dUTP following the manufacturer's protocol (DIG DNA labeling Kit, Roche).

15 µl of purified PCR product was denatured by heating at 95°C for 10 min and then quickly chilled on ice. After that 2µL hexanucleotide mix, 2µL dNTP mix and 1µL Klenow enzyme were added to it. Finally, the tubes were subjected to momentary spin and incubated overnight at 37°C. The following day reaction was stopped by heating at 65°C for 10 min.

3.3.20.2 RNA transfer to Nylon membrane

- RNA was loaded in 1.3% agarose gel (as previously described in 3.3.7) and electrophoresed at 40 volt until they reached the edge of the gel.
- The gel was soaked in 2X SSC buffer for 15 min at room temperature with constant but gentle agitation.
- RNA fragments were blotted to positively-charged nylon membrane with 10X SSC buffer. Upward capillary transfer of RNA was performed at room temperature for 18 h.
- After transfer of RNA, the membrane was soaked in 2X SSC for few min and allowed to air dry.
- RNA was fixed by UV crosslink for 3 min, followed by baking in an oven for 30 min.

3.3.20.3 Pre-hybridization and Hybridization

Pre-hybridization, hybridization, washing and detection were performed essentially in the same manner as for Southern blot analysis (as previously described in 2.3.15) with the exception of hybridization buffer. DIG Easy Hyb Granules was used as hybridization buffer following the manufacturer's protocol (DIG High Prime DNA Labeling and Detection Starter Kit II, Roche, Germany).

3.3.21 DATA Analysis

Sequence analysis was performed using the BLAST search program (www.ncbi.nih.gov/blast) and Multiple sequence alignment programs (www.ebi.ac.uk/tools/clustalw). The conceptual translation of nucleotide sequence was determined by expasy server (www.expasy.ch/tools/dna.html). Softberry server was used (http://www.softberry.com/berry.phtml) to find the exon/intron boundaries, untranslated regions (UTRs), and poly-A tail. To determine the conserved amino acid residues BOXSHADE 3.21 (www.ch.embnet.org/software /BOX_form.html) was used. 3D model of jute XTH gene sequence was constructed using CPHmodels-3.0 server (www.cbs.dtu.dk/services/CPHmodels) and

represented using Swiss-Pdb Viewer (www.spdbv.vital-it.ch). VecScreen program (www.ncbi.nlm.nih.gov/ VecScreen/VecScreen.html) was used for removal of vector sequences from sequence results. To assemble a set of contiguous sequences (contigs) Cap3 sequence assembly program (www.pbil.univ-lyon1.fr/cap3.php) was used.

Phylogenetic analysis was performed with all full length *At*XTH sequences publicly available for *Arabidopsis thaliana* and jute. The tree was generated by MEGA 4.1 (Tamura *et al.*, 2007) using neighbor-joining method.

3.4 RESULTS

Identification of mRNA transcripts that have different expression patterns between *Macrophomina phaseolina* sensitive and resistant jute species were carried out by differential display. Each experiment was repeated by using total RNA from at least two independent preparations.

3.4.1 Culture of *Macrophomina phaseolina*

Macrophomina phaseolina were grown in potato dextrose agar (PDA) solid media. It took at least 3-4 days for optimum growth on the solid medium (Figure 3.4.1). This fungus was used as the inoculum for infection of jute seedlings.

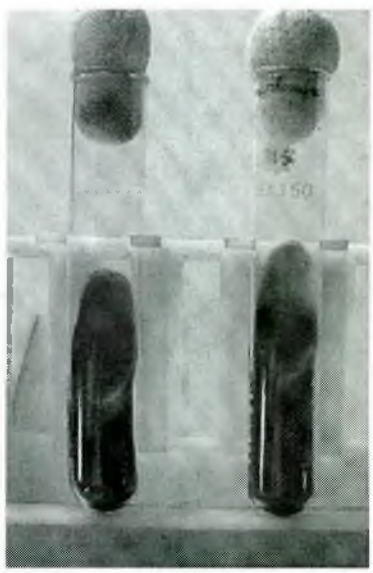


Figure 3.4.1: Growth of *Macrophomina phaseolina* in PDA media.

3.4.2 Infection of jute with *Macrophomina phaseolina*

Seeds of two jute species (*Corchorus olitorius* Var. O-72 and *C. trilocularis*) were germinated on petri dishes. *C. olitorius* Var. O-72 showed better germination compared to *C. trilocularis*. Seeds of *C. trilocularis* were smaller and required prior rubbing to roughen up the surface for loosening of the hard seed coat.

When the fungal suspension was sprayed on the seedlings clear changes were visible in the *C. olitorius* seedlings, particularly in the root. The roots became yellowish-

brown after overnight fungal exposure and the change increased with time. On the contrary, all *C. trilocularis* seedlings showed normal growth.

3.4.3 Total RNA from sensitive and resistant plant

Total RNA was isolated from both sensitive and resistant jute species. There were six samples in total for differential display study. One sample from each species was uninfected and the other two were predisposed to *M. phaseolina* for different time periods. Table 3.4.1 shows the samples and their respective incubation period with *M. phaseolina*. Total RNA was isolated from treated and untreated samples and were quantified with a spectrophotometer, SPECORD ® 50, using software WinASPECT® (Germany). WinASPECT® provided a tentative assessment of RNA quality and quantity.

Table 3.4.1: Different samples for differential gene expression study.

Species	Sample	Age before RNA isolation
C. olitorius (BJRI tossa pat-4; O-72)	S_0	3 days uninfected + 15 hours of uninfected growth
	S_{15}	3 days uninfected + 15 hours of infected growth
	S_{24}	3 days uninfected + 24 hours of infected growth
C. trilocularis (Wild species)	T_0	3 days uninfected + 15 hours of uninfected growth
	T_{15}	3 days uninfected + 15 hours of infected growth
	T_{24}	3 days uninfected + 24 hours of infected growth

Each of the RNA sample were run on MOPS containing agarose gel to check the integrity of RNA. The gel photograph of RNA showed good quality un-degraded RNA (Figure 3.4.2).

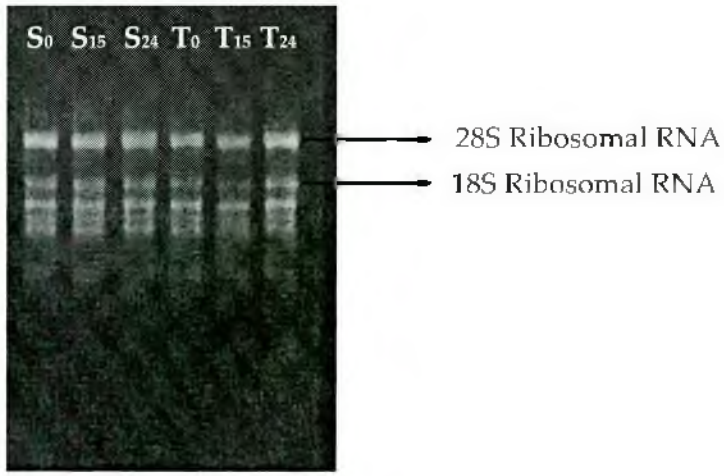


Figure 3.4.2: Agarose gel electrophoresis to check the quality of RNA.

3.4.4 Reverse transcription of RNA and amplification of cDNA were performed using anchor and arbitrary primers

cDNA synthesis by reverse transcription was done according to manufacture protocol (section 3.3.10). Later using these cDNA as template PCR was done using both anchor and arbitrary primer. The following table shows the combination of arbitrary and anchor primers used for differential display.

Table 3.4.2: Combinations of anchored and arbitrary primers used for differential display.

Anchored primers	Anchored primers sequence, 5'-3'	Arbitrary primers	Arbitrary primers sequence, 5'-3'
$T_{12}A$	TTTTTTTTTTTA	ARB-11	CTGGCGTGTCA
$T_{12}G$	TTTTTTTTTTTG	ARB-11	CTGGCGTGTCA
$T_{12}C$	TTTTTTTTTTTC	ARB-11	CTGGCGTGTCA

Amplified products were separated in 10% polyacrylamide gel and visualized by silver staining. Separation on agarose gel was not good enough to allow identification of differentially amplified products.

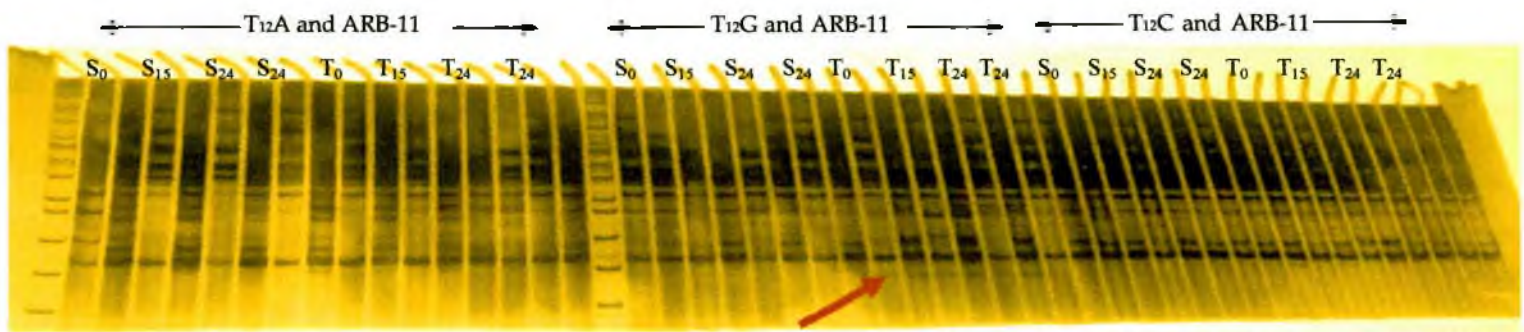


Figure 3.4.3: cDNA differential display using $T_{12}A$ and ARB 11, $T_{12}G$ and ARB 11 and $T_{12}C$ and ARB11 primers (S_0= Susceptible plant without infection, S_{15}= Susceptible plant after 15 hours of infection, S_{24} = Susceptible plant after 24 hours of infection, T_0= Tolerant plant without infection, T_{15} = Tolerant plant after 15 hours of infection, T_{24}= Tolerant plant after 24 hours of infection)

Different primer combinations were used in two PCR events significant number of amplified products were observed which allowed us to monitor the differential expression pattern of the treated samples (Table 3.4.3).

Table 3.4.3: Number of DNA fragments amplified during two PCR events.

Anchored primer		Arbitrary primer	Total bands (number)	Average bands (number)
$T_{12}A$	1st PCR	ARB-11	86	87
	2nd PCR	ARB-11	88	
$T_{12}G$	1st PCR	ARB-11	90	90
	2nd PCR	ARB-11	90	
$T_{12}C$	1st PCR	ARB-11	96	94.5
	2nd PCR	ARB-11	93	

During these two PCR events, almost equal number of bands was produced, indicating the reproducibility of the result. There were some bands which amplified only in the three samples from the susceptible species (S_0, S_{15} and S_{24}). The opposite also happened. In some other cases, bands appeared only in samples which were infected with fungus for a 15 and 24 hours. Therefore, both constitutive and induced pattern of differential gene expression were discernable using this differential display technique. However, from the observed polymorphic display one polymorphic band was selected for cloning and molecular characterization. The selected polymorphic band was constitutively expressed in tolerant plants and inducibly expressed in susceptible plants. That particular band was eluted from PAGE and checks it to the agarose gel (Figure 3.4.4).

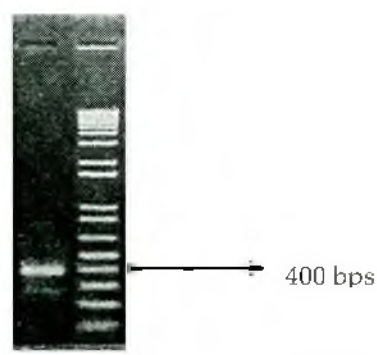


Figure 3.4.4: Differentially expressed band eluted from PAGE and check in agarose gel.

3.4.5 Cloning of differentially expressed polymorphic band

Cloning of the differentially expressed band was performed using Invitrogen's pCR®8/GW/TOPO® TA Cloning® Kit according to the manufacturer protocol. The resulting ligated construct was transformed into chemocompetent *E. coli* (DH5α) cells.

3.4.6 Colony screening and sequencing

Plasmids were isolated from selected colony and PCR performed with M13 Forward and M13 Reverse primer pair. Finally PCR product was sent to molecular service centre of 1ST BASE, Malaysia for sequencing. Sequence is given below:

>Sequence (264 bp)

CTGGCGTGTCAGCATGAAGATCAAGCTCGTCCCCGGTGACTCTGCCGGAACAGTCACCGCCTTTTATCTGAACTCC
GACACAGATACTGTAAGAGACGAACTGGATTTTGAGTTCTTGGGAAACCGAAGTGGGCAGCCATACACTGTCCAAA
CAAACGTGTTCGCACATGGAAAGGGTGACAGGGAACAAAGGGTTAATCTCTGGTTTGATCCATCTCAAGATTTCCA
TGATTACCAAATTAGATGGAACCATAAGGAAATTGT

BLASTn and BLASTx output indicated (Table 3.4.4 and 3.4.5) that the cloned sequence has high homology to Endoxyloglucan transferase (XTH), an enzyme responsible for rearrangement of cellulose/xyloglucan framework in plant cell wall (Albert et al., 2004).

3.4.7 Isolation of full length gene from both resistant and sensitive jute species

Primer based gene walking, 3' RACE and 5' RACE were performed to obtain full length sequence of Xyloglucan endotransglycosylase gene.

3.4.7.1 Primer based gene walking

A downstream degenerate primer (D2 Rev) was designed and PCR was performed using a gene specific primer (EXTGF) and D2 Rev primer (primer list are given in Table 3.3.1). Primer based gene walking yielded a 672 bps fragment of putative XTH gene (Figure 3.4.5).

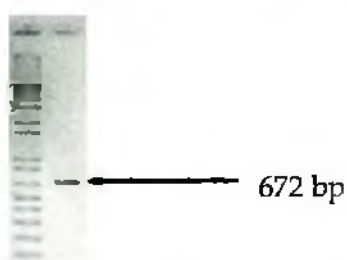


Figure 3.4.5: PCR product of "EXTGF and D2 Rev" primer pair.

The 672 bp product was assembled with the initial 264 bp sequence using CAP 3. Assembled sequence is given below:

>Sequence (EXTGF and D2 Rev, 672 bp)

GTCCAAACAAACGTGTTCGCACATGGAAAGGGTGACAGGGAACAAAGGGTTAATCTCTGGTTTGATCCATCTCAAG
ATTTCCATGATTACCAAATTAGATGGAACCATAAGGAAATTGTGTAAGTAAAATTTCAAAACAATAATGCCTATAG
CTACAGAAATTTTTACATAGCAGCCGACACTTGTATGGTAATGCAATATGTGAGAGTGAGAGAGAGAAAAATATCT
ATCAAATCAGTAAAAAGTTGATACATCAACTTTATTATTAAAGTCAAGCATATAGTATGATTTAATTGGGATATTA
ATAACTTTTTAATTGTGTTAAAATGAGTAATGGGTGGTTAAAAAAGTTTGTAATTTTACAGGTTTTTGGTAGATGA
TACGCCAATCAGAGTTTACAAGAACAATGAAGCAAAAGGTGTCCCATACCCAAAATCCCAACCCATGGGAGTATAT
TCAACACTTTGGGAAGCCGATGATTGGGCGACAAGGGGTGGATTGGAGAAAATTGATTGGAGCAAAGCTCCTTTCT
TAGCTTATTACAAGGATTTCGACATCGAAGGTTGTGCTGTTCCTGGACCAACTAATTGTGCCTCAAACCCTAGGAA
TTGGTGGGAAGGCACTGCTTATCAAGGCCTTAATGCCTTGGAGGCTCAAGGATACCGGTGGGGT

3.4.7.2 3'-RACE of XTH

To explore the 3' end of the putative XTH gene, 3' RACE was performed. cDNA was synthesized by using adapter primer AP (Provided by 3' RACE Kit, Invitrogen™). The RACE yielded 771 bp of XTH gene fragment (Figure 3.4.6).

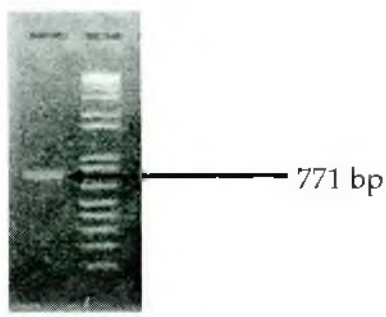


Figure 3.4.6: 3' RACE product using "EXGT 3' end For and AP" primer pair.

>Sequence of 3' RACE product

GCAGCCGACACTTGTATGGTAATGCAATATGTGAGAGTGAGAGAGAGAAAAATATCTATCAAATCAGTAAAAAGTT
GATACATCAACTTTATTATTAAAGTCAAGCATATAGTATGATTTAATTGGGATATTAATAACTTTTTAATTGTGTT
AAAATGAGTAATGGGTGGTTAAAAAAGTTTGTAATTTTACAGGTTTTTGGGAGAGGATACCCCAATCAGAGTTTAC
AAAAACAAAGAAGCAAAAGGGGTCCCATACCCAAAATCCCAACCCATGGGAGGATATTCAACACTTTGGGAAACCG
ATGATTGGGCGACAAGGGGGGGATTGGAGAAAAATGATTGGAGCAAAACTCCTTTCTTAGCTTATTACAAGGATTT
CGAAATCGAAGGGTGGGCTGTTCCCGGACCAAATAATTGTGCCCCAAACCCTAGGAATTGGGGGGAAGGCACTGCT
TATCAAGGCCTTAAACCCTTGGAAAATAAAAAAAACAGGGGGGTTCGTATGAACCAAATGATCTATGATTATTGCA
CCGACAAATCCCGGGACCCGGTTCCCCCACCGGAAAGCACCCCCGGCATCTAAAAATCCTATTTGGAAAATTCCTT
ATCCAAGCTCAAATGTTCAGGACATTTGAAAAAAAAAGTTTCATTTGTCATCAAACTTTTTACGTACAAGCAGCCC
CCCTTTGTACAAAAGCTATATATATAGCCTTATAAGAGAGCCATGCAAAAAAAAAAAAAAAAAGTACTAGTCGCGC
GGTGGGCACAA

3.4.7.3 5'-RACE of XTH

5' end of the gene was generated by 5' RACE. One primer was designed for cDNA synthesis from RNA (GSP) and another gene specific primer GSP2 was used for confirmation by nested PCR. RACE yielded 470 bp fragment of XTH gene.

