GENETIC TRANSFORMATION OF LENTIL (*Lens culinaris* M. cv.Sultan.1) WITH A TRANSCRIPTION FACTOR REGULATOR (MBF1c) AND ANALYSIS OF TRANSGENIC PLANTS

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ABSTRACT

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AND
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In this study, Agrobacterium mediated genetic transformation of lentil Sultan 1 cultivar with MBF1c and evaluation of transgenic plants was aimed.

The study was initially based on optimized protocol with Agrobacterium tumefaciens KYRT1 strain and pTJK136 binary plasmid. Based on this protocol and transient marker gene expression in embryo apex, 15% stable transformation efficiency was aimed. However limited knowledge about pTJK136 and problem with curing KYRT1 leaded us to use Agrobacterium tumefaciens C58C1 strain and also to engineer an alternative binary plasmid; pPZP101. Hence, scope of this study became construction of a plant binary transformation vector and lentil transformation optimization with C58C1 strain.

First plant transformation vector designed in this study was pPZP101ManA-MBF1c. Transformations with C58C1::pPZP101ManA-MBF1c were carried out with a reformulated co-cultivation media. Cotyledonary nodes were isolated from three days old lentil seedlings germinated with phytormone (BAP/TDZ) induction. Isolated nodes were either injured and pre-incubated in co-cultivation media or pre-incubated and then injured prior to transformation. Regeneration and necrosis behaviors of the transformed explants leaded us to the conclusion that explant preparation is the critical step of transformation. And data suggest that explants isolated from 2mg/l BAP, pre-incubated two days in co-cultivation media, injured and transformed performed significantly better scores for necrosis shoot regeneration and callus formation parameters.

Transformed explants that survived in subsequent sub-cultures in mannose selection raised shoots. These shoots were grafted and regenerated into plantlets. The putative transgenic plantlets were screened for transgene with PCR. Initial amplification signals fainted and lost as grafts grew. In order to make a diagnosis of this fainting behavior the second plant transformation vector pPZP101ManA-GUSint-MBF1c was constructed and transient GUS expression analysis were made.

Keywords: *Lens culinaris*, MBF1c, *Agrobacterium*,

ÖZ

MERCİMEĞİN (*Lens culinaris* M. cv. Sultan.1) TRANSKRİPSİYON FAKTÖR REGÜLATÖR PROTEİNİ (MBF1c) İLE GENETİK TRANSFORMASYONU
VE
TRANSGENİK BİTKİLERİN ANALİZİ

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Bu çalışmada Agrobakteri yoluyla mercimek Sultan 1 varyetesinin genetik modifikasyonu ve MBF1c eksprese eden transgenik bitkilerin değerlendirilmesi hedeflenmiştir.

Çalışma Agrobacterium tumefaciens KYRT1 suşu ve pTJK136 ikincil plazmidi ile optimize edilmiş Sultan 1 transformasyon protokolü ile başlatılmıştır. KYRT1::pTJK136 tabanında embriyonik apeks meristeminde bakılan geçici GUS geni ifadelerine dayanarak 15% stabil transformasyon oranı hedeflenmiştir. Fakat pTJK136 plazmidi ile ilgili yetersiz veri ve KYRT1 suşunun boşaltılamamış olması nedeniyle, çalışma C58C1 Agrobakteri suşunun kullanıldığı ve yeni ikincil plazmid yapımı çalışmalarını esas alan optimizasyon çalışmaları şeklini almıştır.

Bu çalışmada ilk olarak dizayn edilen bitki transformasyon vektörü pPZP101ManA-MBF1c dir. Transformasyon deneyleri C58C1::pPZP101ManA-MBF1c tabanında, yeniden formülize edilen agrobakteri-bitki eş-kültür ortamında yapılmıştır. Bu deneylerde pitohormonla beraber filizlendirilmiş üç günlük mercimeklerden elde edilen çenek boğumu dokuları kullanılmıştır. Elde edilen dokular transforme edilmeden evvel ya ön-inkübasyona tabi tutulup yaralanmış ya da yaralandıktan sonra ön-inkübasyona tabi tutulmuştur. Elde edilen verilere göre transforme olan dokuların nekroz ve re-jenerasyonunda, dokunu yaralanmasından daha çok dokunun elde edilmesi ve transformasyona hazırlanması etkilidir. Ayrıca 2mg/l BAP ile çimlendirilmiş mercimeklerden elde edilen dokuların öncelikle 2 gün ön-inkübasyona alınması ve sonra yaralanarak transforme edilmesi dokuların nekrozu, re-jenerasyonu ve kallus formasyonu ele alındığında en iyi sonucu vermiştir.

Bu şekilde transforme edilen ve seçici ortamda alt kültüre alınan dokulardan çıkan sürgünler aşılanıp büyümeye bırakılmıştır. Büyüyen bitkilerden yapılan PZR analizlerinde başlangıçta elde edilen sinyaller bitki büyüdükçe kaybolmuştur. Bu durumun nedenlerini araştırabilmek üzere GUSint markör genini taşıyan ikinci bitki transformasyon vektörü pPZP101ManA-GUSint-MBF1c dizayn edilmiş ve bu vektörle mercimekte geçici GUS geni ifadesine bakılmıştır.