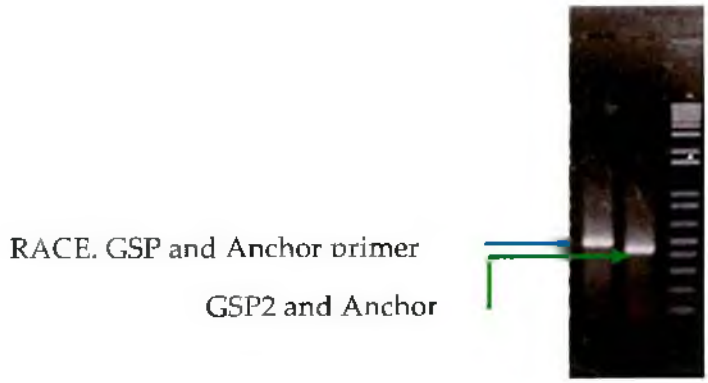


Figure 3.4.7: 5' RACE using GSP and Anchor primer and confirmation by nested PCR using GSP2 and Anchor primer.

> Sequence of 5' RACE product (446 bp)

ATCTGTAAACCAAAAACTCAATATTTGTAATTCAAAACACGACAATGGGTTTAAATTTAAACAATGGTTTTCTTTT
GCTCTTCTCTTGCATTATTCTTGCAATTTCCCTCTCTGTTTCGGGTCGACCCGCCACATTTCTTGAAGATTTCCGA
GTCACTTGGGCTGATTCCCATATTAAGCAACTCGATGGAGGGAGGACCATCCAACTTGTTCTAGACCAAAATTCAG
GATGTGGATTTGCTTCCAAAAGCAAGTATTTGTTCGGACGTGTCAGCATGAAGATCAAGCTCGTCCCCGGTGACTC
TGCCGGAACAGTCACCGCCTTTTATCTGAACTCCGACACAGATACTGTAAGAGACGAACTGGATTTTGAGTTCTTG
GGAAACCGAAGTGGGCAGCCATACACTGTCCAAACAAACGTGTTCGCACATGGAAAGGGTGACAGG

3.4.8 Assembled sequence from both resistant and sensitive jute species

The initial sequence, primer based gene walking sequence and RACE sequences were assembled using Cap3 software.

3.4.8.1 Sequence from resistant specie, *Corchorus trilocularis* L.

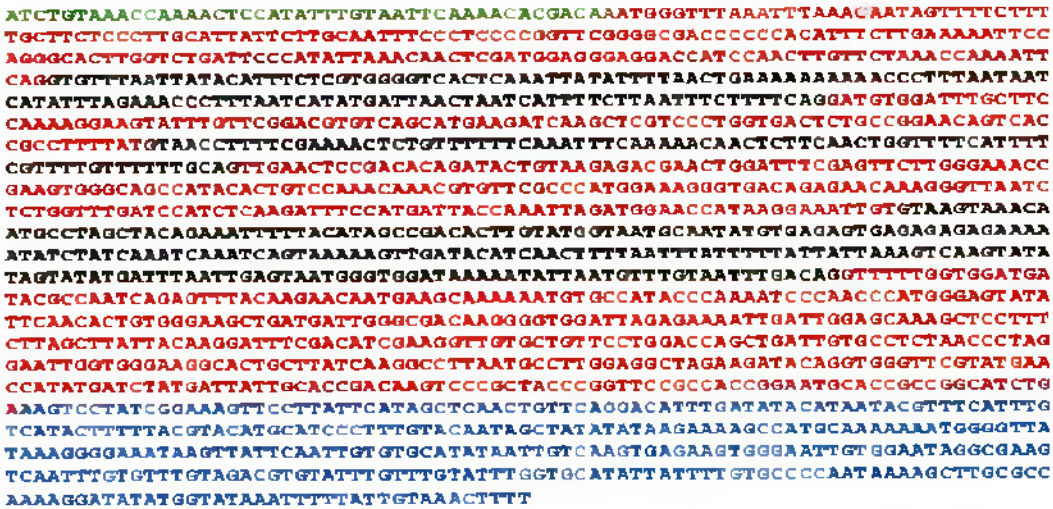
>*Ct*XTH

ATCTGTAAACCAAAAACTCAATATTTGTAATTCAAAACACGACAATGGGTTTAAATTTAAACAATGGTTTTCTTT
TGCTCTTCTCTTGCATTATTCTTGCAATTTCCCTCTCTGTTTCGGGTCGACCCGCCACATTTCTTGAAGATTTCC
GAGTCACTTGGGCTGATTCCCATATTAAGCAACTCGATGGAGGGAGGACCATCCAACTTGTTCTAGACCAAAATT
CAGGTGTTTATATATATACATTTCTCGTGGGGTCACTCTAATTATATTTTAAGTGAAAAAAAAAAAAAAAAAAAA
AACCTTTAATCATATTTAGAAACCCTTTAATCATATGATTAACTAACCATTTTCTTAATTTGTTTTCAGGATGTG
GATTTGCTTCCAAAAGCAAGTATTTGTTCGGACGTGTCAGCATGAAGATCAAGCTCGTCCCCGGTGACTCTGCCG
GAACAGTCACCGCCTTTTATGTAAACCTTTCGAAAAACTCTGTTTTTCAAATTTCAAAAACCAAACTCTGTTTAA
TCATCAGCTTCTTAATTAACTTGTTTCCATTTGCAGCTGAACTCCGACACAGATACTGTAAGAGACGAACTGGAT
TTTGAGTTCTTGGGAAACCGAAGTGGGCAGCCATACACTGTCCAAACAAACGTGTTCGCACATGGAAAGGGTGAC
AGGGAACAAAGGGTTAATCTCTGGTTTGATCCATCTCAAGATTTCCATGATTACCAAATTAGATGGAACCATAAG
GAAATTGTGTAAGTAAAATTTCAAAACAATAATGCCTATAGCTACAGAAATTTTTACATAGCAGCCGACACTTGT
ATGGTAATGCAATATGTGAGAGTGAGAGAGAGAAAAATATCTATCAAATCAGTAAAAAGTTGATACATCAACTTT
ATTATTAAAGTCAAGCATATAGTATGATTTAATTGGGATATTAATAACTTTTTAATTGTGTTAAAATGAGTAATG
GGTGGTTAAAAAAGTTTGTAATTTTACAGGTTTTTGGGAGAGGATACCCCAATCAGAGTTTACAAAAACAAAGAA
GCAAAAGGGGTCCCATACCCAAAATCCCAACCCATGGGAGGATATTCAACACTTTGGGAAACCGATGATTGGGCG
ACAAGGGGGGGATTGGAGAAAAATGATTGGAGCAAAACTCCTTTCTTAGCTTATTACAAGGATTTCGAAATCGAA
GGGTGGGCTGTTCCCGGACCAAATAATTGTGCCCCAAACCCTAGGAATTGGGGGGAAGGCACTGCTTATCAAGGC
CTTAAACCCTTGGAAAATAAAAAAAACAGGGGGGTTCGTATGAACCAAATGATCTATGATTATTGCACCGACAAA
TCCCGGGACCCGGTTCCCCCACCGGAAAGCACCCCCGGCATCTAAAAATCCTATTTGGAAAATTCCTTATCCAAA
GCTCAAATGTTCAGGACATTTGAAAAAAAAAGTTTCATTTGTCATCAAACTTTTTACGTACAAGCAGCCCCCCTT
TGTACAAAAGCTATATATATAGCCTTATAAGAGAGCCATGCAAAAAAAAAAAAAAAAAAGTACTAGTCGCGCGGTG
GGCACAA

The green region shows the 5' UTR and the blue region shows the 3' UTR, the black region shows the intron and the red region shows the coding sequence.

3.4.8.2 Sequence from resistant specie, *Corchorus olitorius* L.

>CoXTH



The green region shows the 5' UTR and the blue region shows the 3' UTR, the black region shows the intron and the red region shows the coding sequence.

3.4.8.3 *Ct*XTH protein sequence

The following protein sequence was determined using Expasy Translate Tool.

```
atgggtttaaatttaaacaatggttttcttttgctcttctcttgcattattcttgcaatt
 M  G  L  N  L  N  N  G  F  L  L  L  F  S  C  I  I  L  A  I
tccctctctgtttcgggtcgacccgccacatttcttgaagatttccgagtcacttgggct
 S  L  S  V  S  G  R  P  A  T  F  L  E  D  F  R  V  T  W  A
gattcccatattaagcaactcgatggagggaggaccatccaacttgttctagaccaaaat
 D  S  H  I  K  Q  L  D  G  G  R  T  I  Q  L  V  L  D  Q  N
tcaggatgtggatttgcttccaaaagcaagtatttgttcggacgtgtcagcatgaagatc
 S  G  C  G  F  A  S  K  S  K  Y  L  F  G  R  V  S  M  K  I
aagctcgtccccggtgactctgccggaacagtcaccgccttttatctgaactccgacaca
 K  L  V  P  G  D  S  A  G  T  V  T  A  F  Y  L  N  S  D  T
gatactgtaagagacgaactggattttgagttcttgggaaaccgaagtgggcagccatac
 D  T  V  R  D  E  L  D  F  E  F  L  G  N  R  S  G  Q  P  Y
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 T  V  Q  T  N  V  F  A  H  G  K  G  D  R  E  Q  R  V  N  L
tggtttgatccatctcaagatttccatgattaccaaattagatggaaccataaggaaatt
 W  F  D  P  S  Q  D  F  H  D  Y  Q  I  R  W  N  H  K  E  I
gtgtttttgggagaggataccccaatcagagtttacaaaaacaaagaagcaaaaggggtc
 V  F  L  G  E  D  T  P  I  R  V  Y  K  N  K  E  A  K  G  V
ccatacccaaaatcccaacccatgggaggatattcaacactttgggaaaccgatgattgg
 P  Y  P  K  S  Q  P  M  G  G  Y  S  T  L  W  E  T  D  D  W
gcgacaaggggggattggagaaaaatgattggagcaaaactcctttcttagcttattac
 A  T  R  G  G  L  E  K  N  D  W  S  K  T  P  F  L  A  Y  Y
aaggatttcgaaatcgaagggtgggctgttcccggaccaaataattgtgccccaaaccct
 K  D  F  E  I  E  G  W  A  V  P  G  P  N  N  C  A  P  N  P
aggaattgggggggaaggcactgcttatcaaggccttaaaccccttggaaaataaaaaaaac
 R  N  W  G  E  G  T  A  Y  Q  G  L  K  P  L  E  N  K  K  N
agggggggttcgtatgaaccaaatgatctatgattattgcaccgacaaatcccgggacccg
 R  G  V  R  M  N  Q  M  I  Y  D  Y  C  T  D  K  S  R  D  P
Gttcccccaccggaaagcacccccggcatctaa
 V  P  P  P  E  S  T  P  G  I  -
```

3.4.8.5 *Co*XTH protein sequence

```
atgggtttaaatttaaacaatagttttcttttgcttctcccttgcattattcttgcaatt
 M  G  L  N  L  N  N  S  F  L  L  L  L  P  C  I  I  L  A  I
tccctccccggttcggggcgacccccacatttcttgaaaaattccagggcacttggtct
 S  L  P  G  S  G  R  P  P  T  F  L  E  K  F  Q  G  T  W  S
gattcccatattaaacaactcgatggagggaggaccatccaacttgttctaaaccaaaat
 D  S  H  I  K  Q  L  D  G  G  R  T  I  Q  L  V  L  N  Q  N
tcaggatgtggatttgcttccaaaaggaagtatttgttcggacgtgtcagcatgaagatc
 S  G  C  G  F  A  S  K  R  K  Y  L  F  G  R  V  S  M  K  I
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 K  L  V  P  G  D  S  A  G  T  V  T  A  F  Y  L  N  S  D  T
gatactgtaagagacgaactggatttcgagttcttgggaaaccgaagtgggcagccatac
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 P  Y  P  K  S  Q  P  M  G  V  Y  S  T  L  W  E  A  D  D  W
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 A  T  R  G  G  L  E  K  I  D  W  S  K  A  P  F  L  A  Y  Y
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 K  D  F  D  I  E  G  C  A  V  P  G  P  A  D  C  A  S  N  P
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 R  N  W  W  E  G  T  A  Y  Q  G  L  N  A  L  E  A  R  R  Y
aggtgggttcgtatgaaccatatgatctatgattattgcaccgacaagtcccgctacccg
 R  W  V  R  M  N  H  M  I  Y  D  Y  C  T  D  K  S  R  Y  P
gttccgccaccggaatgcaccgccggcatctga
 V  P  P  P  E  C  T  A  G  I  -
```

3.4.9 BLASTn of *Ct*XTH and *Co*XTH

The result of the BLASTn is given in Table 3.4.4.

Accession	Description	E value	Max identity
AY189971.2	*Gossypium hirsutum* xyloglucan endotransglycosylase mRNA	0.0	84%
EU494963.1	*Malus x domestica* xyloglucan endotransglucosylase/hydrolase	2e-174	76%
AF093507.1	*Medicago truncatula* xyloglucan endo-transglycosylase-like protein	9e-153	75%
AK227046.1	*Arabidopsis thaliana* mRNA for xyloglucan endotransglycosylase	4e-132	74%
D16455.1	*Glycine max* mRNA for endo-xyloglucan transferase	5e-26	67%

The second column represents the name of the sequences of the other five species with which the query sequence showed highest identity. *Ct*XTH and *Co*XTH showed high homology with *Gossypium hirsutum* XTH.

3.4.10 BLASTx of *Ct*XTH and *Co*XTH

The result of the BLASTx is given in Table 3.4.5.

Matched with	E value	Bit score
Xyloglucan endotransglycosylase/hydrolase [*Gossypium hirsutum*]	3e-127	458
Xyloglucan endotransglycosylase [*Brassica rapa subsp. pekinensis*]	1e-120	436
Xyloglucan endotransglycosylase hydrolase 1 [*Medicago truncatula*]	1e-118	430
EXGT-A4 (ENDOXYLOGLUCAN TRANSFERASE A4) xyloglucan:xyloglucosyl transferase [*Arabidopsis thaliana*]	6e-76	288
Endo-xyloglucan transferase (EXGT) [*Nicotiana tabacum*]	6e-76	288

The first column represents the name of the sequences of the other five species with which the query sequence showed highest bit score.

3.4.11 Sequence analysis

Both the genes have three introns and the coding region has 873 bps (Figure 3.4.8). These two sequences have a similarity of 92% (BLAST2seq). SignalP (Bendtsen *et al.* 2004) analysis shows that for both the proteins, the first 26 amino acids act as signal peptide to guide it to the cell wall.

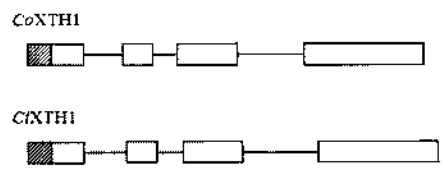


Figure 3.4.8: Schematic diagram of *Ct*XTH1 and *Co*XTH1 gene structure, the white boxes represent exons, lines as introns and the shaded boxes are the putative signal peptide sequences.

3.4.11.1 Multiple Sequence Alignment

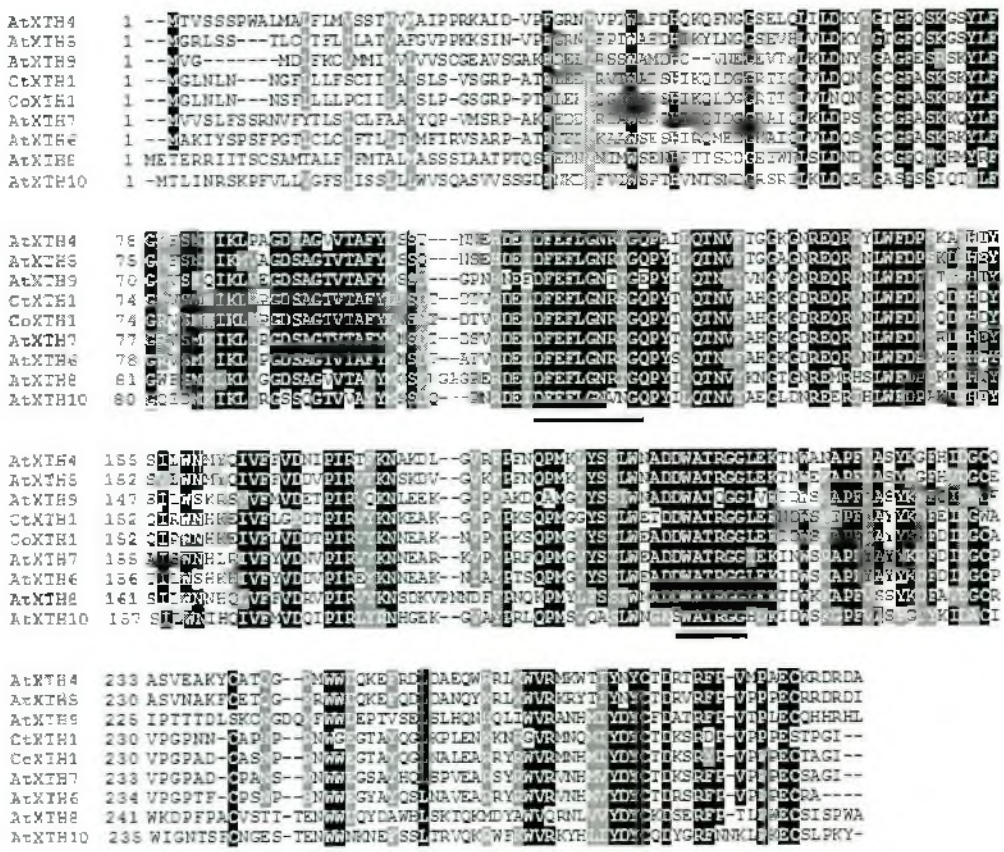


Figure 3.4.9: Alignment of the amino acid sequences of seven *Arabidopsis* group I XTH genes and *Ct*XTH1 and *Co*XTH1, constructed using ClustalW program and drawn with the Boxshade program. The DELDFELFG and DWATRGG motifs are underlined.

3.4.11.2 Phylogenetic analyses

The evolutionary history *Ct*XTH1 and *Co*XTH1 gene sequences were inferred using the UPGMA method (Sneath and Sokal, 1973). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. Phylogenetic analyses were performed using MEGA4.1 (Tamura *et al.*, 2007).

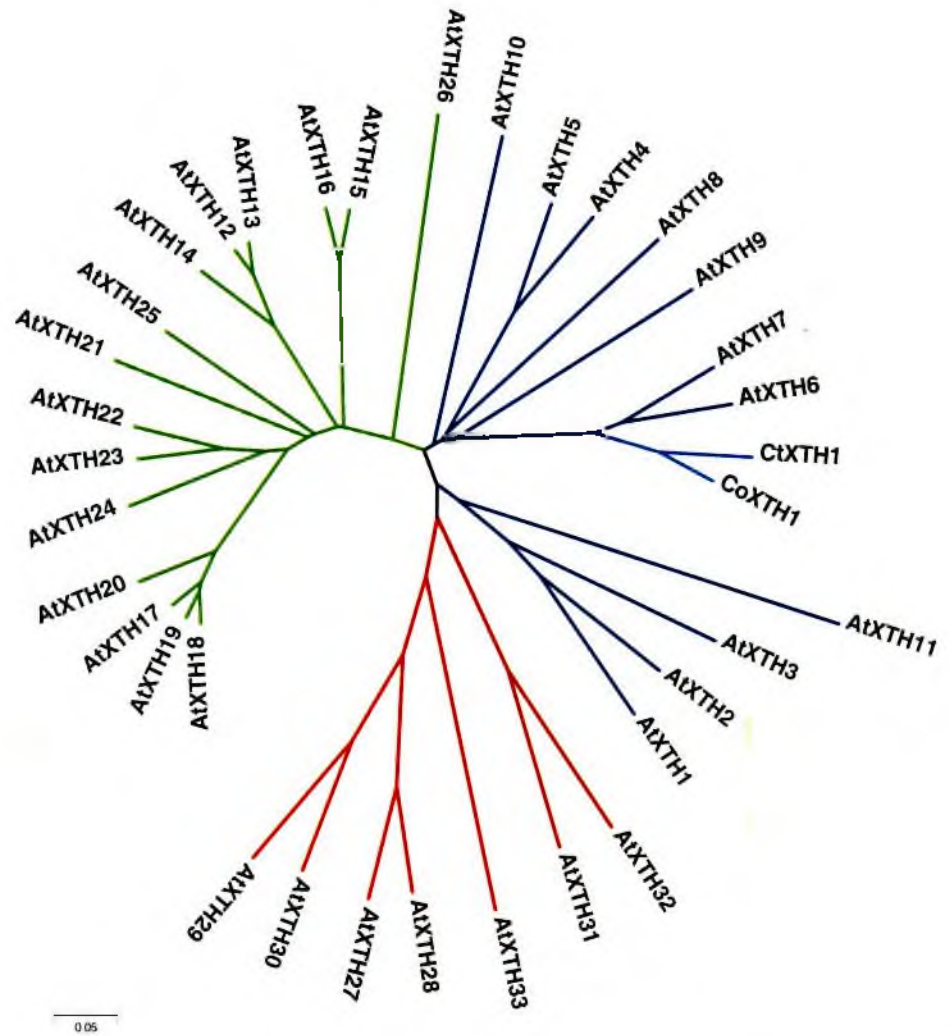


Figure 3.4.10: Phylogenetic relationship of *Co*XTH1 and *Ct*XTH1 with *Arabidopsis* XTH genes. The tree was generated by MEGA 4.1 (Tamura *et al*., 2007) using neighbor-joining method and *p*-distance correction. The *Arabidopsis* genes from Group 1, 2 and 3 are shown in blue, green and red, respectively.

3.4.12 Southern blot analysis

To determine copy number of XTHs in *C. olitorious* and *C. trilocularis*, gDNA from both the plant species were digested with *Eco* RI and *Bam* HI as these restriction enzymes do not have recognition sites within *Ct*XTH1 and *Co*XTH1 genes. Digested gDNA was hybridized with cDNA derived probes. For both the species several hybridizing bands were detected (Figure 3.4.11). This result indicated that jute might have multiple members of this gene family and different members are present in the species under study.

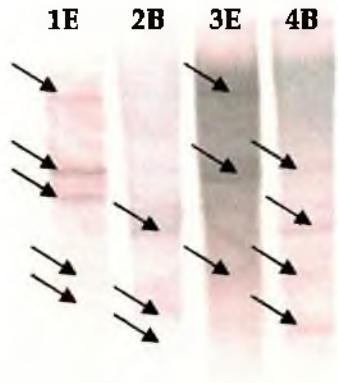


Figure 3.4.11: Southern hybridization analysis of genomic DNA from *C. olitorius* (Lane 1 & 2) and *C. trilocularis* (Lane 3 & 4) digested with EcoRI (E) and BamHI (B). Each arrow shows a signal and multiple arrow indicates the presence of multiple gene in jute genome.

3.4.13 Gene expression

The expression of gene *Co*XTH and *Ct*XTH were determined by semi-quantitative PCR and Northern blot analysis.

3.4.13.1 Semi-quantitative Reverse Transcription PCR

RT-PCR with mRNA of S_0, S_{15} and S_{24} (*C. olitorious*) and T_0, T_{15} and T_{24} (*C. trilocularis*) was performed using EXGT 5F and EXGT GR Rev primers. The *Co*XTH gene showed elevated expression after fungal infection. However, no significant change of *Ct*XTH expression was observed after 24 hours of infection (Figure 3.4.12).

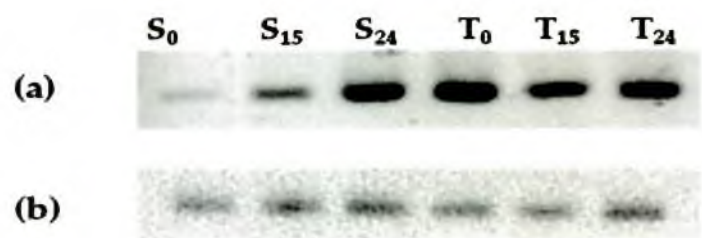


Figure 3.4.12: (a) RT-PCR expression analysis of three *C. olitorious* samples (S_0, S_{15} and S_{24}) and three *C. trilocularis* samples (T_0, T_{15} and T_{24}) using XTH 3'F and XTH 3'R primers. (b) The housekeeping gene β-actin was used as acontrol.

3.4.13.2 Northern blot analysis

Northern blot analysis (Figure 3.4.13) confirmed the differential expression of XTH gene in fungus resistant (*Corchorus trilocularis)* and susceptible (*Corchorus olitorius*) jute species. Result of this blot indicated that the expression increased after fungus infection in sensitive jute species, whereas expression remained almost the same in resistant jute species.

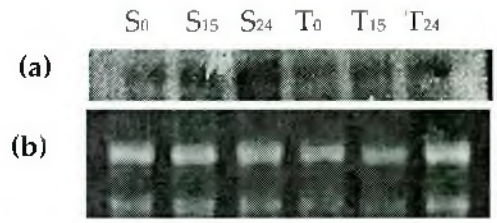


Figure 3.4.13: (a) Northern blot analysis of *M. phaseolina* infected (S_{15}, S_{24}, T_{15} and T_{24}) and uninfected (S_0 and T_0) seedlings. (b) rRNA is shown below as a control for equal loading (c and d).

3.4.14 3D modeling of *Ct*XTH

3D structure of *Ct*XTH was predicted by CPHmodels-3.0 server (http://www.cbs.dtu.dk/services/CPHmodels) and was rendered using Swiss-Pdb viewer (http://spdbv.vital-it.ch/). CPHmodels-3.0 is a protein homology modeling software. The template recognition is based on profile-profile alignment guided by secondary structure and exposure predictions. The model shows that it has only one helix which is observed in the crystal structures of GH16 family proteins.

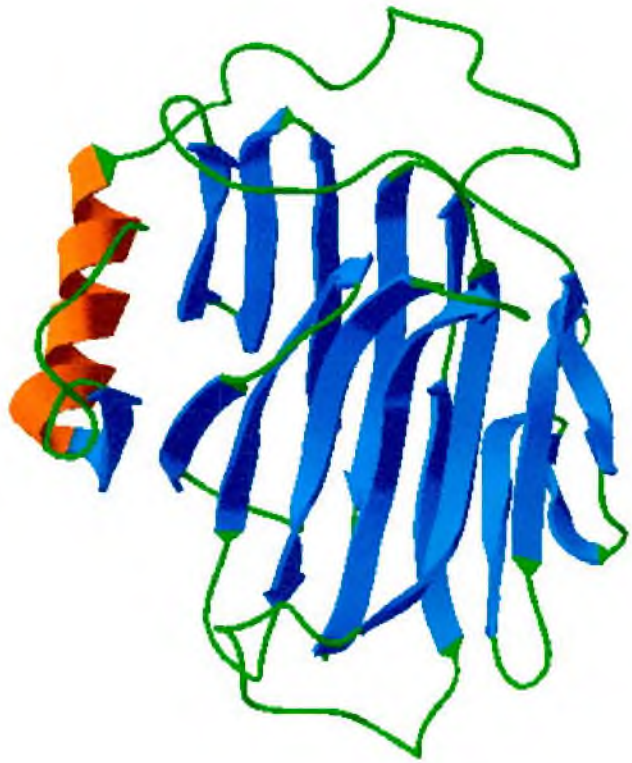


Figure 3.4.14: 3D model of *Ct*XTH1 constructed using CPHmodels-3.0 software and represented using Swiss-Pdb Viewer (Guex and Peitsch, 1997). The model shows β-jellyroll-type structure typical of other GH16 family enzymes (Johansson *et al.*, 2004)

3.5 Discussion

Biotic stresses caused by pathogen, insect and weeds affect both agricultural production as well as quality. About 12% of the agricultural production decreases per year due to the plant pathogen (Cook, 2006).

In the present study, the technique of differential display was used to identify gene(s) related to tolerance to a fungal pathogen. Differential gene expression was studied between a fungus (*Macrophomina phaseolina*) resistant (*C. trilocularis*) and a fungus susceptible (*C. olitorius*) jute species, under both uninfected and infected conditions at different time intervals to identify both constitutive and inducible changes in expression.

Total RNA from these plants were collected and the quality and quantity were determined. Quality of isolated RNA was checked by electrophoresis. Distinct bands of ribosomal RNAs (Figure 3.4.1) indicated presence of un-degraded RNA in samples.

Although most of the published technical modifications and refinements aim to reduce the amount of spurious bands and improve the sensitivity of the differential display, false positives do sometimes occur (Luce and Burrows, 1998). False positive results in differential display may result from a number of intrinsic and extrinsic factors intimately linked to the technique. Factors, such as the systems being compared, experimental designs, appropriate internal controls, criteria for picking bands, reaction setup, type of PCR tube, type of thermocycler, variation in the quality of RNA samples, primers, Moloney murine leukemia virus reverse transcriptase, *Taq* polymerase, and PCR tubes and, of course, the training and experience of a researcher, all can contribute greatly to the rate of false positives (Liang, 1998). Therefore, in all of the reactions, parameters were strictly kept the same. Particularly, each of the isolated RNA samples were aliquoted to a number of micro-centrifuge tubes to avoid repeated thawing before use, as this severely damages RNA. The same thermocycler was used for all RT-PCRs in this experiment.

Different PCR reaction tubes have been reported to have a drastic effect on the results of PCR amplification in differential display. Thin-walled tubes generally perform better than regular tubes in terms of band intensity and reproducibility (Liang and Pardee, 1995). Therefore, in all reactions thin walled PCR tubes were used.

Although the RNA samples of both the species were collected separately, with the exception of very few spurious bands, most bands were reproduced at different time intervals (Table 3.4.3). In this experiment, susceptibility of *C. olitorius* Var. O-72 to *M. phaseolina* was evident from the symptomic response (browning) of seedling roots which increased with infection period. However, *C. trilocularis* remained uninfected.

Resistance of *C. trilocularis* to *M. phaseolina* may be due to constitutive or induced expression of some genes in response to infection. So, this study aimed at identifying differentially expressed genes that show constitutive as well as induced expression (Figure 3.4.3). The selected polymorphic bands were reproduced in all three *C. trilocularis* samples (T_0, T_{15} and T_{24}). The same master mixture for PCR was used for all six samples of both species.

In this study, simpler oligo-dT primers were not used as these cause smearing by binding to different positions of poly-A tail of the same mRNA. 20 bases or longer primers are used to amplify specific genes and shorter primers are used to amplify multiple genes, longer primers at low temperature (38°- 42°C) when used for differential display study produces complex and non-reproducible results (Liang, 1998). For cloning purposes, TA cloning vector (Invitrogen™) was used as no restriction enzymes or post-PCR events were required for cloning.

The identified sequence turned out to be XTH (xylogluxcan endo-transglucxosylase/hydrolase), which like other XTHs, belongs to a multigene family-GH16 glycoside hydrolase (Figure 3.4.14). In *Arabidopsis* thirty three and in rice twenty nine ORFs, which potentially encode XTH proteins, have been identified (Yokoyama and Nishitani, 2001). To investigate the evolutionary relationship of *Ct*XTH with other members of the group, a phylogenetic tree was constructed

(Figure 3.4.10). Rose *et al.*, (2002) classified *Arabidopsis* XTHs in three major groups. Phylogenetic analysis with 33 *Arabidopsis* XTHs puts *Ct*XTH1 and *Co*XTH1 with group I genes. As shown in Figure 3.4.10, they were placed in the same clade with seven *Arabidopsis* group I genes (*At*XTH 10, *At*XTH 5, *At*XTH4, *At*XTH 8, *At*XTH 9, *At*XTH 7 and *At*XTH 6). Michailidis *et al.*, (2009) classified the rest of the group I genes (*At*XTH 1, *At*XTH 2, *At*XTH 3 and *At*XTH 11) as ancestral group and they reside in a different clade.

In the multiple sequence alignment, the conserved motif DEIDFELFG, which has been described as the catalytic site (Xu *et al.*, 1996) appeared in *Ct*XTH1 and *Co*XTH1 as DELDFELFG, whereas, the sequence of the motif for the seven *Arabidopsis* group I genes was found as DE(I/F/L)DFELFG (Figure 3.4.9). In addition, the motif DWATRGG that ensures the hydrophobic stacking interactions with the glucose unit (Baumann *et al.*, 2007) was also present in all species.

*Co*XTH and *Ct*XTH genes are probably equivalent of *Gossypium* as they are of same size in jute and these genes are found to share high sequence identity with its *Gossypium* ortholog 84%, (Table 3.4.4). The existence of this level of structural similarity is not unexpected for XTHs, as data from *A. thaliana* have shown that there is a striking level of both structural and functional similarity between its 33 XTH family members (Yokoyama and Nishitani, 2001).

Expressions of these two (*Co*XTH and *Ct*XTH) genes were studied using semi-quantitative RT-PCR and Northern blotting (Figure 3.4.12 and 3.4.13). Both experiments showed an increase of XTH expression in the sensitive plant (*Co*XTH) after fungal infection whereas expression remained more or less same in the resistant plant (*Ct*XTH).

Although there are a number of studies on XTH genes, relationship between expression pattern and function is still a paradox. Previous studies have reported that the 33 *Arabidopsis* XTH genes exhibit different organ specific expression pattern and respond differently to different sets of plant hormones (Albert *et al.*, 2004; Yokoyama and Nishitani, 2000 and Yokoyama and Nishitani, 2001). XTHs are

thought to play two distinct roles (Smith and Fry, 1991, Nishitani, 1998) that are necessary for cell wall expansion. One is 'integrational' transglycosylation and the other is 'restructuring' transglycosylation. The 'integrational' transglycosylation is a wall strengthening function of XTH where a newly synthesized and secreted xyloglucan chain is grafted to existing wall bound xyloglucan (Thompson *et al.*, 1997). On the other hand, 'restructuring' type of transglycosylation leads to cutting and joining of previously wall bound xyloglucan chains (Albert *et al.*, 2004). Cosgrove (2005) termed XTH as 'wall loosening agent' as transglycosylation leads to irreversible weakening of the cell wall (Smith and Fry, 1991; Nishitani, 1998). It has also been reported that XTH genes show a broad range of responses to biotic and abiotic environmental stresses (Braam and Davis; 1990, Peschke and Sachs, 1994; Xu *et al.*, 1996; Burstin, 2000). XTH expression is also correlated with cell elongation, such as in *Arabidopsis* shoots (Antosiewicz *et al.*, 1997; Xu *et al.*, 1996) and *Arabidopsis* root tips (Vissenberg *et al.*, 2000). Study from Albert *et al.* (2004) showed that the tomato XTH (*Le*XTH1) expression level was increase when attacked by a parasite and not when wounded mechanically. Maldonado-Mendoza *et al.* (2005)also findout that XTH activity was induced systemically in mycorrhizal roots.

From the above discussion and finding of the study, an explanation can be drawn from the expression pattern of *Ct*XTH1 and *Co*XTH1. Although both the enzymes show high homology at the sequence level, the different expression pattern indicates distinct pattern of their regulation. The different regulatory pattern of XTH genes might come from gene duplication and evolutionary diversification that ensures their tissue and hormone specific expression pattern (Xu *et al.*, 1996). Compared to its high yielding counterpart (*C. olitorious*), *C. trilocularis* is relatively sturdy and more adaptive (Ahmed, 1968). As *C. trilocularis* comes in contact with the fungus *Macrophomina*, the level of *Ct*XTH1 decreases over time leading to less wall-loosening ensuring resistance against fungal damage. On the other hand, *Co*XTH1 might be under different regulatory circuit hence its expression shows the opposite pattern. Thus it is likely that *Co*XTH1 expression with change of time course after fungal

infection might lead to increased level of 'restructuring' of the cell wall leading to its weakening.