Anahtar Kelimeler: *Lens culinaris*, MBF1c, Agrobakteri

To the center of comprehension and meaning

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ABBREVIATIONS

ManA	mannose A gene from E.coli
MBF1c	multiprotein bridging factor 1c
TNOS	nitric oxide synthase terminator sequence
PCaMV35S	cauliflower mosaic virus 35S promoter
pBlueSK+	plasmid, cloning vector pBluescript SK+
BAP	benzylaminopurine
GUS	β-glucuronidase
MES	2-[N-Morpholino] ethanesulfonic acid
MS	murashige and Skoog
npt-II	neomycin phospho transferase type II
OD	optical density
T-DNA	transferred DNA
Ti	tumour inducing
vir	virulence
YEB	yeast extract broth

CHAPTER I

INTRODUCTION

1.1 General Information about Lentil

Lentil is in the legume family which is broadly defined by their unusual flower structure, pod shaped fruit, and 88% of the species (examined to date) are able to form nodules with *Rhizobia*. Also they are only second after the *Graminiae* in their importance to humans. The 670 to 750 genera and 18,000 to 19,000 species of legumes include important grain, pasture, and agro-forestry species. In such species diversity, lentil is among the first domesticated plants along with wheat and barley.

It is suggested that lentil was first cultivated in the Near East including Southern Turkey where Neolithic agriculture developed about 9,000 years ago (Zohary and Hopf 1988). By the Bronze Age, the plant had been spread throughout the Mediterranean region, Asia and Europe, which was followed by the New World including Latin America. *Lens culinaris* ssp. *orientalis*, which closely resembles the cultivated species *L. culinaris*, is widely accepted as the progenitor species. The conclusion that the cultivated lentil originated in the Near East arc from *L. culinaris* ssp. *orientalis* is based on discoveries of carbonized remnants of apparent cultivated lentil in the same region over which *L. culinaris* ssp. *orientalis* is distributed (Muehlbauer et al. 1995).

This annual, herbaceous pulse (grain legume) crop and is probably as old as the agriculture itself. Since its domestication in the Near East, lentil (*Lens culinaris* Medicus) remained as an important source of dietary protein in the area.

Despite its importance as a source of protein through ages, the crop has received little attention to improve its yield and quality. Organized collection of germplasm and crop improvement programs have only started at early 1980's with the establishment of international agriculture centers including ICARDA (International Center for Agricultural Research in Dry Areas).

1.2 Botanical Description and Crop Agronomy

Lentil is under the order *Rosales*, suborder *Rosineae*, family *Leguminosae*, subfamily *Papilionaceae*, and tribe *Vicieae*. After a complex taxonomic history, lentil is eventually placed in the genus *Lens* Miller. The name *Lens* describes the shape of the seed of the cultivated form of lentil. Analyzing previous findings based on origin and spread, morphological, cytological, cytogenetical observations and more recently on the basis of isozyme and molecular studies (Ferguson and Robertson 1996), *Lens* was re-classified by Ferguson *et al.* (2000) into seven taxa split into four species:

Lens culinaris Medikus subsp. *culinaris*

subsp. *orientalis* (Boiss.) Ponert

subsp. *tomentosus* (Ladiz.) Ferguson *et al.* (2000)

subsp. *odemensis* (Ladiz.) Ferguson *et al.* (2000)

Lens ervoides (Brign.) Grande

Lens nigricans (M. Bieb.) Godr.

Lens lamottei Czefr.

Most lentil researchers now accept this latest classification (Sarker and Erskine 2006). *Lens orientalis* is the presumed progenitor of cultivated *Lens culinaris* Medik. The last name is for Medikus, a German botanist-physician who has given the name to the plant in 1787.

Lentil plants are typically short, slender, semi-erect annuals varying between 15 to 75 cm height depending on the genotype and en*vir*onmental conditions. Individual plants may bear single stems or may be multi-branched. Branches may arise directly

from the main stem or from the cotyledonary node below ground or they may rise from other branches depending on the available space in the field and en*vir*onmental conditions (Saxena and Hawtin 1981).

The plants have slender tap roots and a mass of fibrous lateral roots. Various types of root systems, ranging from shallow branched roots to deep tap roots, are recognized depending on the texture of soil and climate in the area (Nezamuddin 1970). The tap root and the lateral roots in the upper layers of the soil carry numerous small, round or elongated nodules which start to decline before the onset of flowering (Saxena and Hawtin 1981).

Lens species are diploid plants with $2n = 14$ chromosomes. They all have similar karyotypes consisting of three pairs of metacentric or submetacentric chromosomes, three pairs of acrocentric chromosomes and one satellite pair of chromosomes (Slinkard 1985).

Lentil plants complete their life cycle in three to four months under optimum en*vir*onmental conditions. These conditions are usually reached in spring-sown crop, but in winter-sown crop, growth duration delays up to 30 to 60 days, especially in the earlier stages of growth, because of the suboptimal temperatures. Optimum temperatures for lentil seed germination is in the range of 15-25°C, but above 0°C seeds can germinate. Optimum temperatures for growth and yield are around 24°C.

Due to the hypogeal germination, in which cotyledons remain below the ground, lentil plants are less likely to be killed by freezing, wind, insect damage and grazing. If the young shoots are damaged, new buds can be initiated from the nodes below ground. The crop shows better tolerance to drought than waterlogging (Hawtin et al. 1980). Throughout the world, a large proportion of the lentil crop is grown in semi-arid regions without the benefit of irrigation. In most of these regions, agriculture depends on water conserved in the soil after fall and winter rains. Lentils show adaptability to a wide range of soil types.

Lentils can be grown in sandy loam soils, alluvial soils, and black cotton soils or in heavier clay soils (Nezamuddin 1970). On soils with very high natural fertility and excessive soil moisture, the crop might make excessive vegetative growth but this reduces seed yield (Saxena and Wassimi 1980).

Lentils grow well on slightly acidic soils (pH 5.5 to 6.5) to moderately alkaline soils (pH 7.5 to 9.0). Delayed nodulation and decreased yields have been obtained when the pH of the soil increased beyond 9 (Bharadawaj 1975). Most genotypes of lentil are very sensitive to soil salinity. Especially in the irrigated lentil growing areas, this becomes a major constraint in obtaining proper yields. Salt tolerance of lentils was reported to be much less than that of most cereals, chickpeas, lupins and faba beans although it is higher than that of common bean, cowpeas and soybeans (Ivanov 1973).

1.3 Lentil Abiotic and Biotic Stresses Overview

Abundant portion of worldwide lentil production is confined to the areas of low rain fall and little irrigation. This also hampers application of fertilizers and hence yield potential of lentil cannot be approached.

Attempts for breeding lentil races with higher yield are carried out through the globe. ICARDA as the leading organization, USA, Canada, Australia and Turkey possess a research background on lentil.

Major lentil producers are India, Turkey, Canada and Australia. Lentil production and consumption commerce is cycling between Canada, Australia, U.S. and Middle East and North Africa countries respectively. Other countries mainly consume their lentil production in their market.

As it might be expected like in other grain legumes domesticated long ago an array of abiotic and biotic stress factors effect lentil production severely. Cold, drought, heat, salinity, nutrient deficiency and nutrient toxicity are abiotic stress factors that affect lentil yields worldwide (Monti et al., 1994; Saxena, 1993; Slinkard et al., 1994).

Among an array of increasing biotic stress factors that affect lentil, Ascochyta blight, Anthracnose, Fusarium wilt, root rots, Scierorinia white mold, rust and numerous aphid transmitted *vir*uses can be listed as major yield threats. Resistance traits against some these stress factors were recovered from lentil germplasm. Lentils are being bred for these resistance traits with the help of molecular markers provided.

As long as lentil production spreads to new farmlands experiencing new en*vir*onments along with new abiotic and biotic stress factors, lentil breeding should be speeded up. Along with modern breeding methods that utilize advanced markers, interspecies identification of new resistance genotypes for both abiotic and biotic stress factors and also piling up transgenics that show genius solutions to these stress factors point out the transgenic technology to be at the center of breeding programs.

1.4 Breeding Problems and Genetic Engineering in Some Legume Species

1.4.1 Genetic Engineering of *Phaseolus* Species

1.4.1.1 Current Status and Potential of Conventional Breeding

The *Phaseolus* genus covers more than 30 species, but only five of them (*P. acutifolius, P. coccineus, P. lunatus, P. polyanthus, P. vulgaris*) were domesticated (Debouck, 1999). Among them the common bean (*P. vulgaris*) is the most widely cultivated one.

Phaseolus species diversity was organized into primary, secondary, tertiary, and quaternary gene pools (Singh, 2001) that do not have intercrossing problems in general. The primary gene pool (most of the cultivated species of the genus *P. vulgaris*), the secondary gene pool (*P. coccineus, P. costaricensis, and P. polyanthus*) and the tertiary gene pool (P. *acutifolius and Phaseolus parvifolius*) can intercross in their own gene pools without problems in the progeny (Broughton et al., 2003).

Phaseolus species have grains with high protein content and high quantity of complex carbohydrates. Also grains show low dietary fiber content, low oligosaccharides, and phytochemicals. They are also important sources of iron, phosphorus, magnesium, manganese. *Phaseolus* grains also provide some calcium, copper and zinc (Broughton et al., 2003)

Traditional breeding of common bean has objectives like stable yield reduced production costs tolerance for biotic and abiotic stresses that are achieved to some extent. Agricultural business seeks for common beans of erect plant canopy with pods that do not touch the soil and would allow mechanical harvesting. Also they look for low loss indices and high grain quality along with earliness. On the other hand consumers are requesting bean cultivars with higher nutritional quality and mineral bioavailability. Reduced phytate, tannin, polysaccharides, and oxalates contents along with lower fiber amounts are accompanying traits for higher nutritional quality and mineral bioavailability. In special iron for example is affected by the amount of tannin and dietary fibers.