Crystallographic studies of the catalytic modules of enzymes in family GH16 (Keitel *et al.*, 1993; Michel *et al.*, 2001) reveal an identical folding topology consisting of two antiparallel β-sheets that stack to form a β-sandwich consisting of one concave and one convex face. The concave face contains the sugar substrate binding sites, with the catalytic side chains located on a single strand that is offset from the center of the sheet (Stahlberg *et al.*, 1996). Despite a striking lack of sequence identity within the family, the enzymes show strong structural similarity (Figure 3.4.14). However, modeled structure of *Ct*XTH shows that it exhibits β-jellyroll-type structure typical of other family GH16 enzymes.

Overall, this study indicates that *Co*XTH expression is correlated with *C. olitorius-M. phaseolina* interaction, but it remains to be elucidated whether it is an essential component of the signaling pathway involved in fungal defense.

CHAPTER FOUR

OVER EXPRESSION OF *kat*E GENE INTO JUTE GENOME FOR CONFERING SALT TOLERANCE

4.1 Introduction

Agricultural crops face different types of biotic and abiotic stresses. Among abiotic stresses, salt stress is one of the most serious environmental factors limiting the productivity of crop plants (Ashraf, 1999). The total global area of salt-affected soils including saline and sodic soils was 931 million hectares in 2005 (Martinez-Beltran and Manzur, 2005). Now in 6 more years the figure is expected to be higher. According to an estimate by FAO (2008; htp//:www.fao.org/ ag/agl/agII/spush) over 6% of the world's land is salt affected. Saline conditions reduce the ability of plants to absorb water, causing rapid reductions in growth rate, and induce many metabolic changes (Epstein, 1980). If tolerance in plants cannot be improved vast amount of land may have to be left uncultivated in the future.

Ingress of salinity is a major problem in Bangladesh. The coastal zone directly affected by salinity is extensive (Karim *et al.*, 1990). Over thirty percent of the net available cultivable lands of Bangladesh are located in the coastal areas. But it has been observed that all the coastal cultivable lands can not be utilised for crop production, mostly due to soil salinity. Tidal flooding during wet season (June-October), direct inundation by saline or brackish water and uptake or lateral movement of saline groundwater during dry season (November-January) are the causes of soil salinity development. In 1973, 1.5 million hectares of land had mild salinity. In 1997, this expanded to 2.5 million hectares. It is believed that the figure may be more than 3 million hectares (FAO, 2007). The severity of salinity problem in Bangladesh is increasing with the desiccation of soil due to shortage of rainfall and rise in temperature.

Global warming and the resultant climate change could have profound effects on the water resources (both surface and ground water) of Bangladesh (Ahmed, 2006). Moreover Bangladesh is affected by different types of natural disasters. In the

foreseeable future, the country is likely to be affected by the biggest ever, long lasting and global scale human induced disaster - the climate change and sea level rise (CCSLR). Bangladesh is thought to be one of the most vulnerable countries of the world to CCSLR. It is believed that the impact of climate change on physical system in combination with the effect of sea level rise would cause a net increase in salinity in the already affected soils in the coastal regions. A GCM modeling approach has indicated that, under changed climate conditions the index of aridity will increase during winter (Ahmed *et al.*, 1996). As a result, an increased rate of desiccation in topsoil leading to higher rates of capillary action would be observed. Hence the salinity problem would be accentuated by the impacts of climate change and sea level rise. The extent of increase in soil salinity in a particular area within the coastal zone would determine the extent of crop loss in the affected areas.

The progressive salinization of soil in Bangladesh (Figure 4.1.1) has made the genetic improvement of salt tolerance an urgent priority for future agriculture. Two basic genetic approaches that are currently being used to improve stress tolerance include: (i) exploitation of natural genetic variations, either through direct selection in stressful environments or through the mapping of quantitative trait loci (Flowers, 2004; Foolad, 2004; Lindsay *et al.*, 2004) and subsequent marker-assisted selection, and (ii) generation of transgenic plants by introducing novel gene(s) or by altering expression levels of the existing gene(s) to affect the degree of salt stress tolerance (Joseph and Jini, 2010).

Improvement of stress tolerance has been one of the major goals of traditional breeding programmes but with a very limited success except for some specific cases (e.g. Ashraf and McNeilly, 1990; Yeo and Flowers, 1986). This has been due not only to the intrinsic limitations of classical breeding techniques but also to the genetic and physiological complexity of tolerance traits (Munns, 2002) which are controlled by a number of salt stress-responsive genes (Zhu 2000; Wang *et al.*, 2003; Ueda *et al.*, 2002). However the mapping of quantitative trait loci (QTL) in tomato (Foolad *et al.*, 2001) and rice (Koyama *et al.*, 2001), and methods such as somatic hybridization (Wei *et al.*, 2001) hold promise as a combination of conventional breeding and a molecular approach.

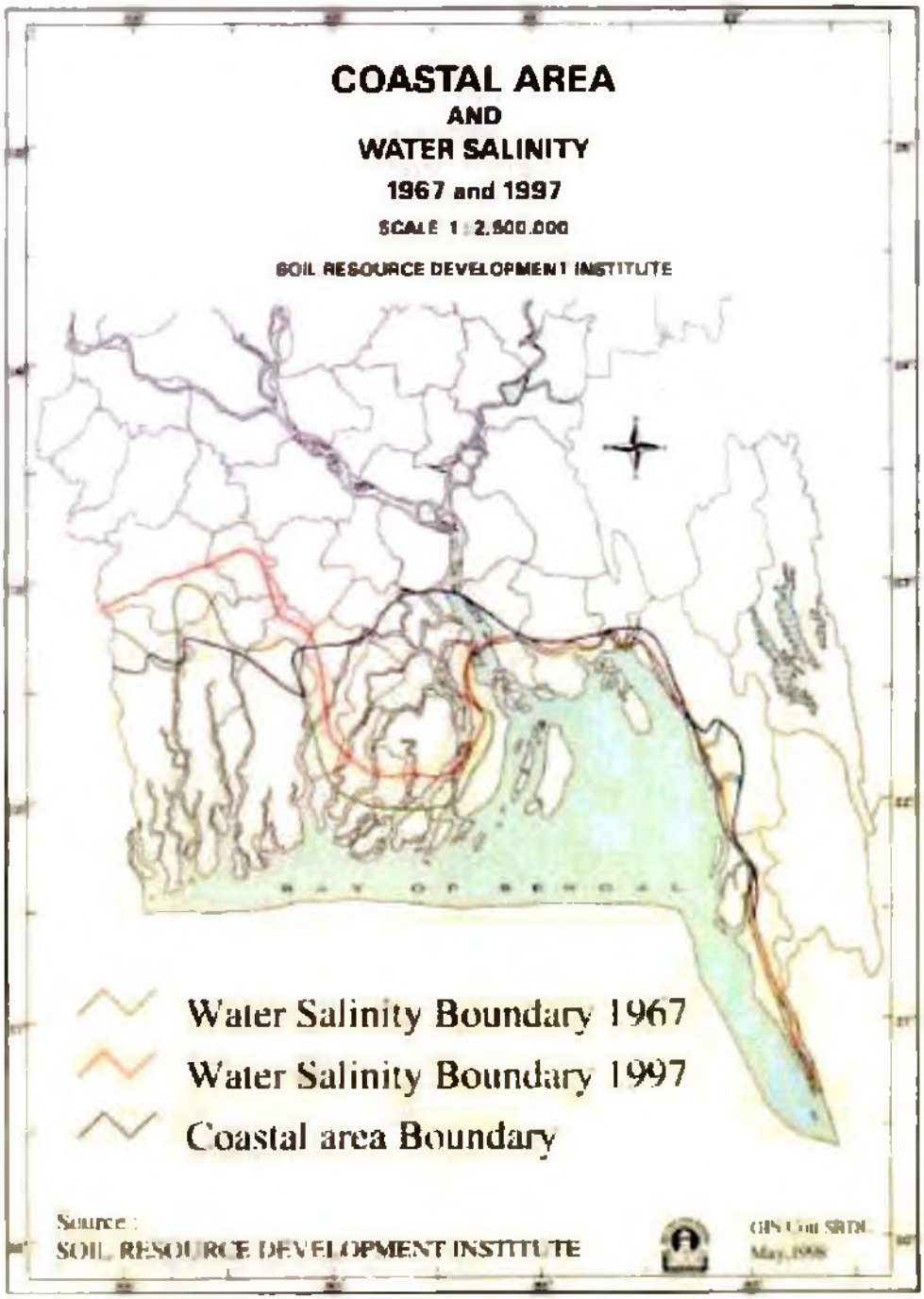


Figure 4.1.1: Water salinity map of Bangladesh (Source: SRDI, 1998)

Genetic engineering of plants has the potential to provide a general and quick method to improve salt tolerance in crop plants (Bajaj *et al.*, 1999). Over the last decade, a large number of papers have been published describing how transformation with different genes confer tolerance to different abiotic stresses, such as low and high temperatures, water or salinity in transgenic plants (Zang *et al.*, 2010; Lal *et al.*, 2008; Moriwaki *et al.*, 2008; Rajagopal *et al.*, 2007; Bourgon *et al.*, 2007). In most cases these genes have been selected because of their likely or demonstrated

participation in the mechanisms of cellular response to stress in plants and microbes. The genes used were those involved in the regulation of ion transport (Apse *et al.*, 1999; Shi *et al.*, 2003; Zhang and Blumwald, 2001), synthesis of osmolytes in the cytoplasm (Kishor *et al.*, 1995; Romero *et al.*, 1997; Sakamoto *et al.*, 1998; Tarczynski *et al.*, 1993) or signal transduction and transcriptional regulation (Kasuga *et al.*, 1999; Piao *et al.*, 2001).

Molecular and biochemical studies indicate that salt stress responses in plants involve significant increases of reactive oxygen species (ROS), including singlet oxygen ($^1O_2$), superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) (Jugklang *et al.*, 2004; Tsai *et al.*, 2004). Excess ROS can seriously disrupt normal metabolism through oxidative damage to lipids, protein and nucleic acids (Fridovich 1986; Davies 1987; Imlay and Linn, 1988). To escape from the toxic action of ROS, plants possess a number of antioxidant systems; for example, superoxide dismutase (SOD) neutralizes the O_2^- and produces H_2O_2, while H_2O_2 neutralization involves other enzymes such as catalase (CAT) and ascorbate peroxidase (APX).

To gain insight into the complex network of genes responsible for salt tolerance, it is important to have information concerning the master genes for salt tolerance. Previously, it was shown that the expression of genes for synthesis of betaine and catalase could confer salt tolerance in the fresh water *Cyanobacterium synechococcus* sp. PCC7942 (Nomura *et al.*, 1995; Deshnium *et al.*, 1995; Kaku *et al.*, 2000). Enzymes that can remove the reactive oxygen species (ROS) produced by the salt stress, play an important role in tolerance. Like the other ROS scavenging enzymes, catalase is up-regulated at the transcriptional level upon exposure to high concentrations of NaCl (Wang *et al.*, 2004). In *Cyanobacteria*, introduction of a catalase gene of *Escherichia coli*, *kat* E, was found to reduce ROS production under salt stress conditions and confer salt tolerance. The result indicates that the quenching of H_2O_2 was an important factor for salt tolerance (Kaku *et al.*, 2000).

The purpose of this study was to introduce the *Escherichia coli kat* E gene in jute (*Corchorus olitorius* L.) and to evaluate the salinity tolerance in these plants. In addition, growth and physiological responses were also studied in both wild type and transgenic plants under stressed and non-stressed conditions.

4.2 Review of literature

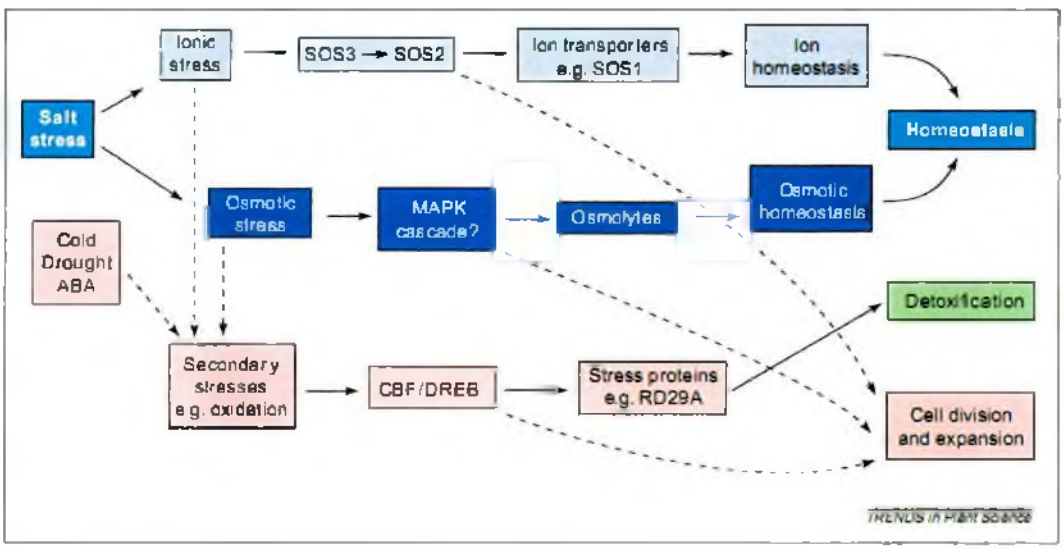
Salinity is one of the major stresses/challenges to the growth of plants and productivity all over the world. According to the FAO Land and Plant Nutrition Management Service, over 800 million hectares of land throughout the world are salt-affected (Munns, 2005). As the salinity of the soils is increasing over the years, it is expected that by 2050, more than 50% of the available land for agriculture will be burdened with salinity (Göl, 2006). Hence the development of salinity-tolerant crops is an urgent priority to sustain agricultural production. Conventional breeding programs aimed at improving crop tolerance to salinity have limited success because of the complexity of the trait (Flowers, 2004). Slow progress in breeding for salt-tolerant crops can be attributed to the poor understanding of the molecular mechanisms of salt tolerance. Therefore, understanding the molecular basis of salt-stress signaling and tolerance mechanisms is essential for breeding and genetic engineering of salt tolerance in crop plants. This chapter reviews the progress to date in understanding salt-stress signaling and efforts to develop salt tolerant plants by conventional breeding and genetic engineering.

4.2.1 Mechanism of salt tolerance

Salinity imposes two kind of stresses on plant tissues, a water-deficit stress that results from the relatively high solute concentrations in the soil, and ion-specific stress resulting from altered K^+/Na^+ ratios and Na^+ and Cl^- concentrations that are inimical to plants (Apse and Blumwald, 2002). Tolerance mechanisms are required to combat the low osmotic potential of the soil solution. The toxicities of high internal Na^+ concentrations also need to be managed through 'exclusion' (i.e. minimize the rate of entry) or compartmentalization of ions into vacuoles within plant cells (Munns, 2002). Salt tolerance requires not only the adaptation to sodium toxicity, but also the acquisition of potassium whose uptake is affected by high external sodium concentration, due to the chemical similarity of the two ions. Therefore, potassium transport systems involving good selectivity of potassium over sodium can also be considered as an important salt tolerance determinant (Rodriguez-Navarro, 2000). In nature plants often develop adaptive strategy to survive a stress period. Adaptive or +presumed adaptive responses may involve: (a) homeostasis that includes ion

homeostasis, which is mainly relevant to salt stress, and osmotic homeostasis or osmotic adjustment; (b) stress damage control and repair, or detoxification; and (c) growth control (Zhu, 2001).

Accordingly, salt and drought stress signaling can be divided into three functional categories: ionic and osmotic stress signaling for the re-establishment of cellular homeostasis under stress conditions, detoxification signaling to control and repair stress damages, and signaling to coordinate cell division and expansion to levels suitable for the particular stress conditions (Selçuk, 2004).



Courtesy: Zhu, 2001.

Figure 4.2.1: Mechanism of salt tolerance.

Three aspects of salt tolerance in plants (homeostasis, detoxification and growth control) and the pathways that interconnect them; homeostasis is broken down into ionic and osmotic homeostasis. The SOS pathway mediates ionic homeostasis and Na^+ tolerance. A mitogen-activated-protein kinase (MAPK) cascade similar to the yeast HOG1 pathways is proposed to mediate osmotic homeostasis. The two primary stresses, ionic and osmotic stresses, cause damage or secondary stresses such as oxidation. Lea type stress proteins such as RD29A are proposed to function in the detoxification or alleviation of damages. CBF/DREB transcription factors mediate some of the stress protein gene expression in response to secondary stresses caused by high salt concentrations, cold, drought or abscisic acid (ABA). The ionic homeostasis, osmotic homeostasis and detoxification pathways are proposed to feed actively into cell division and expansion regulation to control growth (Zhu, 2001).

Homeostasis signaling negatively regulates detoxification responses because once cellular homeostasis is reestablished, stress injury would be reduced, and failure to reestablish homeostasis would aggravate stress injury. Homeostasis and detoxification signaling lead to stress tolerance and are expected to negatively regulate the growth inhibition response, i.e., to relieve growth inhibition of the stress factors (Zhu, 2002).