Breeding superior cultivars of common bean narrowed down the allelic variations for many traits. Today new methods of conventional breeding are employed for recovering commercial cultivars from genetic bottleneck. But trait assessments with already prepared genetic markers remain controversial. Also the long period of breeding practice cannot accommodate with the newly appearing pathogen races. Besides all of these constraints the crucial point raised is identification and evaluation of new tolerance genes and alleles from other species. These traits cannot be transferred with conventional breeding due to crossing barriers. Hence urgent advancements in genetic background of common bean require the genetic engineering technology.

1.4.1.2 Common Bean Tissue Culture

Common bean tissue culture actually follows the transformation attempts. Initial genetic engineering works that showed transgene expression was all nullified due to absence of proper regeneration procedures. The tissue culture and regeneration of *Phaseolus* were extensively covered through 1997-2005.

Phaseolus tissue culture studies reported multiple shoot regeneration from mature embryo meristem regions under cytokinin induction. A special case study that may be noted here is multiple shoot regeneration from the meristem peripheral sections (Aragão and Rech, 1997). These regeneration works that employ embryo apex or axillary meristems however did not produce satisfactory results (McClean and Grafton, 1989; Malik and Saxena, 1992; Mohamed et al., 1992, 1993). Optimization studies over these works gave satisfactory and reproducible regeneration scores thereafter. Regeneration was induced at thin sections of meristems (transverse thin cell layers; tTCL) cultured under cytokinin (Carvalho et al. 2000). The same author also showed that AgNO3 with BAP together enhanced shoot number per explant and increased shoot elongation.

1.4.1.3 Common Bean Genetic Engineering

1.4.1.3.1 Bombardment Mediated Genetic Transformation

Initial genetic modification studies for common bean leans back to the *Agrobacterium* susceptibility analysis. Then generation of transgene expressing tissues was undertaken but regeneration from these tissues was not possible. (Lippincott et al., 1968; McClean et al., 1991; Franklin et al., 1993; Becker et al., 1994; Lewis and Bliss, 1994; Brasileiro et al., 1996; Nagl et al., 1997; Karakaya and Ozcan, 2001). At the mean time as an emerging trend, electroporation & PEG mediated transformation events were also carried out in bean protoplasts (Crepy et al., 1986; Bustos, 1991; Leon et al., 1991; Giovinazzo et al., 1993). All of these bean transformation works were obstructed due to lack of an efficient tissue culture and regeneration system that was just stated above in the preceding section.

Due to regeneration concerns and for evaluation of particle bombardment applicability to bean genetic engineering initial microprojectile delivery of marker genes targeted to the embryo apex meristems (Genga et al., 1991; Aragão et al., 1992, 1993; Russel et al., 1993). And consequently first transgenic common bean was generated through microprojectile bombardment method on navy beans (cv. Seafarer) (Russel et al., 1993). Researchers noted a very low (0.03%) transformation efficiency and transgene expression in progeny. However the method applied did not produce transgenics for other cultivars.

In depth evaluation of the bombardment mediated meristem transformations introduced meristem apex functional organization concept to the transformation studies. Faith of the microprojectiles delivery to meristem L1, L2, and L3 functional were considered for efficient production of transgenic plants. It was noted that microprojectiles that hit to the L2 and L3 layers may possibly give rise to transgenic plants (Aragão et al., 1993).

During ongoing studies with microprojectile bombardment for some common bean species it was also noted that meristems hindered with leaf initials had drastically reduced number of microprojectiles penetrating deep into the meristems. Also de novo shoot regeneration induced by cytokinins from the transformed apical meristems was shown to be confined to the peripheral layers of the meristematic ring (McClean and Grafton, 1989; Franklin et al., 1991; Malik and Saxena, 1992; Aragão and Rech, 1997). As a result a notion claiming that "apical meristems are noted not to be suitable for microprojectile mediated transformation" was raised.

Besides all these works a reproducible bean transformation work was elicited by Aragão et al. and Aragão and Rech at 1996, 1997 respectively. In their work bombarded embryonic axes raised transgenic plants with and average frequency of 0.9% transformation frequency. With this method researchers were able to transform different *P. vulgaris* varieties including some of the recalcitrant ecotypes.

Although successful common bean transformation methods were noted, the in-efficient selection system was an existing problem. A few number of cells transformed at the meristem regions in each transformation event could not be selected properly with the antibiotics, herbicides used in selection (Aragão et al., 1996, 2002). For this purpose a novel agent named "imazapyr" was discovered. Imazapyr is an herbicide that actively deposits at apical meristems. In an efficient transformation study, sequentially bombarded meristems selected with imazapyr generated significant increase transformation rates. And the rate of transgenic progeny was comparable to that of the soybean transformation. Also with this method occurrence of chimeric plants were avoided. (Aragão et al., 2000).

1.4.1.3.2 *Agrobacterium* Mediated Genetic Transformation

Although common bean transformation studies were started the only *Agrobacterium* mediated genetic transformation on common bean was reported in 2005 by Liu et al. In this study Liu et al. reports transformation of Kidney beans through a procedure that is based on sonication and vacuum infiltration of *Agrobacterium* cells (LBA4404) with germinating beans. Potting of the transformed seedlings gave rise to 16 transgenic plants with a rate of 3% transformation efficiency. The transgene expression was demonstrated till the second generation.

1.4.2 Cowpea

1.4.2.1 General Information about Cowpea

Cowpea is the representative legume with high quality protein content and is used as food and feed in African continent. This important crop with the species name *Vigna unguiculata* (var. *unguiculata*) is also known as black-eyed pea. Adaptation to hot climate and low, unsteady rainfall are the two distinguishing features of cowpea. Adaptation to drought can be attributed to its lower canopy covering the soil surface, trapping the moisture in the ground in the semi-arid zones. Traditionally cultivated cowpea is usually intercropped with sorghum, millet, maize, or cassava and generally not harvested for commercial concerns. This plant shows a wide

variety of growth habits and morphology. Its extended tap root that recovers the scarce water during drought season maintains its survival (Bado et al., 2006).

Both foliage and grains of cowpea are proper sources of protein. Grains contain about 23–25% protein by weight (Ohler et al., 1996). Prior to flowering fresh leaves were harvested and used as food during the drought period when the food is scarce. Enhanced lysine content and other essential amino acids makes it good complement to cereals as it is the general case for grain legumes. Also grains of cowpea contain approximately 60% carbohydrates composed of starch mainly. Grains of cowpea also serve as a quite good source of essential vitamins including thiamin, riboflavin, ascorbic acid, niacin, and folic acid. Cowpea harvested in Africa is also consumed within the boundaries of the continent.

Classification of cowpea seems rather complex. Cowpea is under family Fabaceae (Leguminosae), subfamily Papilionoideae and genus *Vigna*. Also cowpea belongs to the subgenus *Vigna* and section *Catiang*. Finally cowpea is classified under species *unguiculata*.

Under the light of morphological, ethnographical and molecular evidences *V. unguiculata* was classified into 11 subspecies (Pasquet 1999). Only one of the eleven species is annual and is cowpea. And Cowpea has two varieties; the cultivated *unguiculata* and the weedy form *spontanea*.

1.4.2.2 Conventional Breeding of Cowpea

In cowpea research an unofficial group named "Network for the Genetic Improvement of Cowpea for Africa" (NGICA) shoulders cowpea improvement including genetic modification. Besides this group cowpea genome sequencing is undertaken by University of *Vir*ginia through a new approach. This new approach was lanced as Gene Spacing under the trade name GeneThresher^{R}. With this sequencing method and through ESTs, SSRs, SNPs extensive data are being generated for cowpea breeding.

Insect is the major cause crop loss for cowpea in the field and during storage (Ogbuinya, 1997). Insecticide use for cowpea was noted to be improper and traditional which was also noted to be main cause failure in insect control (Murdock 2002). Also use of proper insecticides for cowpea harvest may impose a bulk of insecticide pollution in mainland Africa each year (Murdock 2002). Compared to the losses by insects crop losses via *vir*al diseases can be noted as insignificant.

Major breeding objectives for cowpea improvement include disease and insect resistance and improvements in physiological characters along with quality traits. Conventional breeding is generally considered for non multigenic heritable traits. Notable improvements were achieved through conventional breeding of cowpea. Constraints of conventional breeding and need for genetic engineering also hold for cowpea improvements.

1.4.2.3 Development of Transgenic Cowpeas

Cowpea genetic pool contains approximately no resistance trait against insect pests that are the main cause for immense yield loses. Cultivars that avoids these insect based loses has been in center of genetic improvement for cowpea (Ehlers and Hall, 1997).

First transformation report on cowpea was recorded by Garcia et al. (1986, 1987). Kanamycin resistant callus without regeneration was the only outcome of this study. Later studies on cowpea transformation were reported by, Penza et al. (1991) on longitudinally sliced mature embryo but stable integration of the transgene was not recorded in regenerated plants. In another study that shared the same faith, Muthukumar et al. (1996) selected the transformant cotyledons with hygromycin that did not produce transgenic progeny. In a similar procedure that used also cotyledons, microprojectile bombarded explants yielded only a small number of progeny expressing the transgene that is not supported with stable integration data in the progeny (Ikea et al. 2003).

The first transgenic cowpea was achieved by Popelka et al., (2006) considered as the main frame of the optimized cowpea transformation procedure. And this procedure has been modified significantly. Major modifications that enhanced transformation rate significantly can be dropped into three sections. First, embryonic axis confined to the cotyledons was used as target tissue for transformation. Second, salt strength of the *Agrobacterium* re-suspension medium was reduced by 10X. And third, sulphur containing compounds (thiosulphate, dithiothreitol and L-cysteine) were added into re-suspension and co-cultivation medium that reduces necrosis of wounded tissue (Olhoft and Somers, 2001; Olhoft et al., 2003).