4.2.2 Soil salinity and crop production

Soil salinity and water salinity are two common hazards that cause unfavourable environment and hydrological situation restricting normal crop production. Salinity acts like drought on plants, preventing roots from performing their osmotic activity where water and nutrients move from an area of low concentration into an area of high concentration (McKenzie, 1998). Therefore, because of the salt levels in the soil, water and nutrients cannot move into the plant roots. Soil salinity imposes ion toxicity, osmotic stress, nutrient (N, Ca, K, P, Fe, Zn) deficiency and oxidative stress on plants. Sodium accumulation in cell walls can rapidly lead to osmotic stress and cell death (Munns, 2002). Ion toxicity is the result of replacement of K^+ by Na^+ in biochemical reactions, and Na^+ and Cl^- induced conformational changes in proteins. For several enzymes, K+ acts as a cofactor and cannot be substituted by Na^+. High K^+ concentration is also required for binding tRNA to ribosomes and thus protein synthesis (Zhu, 2002; Tester and Davenport, 2003). Ion toxicity and osmotic stress cause metabolic imbalance, which in turn leads to oxidative stress (Hernandez, 2001). Soil salinity often leads to the development of other problems in soils such as soil and sodicity alkalinity. Soil sodicity is the result of the binding of Na^+ to the negatively charged clay particles, which leads to clay swelling and dispersal. Hydrolysis of the Na-clay complex results in soil alkalinity (Chinnusamy *et al.*, 2006). Thus, soil salinity is a major factor limiting sustainable agriculture.

Salinity affects almost all plant processes starting with germination (Esechie *et al.*, 2002), through to growth and production (Murillo-Amador *et al.*, 2001; Fricke and Peters, 2002). Plants vary tremendously in their ability to tolerate salt in water (Ashwath, 1987). Based on plant tolerance to salinity, plants are classified as halophytes, which can grow and reproduce under high salinity (>400mM NaCl), and glycophytes, which cannot survive high salinity. Most grain crops and vegetables are glycophytes and are highly susceptible to soil salinity even when the soil electrical

conductivity (Ec) is 4 dS m^{-1} (Chinnusamy *et al.*, 2005). Different threshold tolerance Ec and different rate of reduction in yield beyond threshold tolerance Ec indicate variation in mechanisms of salt tolerance among crop species (Table 4.2.1). Most crop plants are susceptible to salinity even when Ec is 3.0 dS m-1. Salt tolerance depends upon various characters of plants (Flowers and Flowers, 2005), which are - morphology, compartmentalization and compatible solutes, regulation of transpiration, control of ion movement, membrane characteristics, tolerating high Na^+/K^+ ratios in the cytoplasm and salt glands.With so many factors involved, it is expected that salt tolerance would depend on the action of many genes.

Table 4.2.1: Many important crops are susceptible to soil salinity* (Maas, 1990).

Crop	Threshold salinity (d sm-1)	Decrease in yield (slope % per ds m^{-1})
Bean (*Phaseolus vulgaris* L.)	1.0	19.0
Eggplant (*Solanum melongena* L.)	1.1	6.9
Pepper (*Capsicum annuum* L.)	1.5	14.0
Corn (*Zea mays* L.)	1.7	12.0
Sugarcane (*Saccharum officinarum* L.)	1.7	5.9
Potato (*Solanum tuberosum* L.)	1.7	12.0
Cabbage (*Brassica oleracea* var. *capitata* L.)	1.8	9.7
Tomato (*Lycopersicon esculentum* Mill.)	2.5	9.9
Rice, paddy (*Oryza sativa* L.)	3.0	12.0
Peanut (*Arachis hypogaea* L.)	3.2	29.0
Soybean (*Glycine max* (L.) Merr.)	5.0	20.0
Wheat (*Triticum aestivum* L.)	6.0	7.1
Sugar beet (*Beta vulgaris* L.)	7.0	5.9
Cotton (*Gossypium hirsutum* L.)	7.7	5.2
Barley (*Hordeum vulgare* L.)	8.0	5.0

* Lack of a direct correlation between the threshold salinity and yield decrease per unit increase in salinity has been attributed to the differences in salt exclusion, uptake, compartmentalization and other mechanisms of salt tolerance among these crop species.

Xiong and Zhu (2002) worked on oxidative stress on plants and they described four detrimental effects on plants due to salinity stress. The first type of negative effect is osmotic stress. Salt stress alters the water potential in the environment and this causes osmotic stress to plants. Because of high salinity, plants lose their turgor. Another negative effect of salinity is nutrient deficiency. As a result of decreasing water uptake from the soil, the entry of essential minerals such as phosphorus, potassium, nitrate, and calcium, for plant growth is lowered. Ion cytotoxicity is another detrimental effect of salinity (Xiong and Zhu, 2002). Injurious concentrations of Na^+, Cl^- and SO_4^{-2} cause ion toxicity. Oxidative stress occurs as a secondary effect of salinity and it is caused by excessive reactive oxygen species (ROS). Hydrogen peroxide, hydroxyl radicals and superoxide anions belong to ROS. In fact, generation of ROS usually involves normal cellular reactions. However, when plants are subjected to stress, the amount of ROS in the cells are also increases (Göl, 2006).

Salinity generally affects growth rate and it results in plants with smaller leaves, shorter stature and sometimes fewer leaves by reducing growth rate. Osmotic stress forms the initial and primary effect of salinity at low to moderate concentrations (Munns and Termaat, 1986). Salinity changes the roots' structure by reducing their length and mass, therefore roots may become thinner or thicker (Shannon and Grieve, 1999). The osmotic effects of salinity result in reduced growth rate and altered leaf color and changes in developmental characteristics including root/shoot ratio and maturity rate. Timing of development is affected by salinity. Onion plants flower early under salt stress, but flowering of tomato plants is delayed by salinity (Pasternak *et al.*, 1979). Moreover, ionic effects of salinity are generally seen in leaf and meristem damage or typical nutritional disorder symptoms (Göl, 2006). Burning of leaves is an effect that is caused by both salinity and nutritional disorders (Shannon and Grieve, 1999).

In spite of negative effects, salinity may have some good effects on yield, quality and disease resistance. For example, Osawa (1963) worked on spinach at low to moderate salinity and found that salinity may have a positive effect on yield

increase. Sugar content of carrot is also known to increase in the presence of salinity (Bernstein, 1959).

4.2.3 Soil salinity and Bangladesh

Bangladesh is thought to be one of the most vulnerable countries of the world due to climate change and sea level rise. There are a number of environmental issues and problems which are hindering development of Bangladesh. Salinity is one of the major problems which is expected to be exacerbated by climate change and sea level rise. Salinity intrusion due to reduction of freshwater flow from upstream, salinisation of groundwater and fluctuation of soil salinity are major concerns of Bangladesh. This problem is occurring mainly in the coastal belt of Bangladesh which covers about 20 per cent of the country's total area and over 30 per cent of the net cultivable area (Haque, 2006). Presently slight to strong soil salinity problems exist in 20 districts of Bangladesh. Salinity levels vary widely across a saline seep. Salinity also varies from season to season. Salinity usually appears on the soil surface just after rainy season. Status of soil salinity in Bangladesh is given in Table 4.2.2.

Table 4.2.2. Salinity affected areas in the coastal and offshore regions of Bangladesh

Description	Total cultivated area	Saline area	Area of each salinity class (ha)				
			dSm^{-1}				
			S_1 (2.0-4.0)	S_2 (4.1-8.0)	S_3 (8.1-12.0)	S_4 (12.1-16.0)	S_5 (>16.0)
Non-saline with very slightly saline	4,25,490	1,15,370 (27%)	82,260 (27%)		1,520 (1%)	0	0
Very slightly saline with slightly saline	4,20,420	3,09,190 (73%)	1,70,380 (55%)	31,590 (72%)	29,420 (10%)	0	0
Slightly saline with moderately saline	2,57,270	2,40,220 (93%)	35,490 (15%)	1,13,890 (47%)	61,240 (26%)	25,870 (11%)	2,650 (1%)
Moderately saline with strongly saline	1,98,890	1,98,890 (100%)	1,630 (1%)	36,060 (18%)	73,400 (37%)	55,130 (28%)	32,750 (16%)

Source: Petersen and Shireen (2001)

4.2.4 Developing salt tolerant crop plants

Two basic genetic approaches are employed to improve salt tolerance include. They are (i) use of natural genetic variation by using direct selection in stressful environments or mapping quantitative trait loci (Foolad, 2004; Flowers, 2004; Lindsay *et al.* 2004) and subsequent marker-assisted selection and (ii) generation of transgenic plants by introducing novel gene(s) or by altering expression levels of the existing gene(s) to affect the degree of salt stress tolerance (Yamaguchi and Blumwald, 2005).

4.2.4.1 Marker-assisted breeding

Attempts to improve the salt tolerance in crops have achieved limited success, due to the complexity of the trait, both genetically and physiologically. Tolerance shows all the characteristics of a multigenic trait, with quantitative trait loci (QTLs) identified in barley, citrus, rice and tomato (Flowers and Flowers, 2005). Furthermore, salt tolerance in plants is regulated developmentally and the tolerance of the plants at one stage of development is not always correlated with tolerance at other stages (Foolad, 2004; Flowers, 2004; Greenway and Munns, 1980; Tal and Shannon, 1983). For example, salt tolerance in tomato, barley, corn, rice and wheat increases with the age of the plant (Foolad, 2004). QTLs associated with salt tolerance at the germination stage in barley (Mano and Takeda, 1997), tomato (Foolad *et al.*, 1999) and arabidopsis (Quesada, *et al.*, 2002) were different from those QTLs associated with salt tolerance at the early stage of growth. The plants selected by their ability to germinate at high salinity did not display similar salt tolerance during vegetative growth. This finding repeated for rice and citrus fruits suggest that if any particular QTL were to be chosen as marker for the purposes of plant breeding, then care should be taken over its choice and the growth conditions. A further limitation to the use of QTL in plant breeding is the fact that QTL may be specific to particular crosses. For example in rice (Flowers *et al.*, 2000) and tomato (Monforte *et al.*, 1997), none of the markers found in one cross showed any association with similar traits in a closely related population of recombinant inbred lines or in selections of a cultivar .

However, there is considerable evidence to support the view that salt tolerance and its sub-traits are determined by multiple QTLs and that both additive and dominance

effects are important in the inheritance of many of the traits associated with salt tolerance (Foolad, 2004; Flowers, 2004; Gregorio *et al.*, 2002). The development of high-density QTL maps that incorporate SSR, RFLP and AFLP and advances in marker-assisted selection techniques will facilitate pyramiding traits of interest to attain substantial improvement in crop salt tolerance.

4.2.4.2 Transgenic approaches for salt tolerance

The response of plants to salt stress is controlled by many genes and the functions of these genes cause a wide variety of biochemical and physiological changes (Göl, 2006). Compartmentalization of toxic ions in the vacuole, activation of detoxifying enzymes, synthesis of late embryogenesis abundant (LEA) proteins and accumulation of compatible solutes are examples of the roles of these genes. This polygenic feature of plant's response to salt stress makes it difficult to transfer individual genes using traditional plant breeding or MAS. Transgenic approaches have been employed to obtain genetically modified plants that are tolerant to salt stress.

Genetic engineering of salt tolerance via different strategies has shown promising results. Over expression of genes involved in tolerance related physiological mechanisms is the main approach used in genetic engineering of this trait (Bajaj *et al.*, 1999; Rontein *et al.*, 2002). Osmotic compatible solutes provide osmotic adjustment and these solutes are helpful in improving plant stress tolerance (Rathinasabapathi, 2000; Rontein *et al.*, 2002). The genes encoding enzymes that have roles in enhancing the synthesis of compatible solutes were engineered. For example, Thomas *et al.*, (1995) worked on mannitol, Lilius *et al.*, (1996) worked on glycine betaine, Zhu *et al.*, (1997) worked on proline and Galston *et al.*, (1997) worked on polyamines. Overexpression of different vacuolar antiporter proteins, which provide the exclusion of toxic ions from the cell cytosol were also studied and transgenic plants have been produced with enhanced stress tolerance (Rajagopal *et al.*, 2007; Verma *et al.*, 2007; Apse *et al.*, 1999; Zhang and Blumwald, 2001; Zhang *et al.*, 2001). Moreover, detoxifying enzymes, which decrease the harmful effects of oxidative stress, were studied and transgenic plants have been produced (Tanaka *et*

al., 1999). Although abiotic stress tolerance is known to be governed by multiple genes, significant increases in salt tolerance can be achieved by single gene manipulations as revealed by SOS1 (Shi *et al.*, 2003) and NHX1- (Apse *et al.*, 1999; Zhang and Blumwald, 2001; Zhang *et al.*, 2001) overexpressing transgenic plants. These transgenics are capable of growing and producing flowers at salt concentrations of up to 200 mM NaCl (20 dS/m), which is lethal to wild-type plants. Transgenic tomato plants over expressing Na+/H+ antiporter protein have been developed. In this study a single gene (AtNHX1) coding Na+/H+ antiporter protein was isolated from *Arabidopsis thaliana* and transformed into the tomato genome (Zhang and Blumwald, 2001).

The assessment of salt tolerance in transgenic experiments as described above has been mostly carried out using a limited number of seedlings or mature plants in laboratory experiments. In most of the cases, the experiments were carried out in greenhouse conditions where the plants were not exposed to those conditions that prevail in high-salinity soils. The evaluation of field performance under salt stress is difficult because of the variability of salt levels in field conditions (Richards, 1983; Daniells *et al.*, 2001) and the potential for interactions with other environmental factors, including soil fertility, temperature, light intensity and water loss due to transpiration (Yamaguchi and Blumwald, 2005).

4.2.5 Salt tolerance and antioxidant genes

Oxidative stress is an important aspect of salinity stress in plants. The formation of reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH) and singlet oxygen ($^1O_2$) is a normal function of aerobic metabolism. The types of cellular activities that generate ROS include photorespiration, oxidation of fatty acids, and mitochondrial and chloroplast electron transport. However, ROS production increases under abiotic stresses including salinity (Xiong and Zhu, 2002). Under normal conditions the negative effects of ROS can be eliminated.

Excessive ROS can damage proteins, lipids and nucleic acids (Halliwell and Guteridge, 1985). Antioxidant compounds (nonenzymatic antioxidants) such as ascorbic acid, glutathione, thioredoxin, carotenoids and ROS scavenging enzymes

such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione peroxidase (GPX) are employed by plants to eliminate ROS. The activity and expression level of genes that encode the ROS scavenging enzymes are increased under abiotic stresses. There are many studies describing the correlation between oxidative stress and detoxifying mechanisms. Superoxide dismutase catalyses the conversion of superoxide anions to hydrogen peroxide and water and is the first step of the defense mechanism (Figure 4.2.2).

SOD plays a crucial role in the defense against oxidative stress. If the superoxide anion is not neutralized at this step, it joins to Fe and Cu, causes oxidation and a hydroxyl radical occurs (GÖL, 2006). There is no elimination mechanism for the OH group. Catalases and several classes of peroxidases catalyse the conversion of H_2O_2 to oxygen and water (Figure 4.2.2).

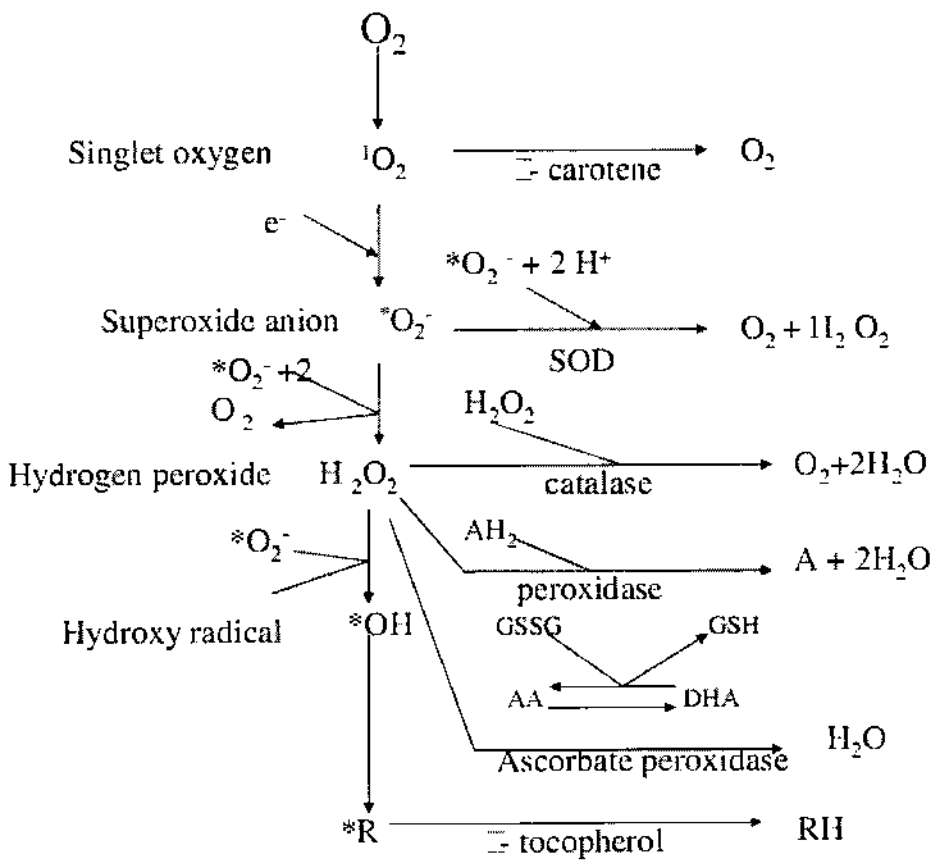


Figure 4.2.2: Mechanisms of reactive oxygen species formation and ROS/ antioxidant signaling pathways.

With the advances in molecular biology and availability of advanced genetic tools considerable progress has been made in the past two decades in improving salt-induced oxidative stress tolerance in plants by developing transgenic lines with altered levels of antioxidants of different crops (Ashraf, 2009). Plants have developed two mechanisms to defend themselves against ROS produced during both normal and abiotic stresses namely, synthesis of antioxidant compounds and antioxidant enzymes (Foyer *et al.*, 1995; Bohnert and Sheveleva, 1998). Key enzymes for detoxifying ROS in plant include superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase etc. SOD catalyzes the disproportionate superoxide radicals into H_2O_2 and oxygen. Catalase and APX are of the primary H_2O_2 scavenging enzymes in plants (Bohnert and Sheveleva, 1998). However, they have distinct catalytic properties. Catalase (Willekense *et al.*, 1994) does not consume reducing power and has a very high reaction rate but only poor affinity for H_2O_2. Alleviation of oxidative damage by scavenging ROS is an important strategy of plants to tolerate stress (Zhu, 2002; Noctor and Foyer, 1998; Hasegawa *et al.*, 2000; Moller, 2001). Hence, several efforts have been made to improve salt tolerance by engineering ROS-detoxifying enzymes.