As it is extensively reviewed for other legume species the cotyledonary node or embryonic axis that are used for direct organogenesis appears to be the most suitable explants for successful genetic transformation (Schroeder et al., 1993; Pigeaire et al., 1997; Olhoft and Somers, 2001; Popelka et al., 2004). On this basis a simplified explant preparation method devised for cowpea transformation produces 1–3 transgenic plants per 1000 cotyledonary nodes processed. In this simplified explant preparation procedure overnight imbibed seeds are first deprived of their hypocotyl and epicotyl segments and then either bisected along the embryonic axis or stripped of their cotyledons leaving the cotyledonary node behind.

Current problems in front of the genetically modified cowpea are the usual concerns for genetically modified organisms. However *Bt*-cowpea would perhaps mean much for the hungry although its economic, ecologic and loyalty concerns are to be resolved prior to committing the transgenics.

1.4.3 Chickpea

1.4.3.1 General Information About Chickpea

Chickpea (Cicer arietinum L.) is a self-pollinating annual legume that is cultivated mainly as food around the globe. It is termed as cool season crop. And it gives proper yields during dry seasons due its deep tap root system.

Grains of chickpea offer a balanced source of protein and carbohydrates. Also it serves some of the essential amino acids for human nutrition along with high amounts of unsaturated fatty acid. Unlike some other grain legumes chickpea shows least amounts anti-nutritional factors. High nutrient digestibility hence bioavailability and uses in herbal medicine, cosmetics and also its nitrogen, organic matter contribution to the soil make chickpea a multifaceted crop that should receive a special attention.

The origin of its wild progenitor *Cicer reticulatum* is located to the southern Turkey (Ladizinsky and Adler 1976). And chickpea is the only domesticated species of the genus *Cicer*. The plant was originally classified in the family Leguminosae and Papilionoideae subfamily and in the tribe Vicieae but depending on the recent evaluations, vascular and pollen structure data leaded its settlement into its own monogeneric tribe, *Cicereae* (Alef).

1.4.3.2 Conventional Breeding of Chickpea and Ground for Transgenics

High yielding cultivars along with tolerance to abiotic stress factors and resistance biotic agents comprise the main aspects of chickpea breeding. Sensitivity to drought and cold are among the first abiotic stress factors to be dealt. Then sensitivity to heat and salinity are considered to be the second important abiotic stress factors. Susceptibility to pests and diseases or competing against weeds during farming practices are also as critical as the abiotic stresses notified.

It is noted that the average yield of chickpea is never reached to its potential due to the diseases encountered during cultivation. And the self-pollinating nature of the chickpea and conventional breeding practices consequently narrowed down the genetic base. As a consequence breeding chickpea for superior lines through conventional breeding is hindered. This insufficient genetic base and crossability problems with far relatives of chickpea in *Cicer* genus enforced breeding through transgenic technology.

1.4.3.3 Tissue Culture and Regeneration

Nearly all of the *in vitro* plant regeneration protocols have been applied to chickpea. Direct organogenesis of shoots from meristems (Bajaj and Dhanju, 1979; Sharma et al., 1979) or immature cotyledons (Shri and Davis, 1992) and embryogenesis from immature cotyledons (Sagare et al., 1993) or leaf callus (Barna and Wakhlu, 1993; Kumar et al., 1994) are representing only some of the regeneration attempts.

A higher frequency of regeneration was shown on embryonic axis with 6-benzylaminopurine (BAP) induction in *in vitro* conditions (Fontana et al. 1993). Later on Kar et al. (1996) used both BAP in combination with α-naphthaleneacetic acid (NAA) and showed high frequency shoot regeneration from embryonic axis explants. Later studies on chickpea became as improvements and reputations of the regeneration works on embryo axis. As the reports and regeneration data piled up highest shoot number per explant was scored on half embryo axis attached to a cotyledons (Sarmah et al., 2004).

1.4.3.4 Genetic Transformation

The first successful transformation work on chickpea was achieved through co-cultivation embryonic axis with *Agrobacterium* cells (Fontana et al., 1993). Transformation rate was considerably low but stable integration of transgene was confirmed.

In two other following transformation attempts the multiple shoot regeneration potential of the chickpea embryonic axis was exploited. In their study Kar et al., (1996) and Krishnamurthy et al., (2000) were able to give evidence for transgenic chickpea progeny.

In chickpea genetic engineering studies performed, selection of the transformed explants were carried out either with kanamycin (neomycin phospho trasnferase II/ *nptII*), (Fontana et al., 1993; Kar et al., 1996) or phosphinothricin (phosphinothricin

acetyl transferase/ *pat*) (Krishnamurthy et al., 2000). Aspartate kinase (AK) is also another selective marker used in chickpea transformation studies but among these three only *pat* selection is reported to be the reliable.

Chickpea transformation studies received many improvements following the work of Fontana et al., (1993). The common point that all of these improvements share was use of embryo axis as explant due to its higher regeneration potential. Different groups had different approaches for embryo axis preparations (Sarmah et al., 2004; Polowick et al., 2004; Senthil et al., 2004; Sanyal et al., 2005). But overall transformation rate was ranged between 0.1 to 1.0%.