As mentioned above catalase (CAT) is a predominant enzyme controlling H_2O_2 level. In Arabidopsis, catalases are encoded by a small multigene family consisting of CAT1, CAT2, and CAT3 (Frugoli *et al.*, 1996, 1998; Zentgraf and Zinkernagel, 1996). While catalases have been extensively studied for many years, growing evidence suggests that catalase and other antioxidative enzymes are closely related to plant biotic or abiotic stress-tolerance, although the signalling processes of stress-induced gene expression of catalase are largely unknown (Beyer and Fridovich, 1987; Dempsey and Klessig, 1994; Foyer *et al.*, 1994; Pinhero *et al.*, 1997; Holmberg and Bulow, 1998; Foyer and Noctor, 2000).

Overexpression of SOD, APX and catalase has been shown to improve oxidative stress tolerance in transgenic plants (Allen, 1995; Roxas *et al.*, 1997). To affirm this, Al-Taweel *et al.*, (2007) examined the influence of salt stress on the repair of PSII and the synthesis of D_1 protein in wild type tobacco (*Nicotiana tabacum* 'Xanthi') and in transformed plants comprising the catalase gene *kat E* from *Escherichia coli*. They observed that

photoinhibition of PSII in leaf discs from both wild-type and *kat E* transformed plants was increased due to salt stress, but the effect of salt stress was not so prominent in the transformed plants than in wild-type plants. The high tolerance of *kat E* transgenic tobacco plants was suggested to be due to increased ability of the chloroplast's translational machinery of these plants to scavenge hydrogen peroxide under salt stress. Such a positive relationship between the activity of catalase and degree of salt tolerance has also been shown in transgenic plants of Arabidopsis (Willekens *et al.*, 1997).

Hamdi *et al.*, (2009) investigated a possible link between catalase (CAT) activity and salinity tolerance in wild type potato and in transgenic lines of potato (cv. 'Désirée') comprising the catalase gene, *kat*E from *Escherichia coli* under salt stress conditions. They found that catalases contribute to salinity tolerance mechanisms in potato. Overexpression of the *E. coli* catalase targeting to the chloroplast of tobacco increased the tolerance of the plants to drought stress (Skikanai *et al.*, 1998).

In an attempt to improve salt tolerance of rice, Nagamiya *et al.*, (2007) introduced *kat E*, into japonica rice cultivar, Nipponbare. They observed that catalase activity in the transgenic rice plants was 1.5 to 2.5-fold higher than non-transgenic rice plants. The transgenic rice plants showed higher tolerance to salt as compared to the non-engineered plants. Similar results were also reported by Moriwaki *et al.*, (2008). They overexpressed *kat E* gene into the indica rice (*Oriza sativa*) and observed that the introduced *kat E* gene was actively expressed in the transgenic rice plants. Catalase activity in T_1 and T_2 transgenic rice was approximately 150% plant height higher than in non transgenic plants. Transgenic rice plants exhibited high salt tolerance compared to non transgenic rice plants. T_2 transgenic plants survived in a 200 mM NaCl solution for 2 weeks, whereas non transgenic plants were scorched after 4 days soaking in the same NaCl solution.

4.3 Materials and methods

4.3.1 *Escherichia coli* strain and *in vitro* culture

The *Escherichia coli* strain, K-12 was used in this experiment. This strain was collected from the Department of Microbiology, University of Dhaka. *E. coli* strain was grown in aerated Luria–Bertani (LB) medium at 37°C. Single colony from this plate was used for isolating the genomic DNA from *E. coli* and finally this DNA was used for isolation of *kat*E gene.

Preparation of Luria–Bertani broth

Materials

For 1000 ml Luria-Bertani Broth

- Yeast extract 5 g
- Peptone 10 g
- NaCl 1 g

Method

- All the above reagents were mixed and adjusted the pH to 7.0 with 1 N NaOH.
- The media so prepared was autoclaved at 121°C for 20 min.
- Broth was allowed to cool and stored at 4°C.

4.3.2 Isolation of *kat*E gene from *Escherichia coli*

4.3.2.1 Genomic DNA isolation

Isolation of chromosomal DNA from *E. coli* was performed as described by Sambrook *et al.*, (1989).

Materials

Lysozyme/RNase mixture

10 mg/ml lysozyme

1 mg/ml RNase

50 mM Tris-Cl (pH 8.0)

Stored at -20°C in small aliquots.

STET buffer

8% Sucrose

5% Triton X-100

50 mM Tris-HCl (pH 8.0)

Filter sterilized and stored at 4°C

Others

4 M lithium chloride (autoclaved)

Phenol: Chloroform: Isoamylalcohol (25 : 24 :1)

Isopropanol

80% Ethanol

TE buffer

Tris-HCl pH 8.0	10mM
EDTA pH 8.0	1 mM

Procedure

- Bacteria were grown overnight in 100 ml LB media at 37°C in a shaker at 200 rpm.
- 15 ml culture was transferred to a screw cap tube and centrifuged at 4000 rpm for 10 min.
- Supernatant was discarded and pellet was air dried completely.
- The pellet was re-suspended with 3 ml STET buffer. After re-suspending 300 µl RNase/ lysozyme mixture was added.
- The solution was boiled for 1 minute and 45 seconds.
- Centrifuged at 14000 rpm for at least 15 min at 4°C.
- Supernatant was transferred into another eppendorf tube and equal volume of Phenol (STET- saturated phenol): Chloroform : Isoamylalcohol (25 : 24 : 1) was added and then centrifuged at 14000 rpm for 10 min at 4°C.
- Aqueous phase was transferred into another eppendorf tube.
- 1/10 volume 4M lithium chloride was added and allowed to sit on ice for 5-10 min.
- Then the solution was centrifuged at 14000 rpm for at least 15 min at 4°C and the supernatant was collected.

- DNA was precipitated with equal volume of isopropanol (ice-cold) by keeping at -30°C temperature for 30 min and centrifuged for 10 min at 14000 rpm at 4°C.
- Supernatant was discarded and the pellet was washed with 500-600 µl of 80% ethanol (ice-cool temperature) and centrifuged for 10 min at 14000 rpm at 4°C.
- The pellet was air dried completely (under laminar hood) and suspended in 30-50 µl of TE buffer.
- Isolated DNA was stored at -20°C.

4.3.2.2 Amplification of *kat*E gene

The *kat* E gene (2262 bp) was amplified by PCR from the *E. coli* chromosomal DNA using the primers **catalase For** 5'-GTTCAATGTCGCAACATAACGA AAAGA-3' and **catalase Rev** 5'-CTATGTAAATCATTTGAGGCGGCGCAAT-3' designed using the sequenced genome of *E. coli* (Blattner *et al.*, 1997). Thirty-five cycles were performed using the following PCR conditions: 94°C for 5 min followed by 35 cycles of denaturation at 95°C for 50 s, annealing at 60°C for 1 min and extension at 72°C for 1 min 30 s and a final elongation step at 72°C for 10 min.

4.3.3 Insertion of *kat*E gene into entry clone

The pCR®8/GW/TOPO® TA Cloning® Kit, which combines Invitrogen's TOPO® Cloning and Gateway® technologies to facilitate cloning of *Taq* polymerase-amplified PCR products into a plasmid vector with high efficiency, was used for cloning of *kat*E gene. A detail of the procedure is described in section 3.3.14.

4.3.4 Construction of expression vector

LR Reaction is a recombination reaction between an Entry Clone and a Destination Vector, mediated by a cocktail of recombination proteins, to create an Expression Clone (Figure 4.3.2). The recombination proteins cut to the left and right of the gene within the attL sites in the Entry Clone and ligate it to the corresponding attR site in the Destination Vector (pH7WGF2), creating an Expression Clone. The resultant 25 bp attB sites [attB1 on the left (N- terminus) and attB2 on the right (C-terminus)] created by the LR reaction are derived from the attL sites (adjacent to the gene), whereas the distal sequences are derived from the attR sites. The LR clonase Enzyme

Mix mediates the GATEWAY LR Reaction and contains recombination proteins Int, Xis, and the *E. coli*-encoded protein IHF. The map of Entry clone and Destination vector is shown in Figure 4.1. For bacterial selection the vector contains gene for spectinomycin resistance and for plant selection it contains hygromycin resistant gene.

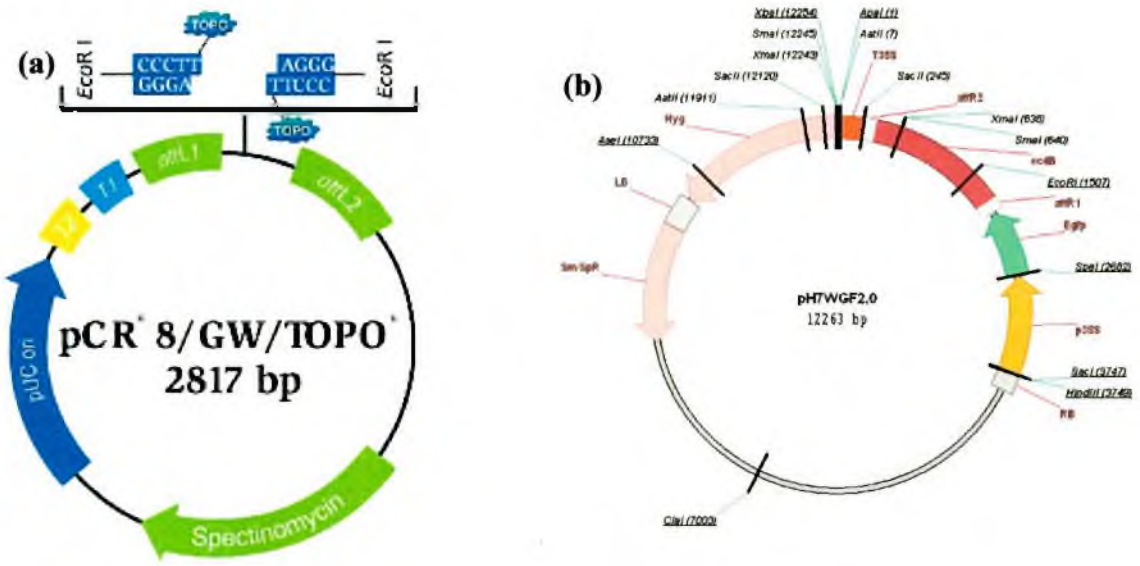


Figure 4.3.1: Map of (a) Entry clone and (b) Destination vector (Courtesy: Invitogen).

LR recombination reaction

Materials

- Entry clone, pCR®8/GW/TOPO® with 2314 bp (*kat* E gene) insert sequence.
- Destination vector, pH7WGF2 (150 ng/µl)
- TE buffer (pH 8.0)
- LR Clonase ™ II enzyme mix

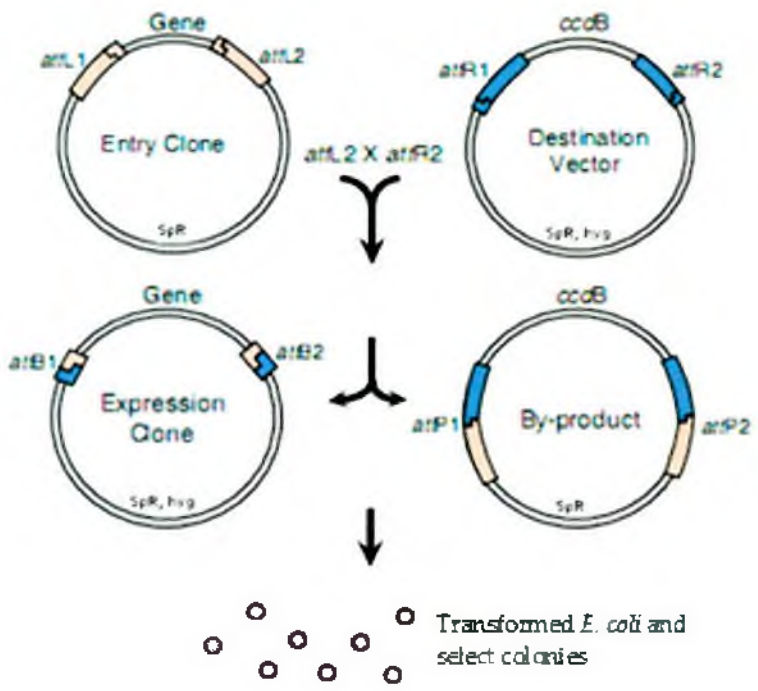


Figure 4.3.2: LR recombination reaction between entry clone and destination vector to create expression clone. Entry clone with attL recombines with a destination vector with attR to form a new expression clone with attB and a byproduct with attP (Courtesy: Invitogen).

Method

- The following components were taken into a 1.5 ml Eppendorf tube at room temperature and mixed:
    i. Entry clone (50-150 ng) 1-7 µl
    ii. Destination vector (150 ng/µl) 1 µl
    iii. TE buffer, pH 8.0 to 8 µl
- 2 µl of LR Clonase ™II enzyme mix was added into an eppendorf tube and mixed well by brief vortexing twice.
- The mixture was incubated at 25°C for 1 hour.
- 1 µl of the Proteinase K solution was added to each sample to terminate the reaction. Following brief vortexing samples were incubated at 37°C for 10 min.

4.3.4.1 Transformation of *E. coli* DH5α cells with destination vector

The focal point of this step is to transform the competent *E. coli* DH5α cells with destination vector containing the desired insert sequence.

Materials

- LB plates with Spectinomycin and Hygromycin
    - Yeast extract 5 g
    - Peptone 10 g
    - NaCl 10 g
    - Agar 1.5%
    - Spectinomycin (100µg/ml)
    - Hygromycin (100µg/ml)
- SOC medium (50ml)
    - Peptone 1.0g
    - Yeast extract 0.25g
    - 1M NaCl 0.5ml
    - 1M KCl 0.125ml
    - 2M Mg^{2+} stock 0.5ml
    - 2M glucose 0.5ml
- High efficiency competent cells (*E. coli* DH5α) were made in the lab.

Method

- The recombinant plasmid i.e., destination vector containing *kat* E gene was added to a sterile 1.5ml microcentrifuge tube on ice.
- Frozen DH5α high efficiency competent cells from -80°C storage was placed on ice and thawed. The cells were mixed by flicking the tube gently.
- 100µl of cell suspension was transferred carefully into a tube.
- The tube was flicked gently to mix and placed on ice for 20 min.
- The cells were heat-shocked for 90 seconds in a water bath at exactly 42°C.
- Immediately the tube was transferred on ice and incubated for 2 min.

- 1000µl of pre-warmed SOC medium was added to the tube containing cells transformed by ligation reactions.
- The sample was incubated for 1.5 hours at 37°C with shaking (~200rpm).
- 100µl of transformation culture was plated onto duplicate LB plates containing Spectinomycin and Hygromycin.
- The plates were incubated overnight (16-24 hours) at 37°C.

4.3.4.2 Confirmation of successfully transformed clone into *E. coli*

4.3.4.2.1 Confirmation by Polymerase chain reaction (PCR)

Insert specific primers, catalase forward 5'-GTTCAATGTCGCAACATAACGAAA AGA-3' and catalase reverse 5'-CTATGTAAATCATTTGAGGCGG CGCAAT-3' were used to confirm the presence of inserted DNA sequence in the transformed *E. coli*. The same PCR profile, which was used to amplify E coli *kat*E (described in section 3.3.2.2), was employed for this reaction.

We also used nested PCR for confirmation of the presence of inserted DNA sequence in the transformed *E. coli*. Here we designed one pair of primers for nested amplification of the *kat*E gene, namely, nested reverse primer 5'- GCACCAGTTCTT CCGGGATAAGTT-3' and nested forward primer 5'-ACGTTTCCACTGGAAACC ACTGGC-3'. By using the insert specific PCR product (as template), PCR was performed using the combination of catalase For with nested reverse primer and nested forward primer with catalase Rev primer.

4.3.4.2.2 Confirmation by sequencing of amplified sequence

QIAquick Gel Extraction Kit® I (from Qiagen) was used for extracting DNA sample for sequencing. The kit was designed to extract and purify DNA of 70 bps to 10 kbps from standard or low-melting agarose gels in TAE or TBE buffer. Up to 400 mg agarose was processed per spin column.

The Gel Extraction process followed was same as that for section 3.3.16.3. The sample was then sent to 1ST BASE®, Malaysia for sequencing.

4.3.5 Transformation of *Agrobacterium tumefaciens* with destination vector

4.3.5.1 Preparation of competent *A. tumefaciens*

After confirming successful transformation of *E. coli*, the destination vector was transferred to *A. tumefaciens* for transformation into the jute plant. For this purpose, *A. tumefaciens* competent cells (LBA4404) was prepared and then transformed with the destination vector pHGWFS7 containing the inserted *kat*E.

Same method (for *E. coli* cells) was followed for preparing competent *A. tumefaciens* as mentioned in section 3.3.14.1.

4.3.5.2 Transformation of recombinant plasmid in plant transformation vector

For jute transformation, the recombinants plasmids were transferred into *Agrobacterium tumefaciens* (LBA4404) by the liquid nitrogen freeze-thaw method. This method is described below-

- About 1 μg of recombinant plasmid DNA was added to the 100μl competent cell.
- The cells were frozen in liquid nitrogen for about 20-30 sec.
- The cells were thawed immediately by incubating the eppendorf tube in a 37°C water bath for 5 min.
- 1 ml of YMB medium was added to the tube and incubated at 28°C for 24 hours with gentle shaking. This period allowed the bacteria to express the antibiotic resistant genes.
- The tubes were centrifuged for 2-5 min at 5000 g.
- The supernatant was discarded and the pellet (cells) was resuspended in 100 μl of YMB medium.
- Cells were spread on YMB agar plate containing 50 mg/L hygromycin and 50 mg/L spectinomycin. Plates were incubated at 28°C for 2-3 days.

4.3.5.2.1 Confirmation of successful transformation of *Agrobacterium tumefaciens*

4.3.5.2.2 Culture of *Agrobacterium tumefaciens*

Method for preparing the culture of *Agrobacterium tumefaciens* is the same as mentioned in section 2.3.3.

4.3.5.2.3 Plasmid isolation from *A. tumefaciens*

The same process as described earlier (section 2.3.13), was applied for plasmid isolation from *A. tumefaciens*.

4.3.5.2.4 Confirmation of the presence of cloned plasmid in *A. tumefaciens*

Successful transformation of *Agrobacterium tumefaciens* with the destination vector was confirmed by PCR and sequencing. The primers and thermal cycling program is same as mentioned in section 4.3.4.2.

4.3.6 Transformation of jute plant

Jute transformation was performed using previously developed protocol which has been described in Chapter 2.

4.3.7 Molecular analysis of transgenic plants

4.3.7.1 Isolation of jute genomic DNA

DNA was isolated from both transgenic and non-transformed jute plants following a procedure modified from Doyle and Doyle (1990) using nonionic detergent Cetyl Trimethyl Ammonium Bromide (CTAB) mentioned in section 2.3.12.

4.3.7.2 PCR confirmation of the presence of *E. coli katE* gene in T_0 jute leaves

Genomic DNA was extracted from young leaf tissues of T_0 jute plants which were infected with engineered *A. tumefaciens*. PCR was performed in a reaction mixture containing 25 ng genomic DNA, 100 μM dNTP mix, 0.2 μM forward and reverse primers, and 1 unit of *Taq* polymerase (Invitrogen). The PCR profile consisted of 35 cycles of 95°C for 40s (denaturation), 60°C for 50s (annealing), and 72°C for 50s (extension). The following primer pair was used: Hyg Forward 5′-GATGTTGGCG ACCTCGTATT-3′ and Hyg Reverse 5′- GCGAAGAATCTCGTGCTTTC-3′.

4.3.7.3 Selection of transformed plants

After the collection of seeds from T_0 plants, transformed plants were selected according to the protocol described in section 2.3.9 except for the antibiotics. Here hygromycin (25 mg/L) was used instead of kanamycin. The surviving seedlings were transferred to earthen pots and grown in a greenhouse for further studies on morpho-physiological expression.

4.3.7.4 Confirmation of successful transfer of transgene to T_1 generation by PCR

Genomic DNA was extracted from young leaf tissues of both T_1 transgenic plants and non-transformed control plants. PCR was performed according to the protocol described in the previous section (4.3.7.2).

4.3.7.5 Confirmation of the presence of *katE* in T_1 generation by Southern blot analysis

Putative transgenic plants were analysed at the molecular level for the presence of the transgene by Southern blot hybridization (Sambrook and Russell, 2001).

Fifteen-microgram aliquots of both transgenic and non-transgenic gDNA were digested with EcoRI and BamHI, and transferred to positively charged nylon membrane (Amersham Hybond™-N) according to manufacturer's protocol. A detailed procedure is given in section 2.3.15.

As a probe, a PCR fragment of 602 bps was amplified from *E.coli* K-12 gDNA using primers Hyg For 5′- GATGTTGGCGACCTCGTATT -3′ and Hyg Rev 5′-GCGAAGA ATCTCGTGCT TTC -3′.

4.3.7.5 Confirmation of transgene in T_1 generation by Reverse-Transcription polymerase chain reaction (RT-PCR)

First Strand Synthesis

First strand cDNA was synthesized from isolated total RNA by using Hyg Reverse primer following the manufacturer's protocol for SuperScript™ III Reverse Transcriptase (Invitrogen, USA). Detailed procedure is described in section 3.3.10.

PCR using first strand cDNA

PCR amplification of the cDNA was carried out for 35 cycles using primers, Hyg forward and Hyg reverse following the aforementioned PCR conditions (4.3.7.2).

4.3.7.7 Leaf Disc Assay

Healthy, fully expanded jute leaves were collected from both transgenic and non-transgenic plants (50 days old) and washed briefly in de-ionized water. Leaf discs (1 cm diameter) were punched and floated on 7 ml of 250 mM NaCl solution and sterile distilled water (used as experimental control) for 10 days. The leaf discs were kept under continuous white light at 25°C ± 2°C. The experiment was repeated thrice with four different transgenic plants.

4.3.7.8 Salt stress treatment of transgenic plants

Previously selected transgenic plants (selections were performed in hygromycin selection medium) were transferred to earthen pots and grown in a greenhouse. After two weeks, the plants were watered with 150 mM NaCl solution until the control plants died. The relative growth of the seedlings in the presence of continuous salt stress was monitored by measurement of morphological parameters.

4.4 Results

4.4.1 Isolation of *kat* E gene from *E. coli* genomic DNA

Gene specific primers were used to amplify full length *kat* E gene from genomic DNA of *E. coli*. The full length of this gene is 2262 bps long. The amplified DNA fragment was 2309 bp long (Figure 4.4.1). This fragment was cloned and sequenced. This fragment contains 5 bps upstream of the initiation codon (ATG) which corresponds to the 5' UTR and a 42 bps fragment downstream to the termination codon (TGA) corresponding to the 3' UTR.

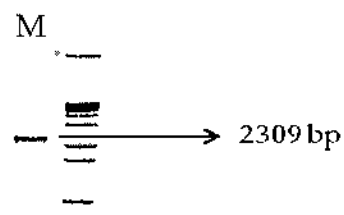


Figure 4.4.1: Amplified PCR product of *kat* E gene from *E. coli* genomic DNA using catalase gene specific primer pairs. Lane M contain *kat* E gene fragment and Lane 1 contain 1 Kb^+ ladder.

4.4.2 Amplified fragment cloned and sequenced

Amplified *kat* E gene (2309 bp) was cloned in entry vector, pCR8 GW TOPO TA™ (Invitrogen, USA). The resulting construct was transformed into chemocompetent *E. coli* (DH5α) cells. Isolated plasmids were sent to molecular service centre of 1ST BASE, Malaysia for sequencing. Sequencing results confirmed the presence and orientation of desired sequence in transformed *E. coli* DH5α.

4.4.3 LR recombination reaction

LR recombination reaction (according to Gateway® technology) was performed to transfer the desired DNA sequence from entry vector to destination vector,

pH7WGF2. After LR recombination reaction between entry vector harboring *kat* E gene and destination vector (pH7WGF2), a resultant vector was created. Then competent *E. coli* DH5α cells were transformed with that resultant vector. As destination vector was so designed that the resultant vector would contain both antibiotic resistance genes for spectinomycin (Sm/Spr) and hygromycin (Hyg), only transformed cells could form characteristic colonies on selective media. After transformation and overnight (16-24 hours) incubation at 37°C on LB plate containing the antibiotics, spectinomycin and hygromycin a few single colonies were obtained (Figure 4.4.2).

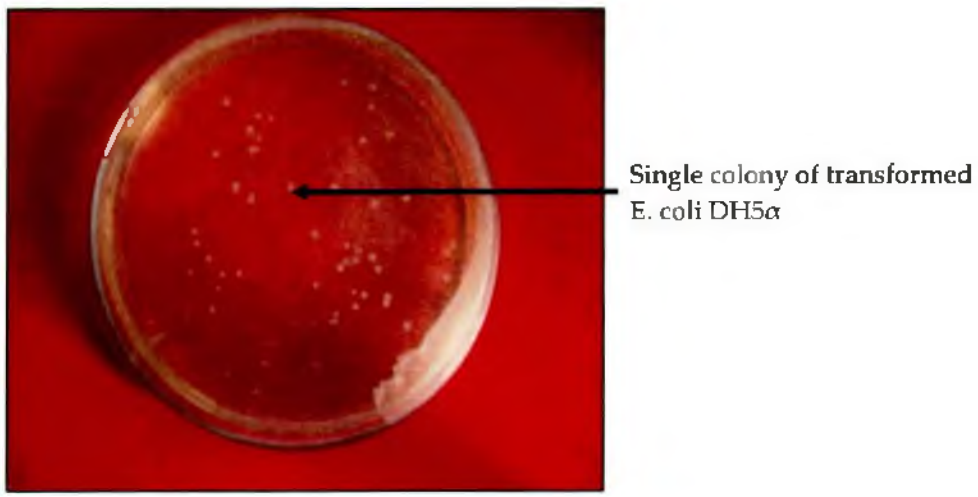


Figure 4.4.2: Colonies of *E. coli* DH5α transformed with pH7WGF2.

4.4.4 Presence of desired sequence in transformed *E. coli* DH5α cells

In the vector constructed, independent construct of antibiotic gene cassettes permitted the transformed cells to survive on the selection medium. But, that did not essentially prove whether transformed cells harbored the desired DNA sequence or not. In order to ascertain its existence, two levels of confirmation were achieved by PCR and sequencing.

4.4.4.1 Validation by PCR

Plasmid DNA was isolated from selected single colony and PCR was performd using gene specific primers. Desired amplification (2309 bps band) was observed in plasmid DNA isolated from a selected single colony (Figure 4.4.3).

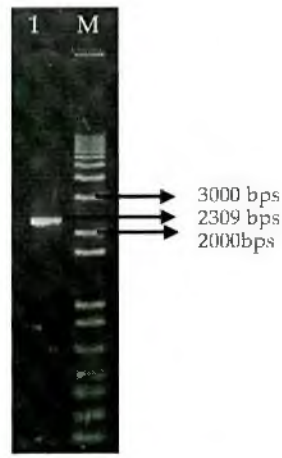


Figure 4.4.3: Presence of *kat* E in *E. coli* selected on antibiotics. Amplification of a band of the desired size from transformed *E. coli* DH5α plasmid DNA using catalase gene specific primer pairs. Lane 1 contains 2309 bps amplified fragment and Lane M contains 1 Kb+ ladder.

Using this PCR product as a template, nested PCR was performed using combinations of catalase forward and nested reverse primer, and nested forward primer and catalase reverse primer. As shown in Figure 4.4.4, primer pair catalase forward and nested reverse yielded the desired PCR product of 1101 bps (Lane 1), while for combination of nested forward and catalase reverse primers an expected PCR product of 1438 bps was obtained (Lane-3). These two PCR products confirmed that the desired sequence was in proper orientation in the plasmid.

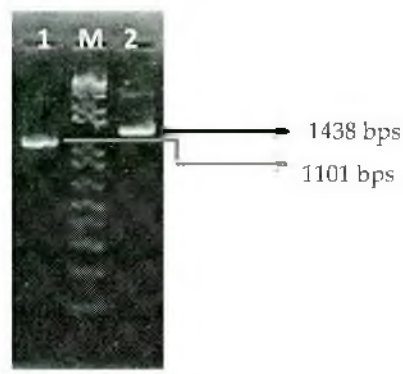


Figure 4.4.4: PCR products confirmed that transformed *E. coli* DH5α cells contained the desired sequence. Lane M was for reference 1kb+ ladder. Lane 1 and 2 contains PCR products from combination of primer pairs "catalase forward and nested reverse" and "nested forward and catalase Rev" respectively.

4.4.4.2 Validation by sequencing

After the presence and orientation of the desired sequence in transformed *E. coli* DH5α was confirmed by PCR, another level of validation was done by sequencing of the PCR product. Sequencing was done by 1ST BASE®, Malaysia. By assembling the two DNA sequences using CAP-3 software a 2309 bps sequence was obtained, which showed an exact match to the *E. coli katE* in analysis with Blast 2 sequence. This confirmed that the transformed *E. coli* DH5α harbored the desired sequence in the correct orientation.

4.4.5 Presence of the desired sequence in transformed *Agrobacterium tumefaciens* LBA4404

Competent *Agrobacterium tumefaciens* LBA4404 was transformed with the plasmid DNA isolated from *E. coli* DH5α which harbored the *katE* gene in the correct orientation. To assess whether any sort of recombination occurred during or after transformation in *A. tumefaciens* LBA4404, PCR reaction followed by sequencing was done.

4.4.5.1 Validation by PCR

Plasmid DNA isolated from transformed *Agrobacterium tumefaciens* was used as template DNA and PCR was performed using combinations of catalase forward and nested reverse primer and nested forward primer and catalase reverse primer.

Figure 4.4.5 shows that, catalase forward and nested reverse primer pairs yielded the desired PCR product of 1101 bps (Lane 3 & 4), while for combination of nested forward and catalase reverse primer, desired PCR product of 1438 bps was obtained (Lane1 & 2). This result confirmed the right orientation of desired DNA sequence in the vector harbored by *A. tumefaciens* LBA4404.

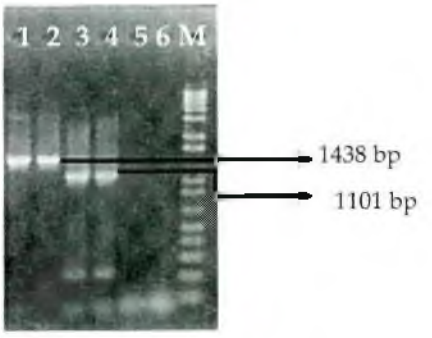


Figure 4.4.5: PCR products confirmed that transformed *Agrobacterium tumefaciens* contained the desired sequence. Lane M is for reference 1kb+ ladder. Lane 5 and 6 contain non-transformed *A. tumefaciens* plasmid DNA as negative control. Lane 1 and 2 contain PCR products from combination of primer pairs "nested forward and catalase reverse" and Lane 3 and 4 contain PCR products obtained from a combination of primer pairs "catalase forward and nested reverse".

For further confirmation, PCR was also performed using nested forward and nested reverse primers. Desired PCR products of 230 bps were obtained as shown in Lane 2, 3 and 4 of Figure 4.4.6.

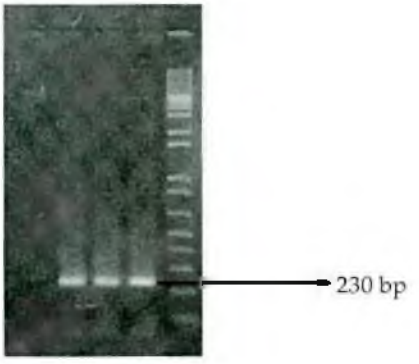


Figure 4.4.6: PCR products confirmed that transformed *Agrobacterium tumefaciens* contained the desired sequence. Lane M is for reference 1kb+ ladder. Lane 1 is for non-transformed *Agrobacterium tumefaciens* plasmid DNA, used as a negative control. Lane 2, 3 and 4 contain PCR products from a combination of nested forward and nested reverse primers.

4.4.5.2 Validation by sequencing

After confirmation by PCR, presence and orientation of desired sequence in transformed *Agrobacterium tumefaciens*, another level of validation was carried out by sequencing of the PCR products. Sequencing was done by 1ST BASE®, Malaysia. Assembling two runs of DNA sequence using CAP-3 software a 2309 bps sequence

was obtained, which showed an exact match to the *kat*E in analysis with Blast 2 sequence, NCBI. So, it was confirmed that the transformed *Agrobacterium tumefaciens* harbored the desired sequence in the correct orientation.

4.4.6 Transformation of jute plants

Agrobacterium mediated jute transformation was performed following the protocol, developed in our laboratory and which has been described in Chapter 2. Prior to analysis at the molecular level, physical characteristics (after-effect of transformation) of the jute plants were observed carefully.

4.4.6.1 Physiological appearance of infected plant

All injected plants (T_0) survived and showed normal growth upon infection with *A. tumefaciens*. However, injection sites appeared swollen or the thickness had increased at the sites of wounding than the surrounding regions (Figure 4.4.7). This could be a characteristic of *Agrobacterium* infection.

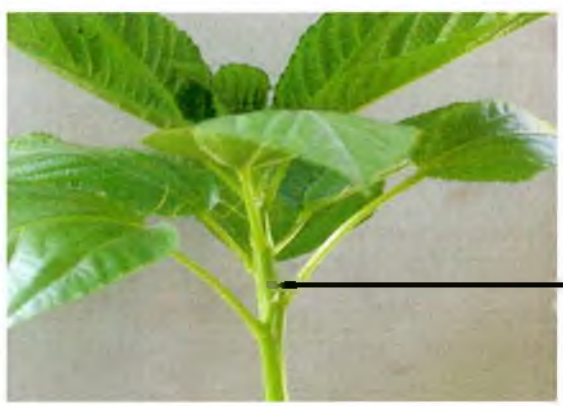


Figure 4.4.7: Physiological characteristic of successful *Agrobacterium* infection.

4.4.6.2 Integration of the transgene in T_0 plants

Genomic DNA was isolated from both control (non-infected plant) and T_0 jute leaves that emerged from the infection points of the infected jute plant and PCR was carried out using *Hyg* gene specific primers. Desired amplification was observed in DNA from transgenic plant (Lane 1 of Figure 4.4.8). Similar band was also observed in destination vector plasmid (pH7WGF2) taken as template while no band was found for genomic DNA isolated from a non-transformed jute plant.

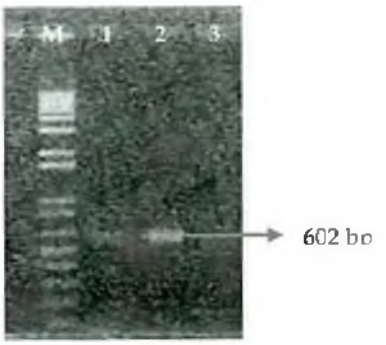


Figure 4.4.8: PCR results confirming the presence of transgene in T_0 plant. Lane M contains 1 Kb$^+$ ladder and Lane 1, 2 and 3 contain transformed plant DNA, destination vector plasmid DNA and non-infected (control) jute plant DNA, respectively.

4.4.6.3 Confirming the passage of transgene to the next (T_1) generation

Hypothetically, through the process of inheritance, the desired DNA sequence should pass on to the next generation, called T_1 generation. Presence of transgene in transformed plant confirmed by growing the germinating the seeds in selective media and at the molecular level by PCR and Southern blotting.

4.4.7 Selection of T_1 transformant plants

T_1 jute seeds were grown on MS medium containing hygromycin (25 mg/l) as a selective agent for transgenic plants. For comparison seeds from non-transgenic control plants from the same species were grown on the same medium. After 10 days, all non-transgenic control seedlings were germinated but subsequently failed to grow. On the other hand transformed plants were germinated and continued to grow photo-autotrophically. These plants were transferred to soil for further growth and seed development for the next generation.