Compared to *Agrobacterium* mediated transformation, microprojectile bombardment mediated transformation studies on chickpea is scarce. Among these studies bombardment mediated transformation of chickpea by Kar et al., (1997) used explant preparation and regeneration procedure that was quiet similar to that of *Agrobacterium* mediated transformation works. Selection of the explants on kanamycin media yielded cryIA(c) expressing shoots that are regenerated into whole plantlets.

These successful transformation procedures summarized are being used and continue to yield substantial numbers of transgenic chickpea and these chickpeas are underway for field trials.

1.4.4 Alfalfa

1.4.4.1 General Information About Alfalfa

The two species *Medicago falcata* and *Medicago sativa* are known as alfalfa. Plant is perennial and cultivated as a main fodder input of around the globe. Central Asia or Middle East is the two possible speciation origins for alfalfa.

The foliage is harvested four times in a year as animal feed. And it serves high quality nutrition for animal feed. Its extensive root system that can penetrate deep

into the soil and nitrogen fixing ability maintains adequate protein, vitamins and minerals for the feed.

1.4.4.2 Problems on Traditional Breeding

Quiet low rates of successful inbreeding and autotetraploidy of the cultivars hampers conventional breeding. And inheritance of multigenic traits becomes more complex due to the autotetraploid nature of alfalfa. Due to this stated genetic nature of alfalfa breeding was carried through selection of phenotypic traits. Complex genetic structure, background and limited crossability were the main cause of limited conventional breeding practices (Brummer, 1999; Lamb et al., 2006). As a consequence alfalfa improving through genetic modification remains as the most plausible tool.

Winter hardiness, salinity tolerance and phytotoxicity are the traits to be concerned for the yield enhancement and digestibility, nutritional modifications and processing are the topics for quality improvement of alfalfa. Achievements on genetic modification of alfalfa had already covered many of the above stated enhancements.

1.4.4.3 Alfalfa Genetic Engineering

An extremely well established transformation method for alfalfa is the main boost behind the numerous transgenic alfalfa plants. Basically transformation procedure resembles tobacco transformation in which leaf discs of alfalfa are co-cultivated with *Agrobacterium* and selected in kanamycin media.

Such a handy tool of transformation protocol also leaded to the promoter function analysis in alfalfa. These promoter activity studies might be accounted for transgenic studies on other plants also.

1.4.4.3.1 Manipulation of Lignin Composition for Forage Quality Improvement

Lignin maintains the structural integrity of the secondary plant cell wall. Digestibility of lignin was attributed to the relative compositions of lignin subunits.

First hypothesis for increased lignin digestibility targeted the ratio of the most abundant lignin subunits. Reddy et al., (2005) altered the ratio of monomethoxylated guaiacyl (G) and dimethoxylated syringyl (S) monolignons through transformation of alfalfa leafs with *M. truncatula* cytochrome P450. Kanamycin selected transgenic alfalfa plants synthesized different levels of lignin did not show any enhancement in fiber digestibility.

Second hypothesis for increasing fiber digestibility was constructed on the relative ratio of low abundant p-hydroxyphenyl (P) lignin subunits (Reddy et al., 2005; Ralph et al., 2006). Alfalfa coumaroyl shikimate 3-hydroxylase (C3H) was down regulated with the antisense of C3H gene from *M. truncatula*. This transformation event resulted in an increase in the ratio of p-hydroxyphenyl (P) units with respect to G and S lignin subunits. Transgenic alfalfa generated with this method showed improved alfalfa feed digestibility.

1.4.4.3.2 Reducing Protein Loss for Forage Quality Improvement

Harvested alfalfa gradually loose its protein content till it is fed. Nonspecific proteases are considered as the principle cause of this gradual protein degradation. And ortho-diphenol was shown to inhibit these nonspecific protease. On this basis alfalfa was transformed with clover poly phenol oxidase (PPO). Transgenic alfalfa with constitutive expression of clover PPO was recovered. And it was shown that transgenic alfalfa plants harvested showed fivefold decrease in nonspecific proteolysis with respect to control plants (Sullivan and Hatfield, 2006).

Ruminant bacteria colonizing the cattle and sheep gut degrades the feed proteins rapidly and serve animal with the bulk bacterial proteins. Rumen bacteria provided protein is low in sulphur amino acids. Schroeder et al., (1991) transformed alfalfa with chicken ovalbumin gene that contains high amounts of sulphur amino acids and resistant to bacterial degradation. Transgenic alfalfa plants recovered through *nptII* selection showed ovalbumin expression constituting 0.001% to 0.01% of the total crude protein.

1.4.4.3.3 Overexpression of Transcription Factor to Improve Drought Tolerance

In order to generate drought tolerant alfalfa Zhang et al., (2005) transformed alfalfa with putative *Medicago truncatula* WXP1 gene AP2 domain. Basta selected transgenic alfalfa showed approximately 34% more cuticlar wax accumulation. Transgenic alfalfa showed increased resistance to drought, better recovery following drought application and decreased chlorophyll bleaching.

1.4.4.3.4 Improvement of Tolerance to Aluminum Phytotoxicity

Main problem with acid soils that maintain higher mineral content to plants is aluminum toxicity.