4.4.7.1 Identification of transgenic (T_1) jute plants by PCR

Three putative transgenic plants were analyzed at the molecular level. DNA was isolated from non-transgenic and transgenic plants and PCR was performed using *hyg* specific primers. Desired amplification was observed in all transgenic plants whereas, no amplification was observed for non-transformed jute plant (Figure 4.4.9).

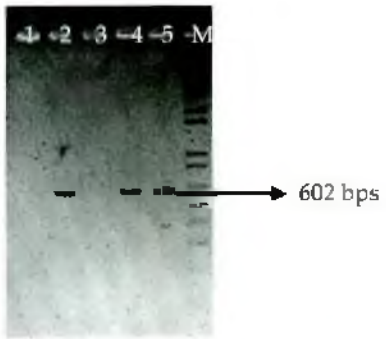


Figure 4.4.9: PCR result confirming the presence of transgene in T_1 jute plant genome. Lane 2, 4 and 5 show PCR products for genomic DNA from different T_1 plants. Lane 1 and 3 are for genomic DNA isolated from non-transformed jute plants (taken as negative control). Lane M is (for reference) 1kb plus ladder.

4.4.7.2 Inheritance of transgene in T_1 generation confirmed by Southern blotting

To determine the inheritance of the transgene from T_0 to progeny, Southern blot analysis was carried out using genomic DNA isolated from the leaves of T_1 plants. Figure 4.4.10 (a, b) shows results of the PCR based Southern blot analysis of plants from T_1 generation to check for the presence of the transgene. A 602 bp *hyg* gene specific probe was used by amplifying DNA from *hyg* gene of the destination vector plasmid. Non-transformed DNA from jute plants was used as a negative control. Fragments of the DNA from the T_1 generation plants (P-1, P-2 and P-3) were found to hybridize with the probe, indicating the presence and inheritance of transgene from progeny to progeny. However, no signal was detected in control plant.

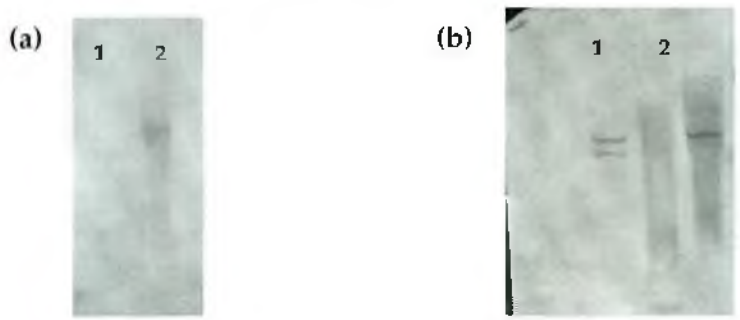


Figure 4.4.10: PCR based Southern blot analysis of transgenic jute plants (T_1) probed for the *hyg* gene (a, b). 15 µg DNA was digested with *EcoRI* and hybridized with the 602 bps *hyg* gene fragment. (a) Lane 1 contains digested DNA from non-transgenic plant used as negative control; Lane 2 contains digested DNA from a T_1 generation plant (P-1 plant). (b) Lane 1 contains digested DNA from a T_1 generation plant (P-2

plant); Lane 2 contains digested DNA from a non-transgenic plant; Lane 1 contains digested DNA from T_1 generation plant (P-3 plant).

4.4.7.3 Expression of *kat* E gene in transgenic plant

Reverse transcription -PCR analysis was performed to determine whether *kat* E gene was expressed in transgenic jute plants. RNA was isolated from leaves of T_1 generation plants and RT-PCR was performed with *hyg* specific primers. The specific band for the *hyg* gene was amplified from transformed plants, but not from non-transformed plants (Figure 4.4.11), which indicated that the transgene was expressed in the transgenic jute plants.

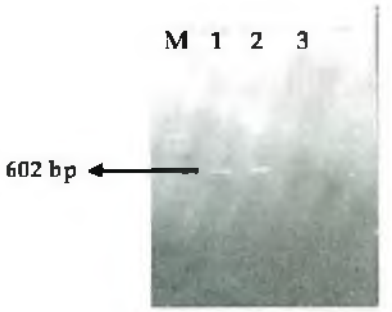


Figure 4.4.11: RT-PCR analysis for expression of transgene in the T_1 generation. Lane 1 and 2 contains RT-PCR product fromT_1 generation plants; Lane 3 is for non-transgenic jute plant.

4.4.8 Leaf disc assay

Leaf disc senescence assay of transformed and non-transformed plants was performed as a bioassay for estimation of salt tolerance potential. Leaf discs from both non-transgenic and transgenics were floated on 250 mM NaCl salt solutions for 10 days to investigate the effect of expression of *kat* E gene in improving the toxic effect of NaCl. Leaf discs floated in water served as the control. Figure 4.4.12 shows that the leaf discs from transgenic plants have less toxic effect than non-transgenic plant at 250 mM NaCl for 10 days. Leaf discs from non-transgenic plants showed strong chlorosis at 250 mM NaCl for 10 days. Whereas no chlorosis was observed for both non-transgenic and transgenic leaf discs when floated in water.

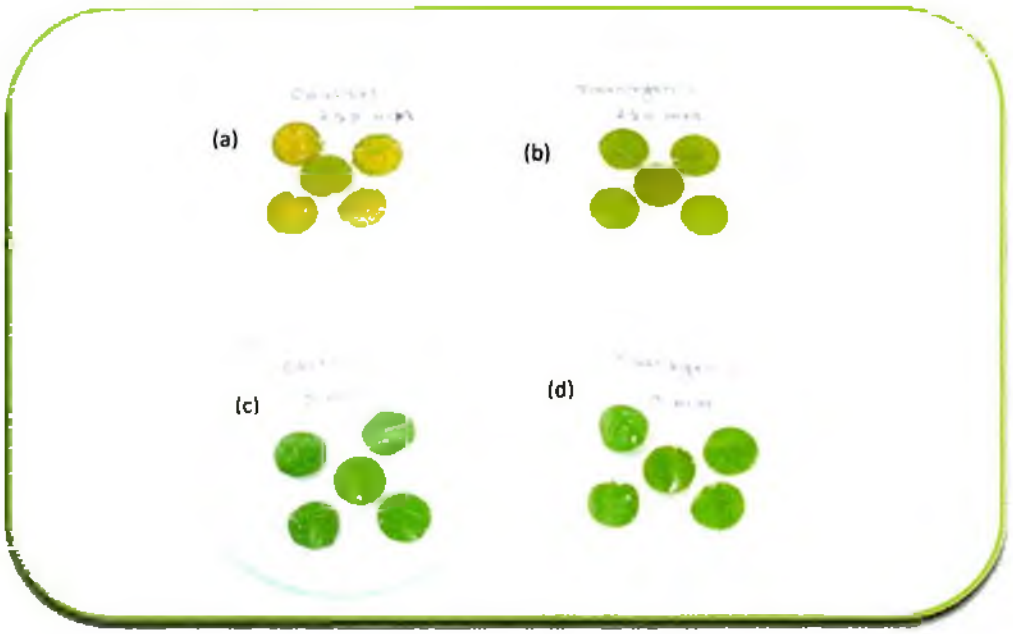


Figure 4.4.12: Leaf disc senescence assay in detached leaves of transgenic jute plants. (a) Leaf discs from non-transgenic plants and (b) leaf discs from transgenic plants in 250 mM NaCl for 10 days; (c) leaf discs from non-transgenic plant and (d) leaf discs from transgenic plant in water for 10 days served as the experimental control.

4.4.9 Salt stress tolerance of transgenic jute

To determine relative salt tolerance, both non-transgenic and transgenic plants were transferred to soil containing pots and grown in a greenhouse. After two weeks of growth under normal condition, plants were watered with 150 mM NaCl until the control plants died. Phenotypic analysis of control (non-transgenic) and transgenic plants for salt tolerance at the whole plant level are shown in Figure 4.4.13. After 3 weeks of 150 mM NaCl treatment, the T_1 transgenic plants showed significant salt tolerance as they grew normally while the control plants showed typical yellowing of leaves as well as growth stunting (Figure 4.4.13a). After another 5 weeks of 150 mM NaCl treatments, all control plants failed to survive while transgenic plants continued to grow normally (Figure 4.4.13b, c). Eventually transgenic plants formed flowers and produced seeds which appeared normal in size and texture. (Figure 4.4.13d).

Control plant Transformed plant

Control plant Transformed plant Transformed plant Transformed plant with normal flower

Figure 4.4.13: Salt tolerance of control (non-transgenic) and T_1 transgenic jute plants at different growth stages. After two weeks of growth under normal condition, plants were watered with 150 mM NaCl. Photograph was taken after (a) 3 weeks of salt treatment; (b) 7 weeks of salt treatment; and (c) 8 weeks of salt treatment. All non-transgenic plants died whereas transgenic plants remained alive (d and e) Transgenic plant displayed normal growth, flower formation and seed setting.

4.5 Discussion

Salinity is one of the major abiotic stresses that adversely affect agriculture and result in crop deterioration (Ouda *et al.*, 2008). Due to wide spread occurrences of this stress, plants are affected in many ways, which ultimately lead to economic losses in agriculture. Although much progress has been made in traditional breeding and new cultivars have been produced, the whole process is time consuming (Ashraf and Akram, 2009). On the other hand, plant genetic engineering provides a less time consuming and direct method of introducing gene of interest into crops to make them tolerant against different threats (Cushman and Bohnert, 2000).

In this study, we transformed popular tossa jute (*Corchorus olitorius* L.) cultivar BJRI tossa pat-4 (O-72) with the *kat*E gene for salt tolerance. *kat*E gene was obtained from *E. coli* and designed to be expressed under the control of 35S cauliflower mosaic virus constitutive promoter. The resultant transgenic jute plants were evaluated for *kat*E expression and salt tolerance.

For construction of expression vector, *kat*E gene was amplified from *E. coli* genomic DNA and the PCR product was ligated to the entry clone (TOPO vector, Invitrogen's TOPO® Cloning and Gateway® technologies). Then LR recombination reaction was performed between the entry clone (containing the inserted *kat*E gene) and destination vector. Here, entry clone with attL recombines with a destination vector with attR to form a new expression clone with attB and a byproduct with attP (Gateway Cloning Technology, Invitrogen). New expression vector with attB is resistant to both spectinomycin and hygromycin whereas the byproduct is resistant only to spectinomycin. Then the resultant vector was transferred into *Agrobacterum tumefaciens* strain LBA4404, using the freeze-thaw method (Singla-Pareek *et al.*, 2003).

The presence and orientation of the desired sequence in the resultant vector was confirmed by PCR and subsequently by sequencing. *Agrobacterium* mediated jute transformation was performed following a protocol developed by us (Sajib *et al.*, 2008) and which has been described in Chapter 2. In this protocol, the apical meristematic regions of young jute plants were infected with *A. tumefaciens* containing *kat*E gene. Morphologically, no noticeable difference was observed in the

transgenic T_0 plants except the thickening at the infection sites. This might possibly be the result of plant defense responses. Some of the seeds collected from these plants showed stable transformation (selected by hygromycin medium) and selected seedlings (T_1 generation) were transferred to soil for further analysis.

Putative transgenic plants were confirmed by PCR using *hyg* gene specific primers, which amplified a 602 bp fragment corresponding to the *hyg* gene (Figure 4.4.8 and 4.4.9). To increase the specificity of PCR amplification long primers (20 bps) with high annealing temperatures (60°C) were designed. The primers were also searched for homology to other sequences using BLAST to avoid getting multiple spurious bands during PCR amplification. Using these primers the amplified products from transgenic plants gave a single and expected size band. These results were further confirmed by Southern analysis of EcoRI digested genomic DNA, probed with a partial *hyg* gene DNA fragment. Under high stringency condition, hybridizing band was detected in all transgenic plants (Figure 4.4.10), indicating the presence and inheritance of transgene over the generations. However, no cross hybridizing band was seen in the non-transgenic jute plants.

Selection on hygromycin medium also provided proof for transformation as only seeds from transformed plants germinated and showed growth. Seeds from non-transgenic plants germinated initially but failed to sustain growth. But seeds of non-transgenic plants showed normal growth in non-selection medium. This eliminated doubts about the vigour of non-transgenic seeds.

Reverse-Transcription PCR was used to determine whether the transgene was actively transcribed in transgenic jute plants. Total RNA was isolated from T_1 transgenic plants and RT-PCR was performed. All T_1 transgenic plants showed amplification derived from the transcript of the transgene (Figure 4.4.11), which indicated that the transgene was expressed in the transgenic jute plants. To exclude the chance of amplification from contaminating DNA in the RNA sample, PCR was performed directly with the same sample of RNA without reverse transcription. However, no amplification was visible.

We examined the effects of salt stress due to NaCl during exposure to light stress of leaf discs from both non-transgenic and *kat*E transformed jute plants (Figure 4.4.12). The transgenic plants containing *kat*E gene showed a clear advantage in overcoming toxic effect from salt stress. Leaf disc from non-transgenic plants showed extensive bleaching due to sodium toxicity. This result indicated the usefulness of expressing *kat*E in transgenic jute plants for survival under high NaCl level.

The T_0 transgenic plants containing *kat*E gene completed their life cycle and set seeds. These seeds were germinated on hygromycin containing MS media to check the stable presence/integration of the introduced transgene. After 10 days, transgenic plants (the ones surviving) were transferred to soil for further analysis. After two weeks of growth under normal condition, plants were watered with 150 mM NaCl to determine the relative growth of both non-transgenic and transgenic plants in the presence of continuous salt stress. After eight weeks of 150 mM NaCl treatment, all non-transgenic control plants died whereas transgenic plant continued to grow and set seeds. These results indicated that transgenic T_1 plants were tolerant to high concentration of salt. Our results are consistent with the earlier report of *kat*E gene over expression in rice (Prodhan *et al.*, 2008). They observed that transgenic plants at a very young stage (3 - 4 days) were able to grow up to 15 days in 100 mM NaCl solution and seven days in 250 mM NaCl solution whereas control plants died within five days in 100 mM and seven days in 50 mM NaCl. Similar results were also observed by Moriwaki *et al.*, (2008). They have introduced *kat*E gene into japonica rice cultivar (BR-5) and found that T_2 transgenic plants survived in a 200 mM NaCl solution for 2 weeks, whereas non-transgenic plants scorched 4 days after soaking in the same NaCl solution. The transgenic rice plants over-expressing *kat*E gene were able to survive upto 250 mM of NaCl, for a period of 14 days and were able to form flower and produce seeds in the presence of 100 mM NaCl (Nagamiya *et al.*, 2007).

In this work, we have been able to enhance salt tolerance of tossa jute, *C. olitorius* L. by introducing *kat*E gene from *E. coli*. Our results provide evidence that *kat*E expression can confer higher levels of salinity tolerance in transgenic jute plants.

CHAPTER FIVE

General conclusion

Jute is a versatile and environment-friendly natural fibre of commercial interest for generating diversified value-added products (Basu *et al.*, 2004). It falls into the category and contains over 100 species, among them only two species, *Corchorus capsularis* L. and *C. olitorius* L., are cultivated widely (Sarker *et al.*, 2007).

These species of jute are self-pollinating and contain very limited genetic variability. *C. capsularis* is comparatively more resistant to flood and drought but slightly more susceptible to diseases and pests (La Farge *et al.*, 1997). It provides white commercial fibre which is not as strong as the fibres of the other cultivated species. *C. olitorius* is relatively tolerant to diseases and pests and produces the stronger commercial fibre. Therefore, a combination of useful characters of these two species in a single genotype is highly desirable.

Therefore, this whole study was conducted for the improvement of jute by introducing useful gene/s. The study was conducted with a view to address three major issues (i) development of an easy and efficient transformation protocol for jute (ii) identification of useful genes from the gene bank at the Bangladesh Jute Reserach Institute for introduction into cultivated species of jute and (iii) development of jute capable of withstanding salinity.

First, we were able to develop a tissue culture independent transformation protocol for *C. olitoius* L. Heritable transmission of the transgene to progeny from genetically modified plants was confirmed by *gus* gene expression by histochemical analysis, selection on kanamycin containing medium, Reverse Transcription-PCR (RT-PCR) and PCR amplification of stably transmitted gene sequence in transformed plants. Efficiency of transformation was determined by selection on medium containing kanamycin, and inheritance of transgene to T_2 generation plants. This simple transformation protocol will surely pave the way for improvement of jute varieties through introduction of useful new traits. This protocol would be of more importance because the two cultivated species of jute do not cross successfully with

each other, possibly due to presence of a strong sexual incompatibility between them (Patel and Dutta, 1960).

Second, under this study, differential gene expression between a fungus (*Macrophomina phaseolina*) resistant wild species (*C. trilocularis*) and a fungus susceptible (*Corchorus olitorius* Var. O-72) jute species were examined in order to find fungus resistant gene(s) from the wild species. One such identified gene was found to encode a xyloglucan endotransglycosylase/hydrolase (XTH) - an enzyme involved in cell wall elongation and restructuring (Albert *et al.*, 2004). Full length coding sequence of the gene was determined using degenerate primer based gene walking, 5' RACE and 3' RACE. Semi-quantitative reverse transcription PCR shows that the expression of *Co*XTH (XTH found in *C. olitorius*) is associated with fungus (*M. phaseolina*) infection. However, it remains to be elucidated whether XTH is an essential component of the signaling pathway involved in fungal defense.

Third, salinity in water or soil is one of the major limiting factors for agricultural production in Bangladesh. The detrimental effects of salinity can be observed at the whole-plant level as death of the plants and/or decrease in productivity. Now-a-days jute is being pushed into marginal lands due to tremendous pressure of growing food crops. Jute therefore has to face various biotic and abiotic stresses. To improve salt tolerance of jute, we have introduced previously characterized salt tolerant *kat*E gene from *Escherichia coli* into tossa jute. The resultant transgenic jute plants expressing *kat*E constitutively were able to grow and form flowers and produce seeds in the presence of 150 mM NaCl showing tolerance in abiotic stress i.e. salt tolerance.

CHAPTER SIX

Reference

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