Solution formulated for this problem was designed as exudation of conjugate bases of organic acids from the roots of alfalfa. Or this purpose nodulary enhanced malate dehydrogenase enzyme coding sequence (neMDH) was transformed into alfalfa (Tesfaye et al. 2001). Transgenic alfalfa expressing neMDH showed enhanced aluminum tolerance with enhanced exudates organic acid conjugates. In hydroponic cultures of transgenic alfalfa (elevated levels of citrate, oxalate, malate, succinate, and acetate were recovered.

1.5 Demand for Genetic Improvement of Lentil

Although conventional breeding methods continue to their developments, improvement of lentil by conventional breeding tools is still delimited by the boundaries of crossability, hybridization and scarcity of novel genes in the lentil germplasm pool.

Breeding through transgenic technology floats over the genetic barriers encountered trough conventional breeding. Transgenic breeding technology can eliminate anti-nutritional factors such as trypsin inhibitors and hemagglutinins in a short time and without loss of any trait as compared to conventional breeding methods. Since lentil is one of the few crops that can be grown in the semiarid regions of the world, nutritional quality improvement can have a significant impact

for balancing of dietary intakes of people living in areas with poor soils and limited rainfall. Hence transgenic technology can also be used for the improvement of nutritional quality of lentil proteins which are characterized by a shortage of methionine and cysteine.

Considering the shortcomings of conventional breeding and on the whole due to its importance as highly nutritional pulse crop of the semi-arid lands there exists a demand for genetic engineering of lentil against abiotic and biotic stress conditions for sustaining crop production and improving its yield.

1.6 World Wide Plausible Transformation Studies on Lentil

From liposome mediated to electroporation, so many methods of transformation strategies were studied in order to figure out an efficient gene transfer system for lentil. As a result of these studies two of the plausible transformation systems turned out to be *Agrobacterium tumefacience* and Microprojectile bombardment mediated gene transfer systems as many of the elaborate transformation studies implied.

Among the pioneers of *Agrobacterium* mediated transformation of lentil was Warkentin and McHughen (1991.) Following their initial *Agrobacterium* strain selection (GV2260) for lentil transformation, in 1992 they were among the first that used p35GUSINT vector construct. *Vir*tually in all of the *Agrobacterium*-mediated transformation optimization studies (Warkentin and McHughen 1992; Warkentin and McHughen 1993; Lurquin *et al*. 1998; Oktem *et al*. 1999; Mahmoudian *et al*. 2002) p35SGUSINT vector containing kanamycin resistance gene (*NOS-NPT*II-*NOS*) and intron containing β-glucuronidase (*GUS*) gene was used. The vector was generated by cloning *GUS* gene cassette with cauliflower mosaic *vir*us 35S promoter and terminator from pGUSINT plasmid into Stratagene pBS vector (Vancanneyt *et al*. 1990).

Transient expression of visual markers on various lentil tissues highlighted meristematic zones (apical meristems, lateral meristems, cotiledoary nodal segments) as efficient explants for gene transfer but, during regeneration from these

tissues expression of visual marker (*GUS*) diminished quiet often. Possible cause was assigned to possible death of transformed zones due to immense wounding of explants prior to *Agrobacterium* infection. Solution devised to the problem was gentile wounding with microprojectile bombardment. Also in later transformation studies the cotyledonary nodal segments were chosen as the target tissue for transforming lentil due to high frequency shoot regeneration from its axillary meristems under cytokinin induction.

Further problems in lentil transformations aroused in the step of selecting putative transformants. In the transformation study by Warkentin and McHughen (1992), selection of transformed tissues was done by the use of kanamycin which was later expressed as very inefficient for the type of explant used. In general, negative selection procedures were considered to be in-efficient during selection of transformed meristematic tissues. It was because small transformed sectors of meristem tissues were thought to be driven to extinction by administration of high amount of selection agent in the culture media killing most of the non-transformed delicate nursing cells which would normally support the transformed section. Since all of the plausible transformation attempts so far depended on direct regeneration of the plantlets from intact mature meristematic tissues (especially cotyledonary node axial meristems), selection of transformants was always a problem in lentil transgenic studies as it is also the same for most of the grain legume transformation studies.

The only plausible report about genetically engineered Lentil was published by Gulati *et al*. in 2002 with viable F1 generation inheriting the transgene with Mendelian ratio. Transformation of lentil cotyledonary nodal segments was mediated by bombardment of pBUC19 plasmid with chimeric SuRA/SuRB Hra acetolactate synthase gene (*ALS*) from tobacco, conferring resistance to sulfonylurea herbicides.

Recovery of low number of transgenic plantlets in this study can mainly be attributed to the efficient selection method used; with sulfonylurea urea herbicide in an all or none fashion

1.7 Overview of Lentil Transformation Studies Made in Our Lab

Our lab initiated the study of lentil transformation with Oktem *et al.* in 1999. In the study *GUS* gene delivery and expression in lentil cotyledonary nodes was mediated through particle bombardment method. Throughout the study an electronic processor controlled GeneboosterTM (Jenes *et al.* 1996) particle delivery system was used. *GUS* expressing sectors were seen on 4% of the regenerating shoots.

Later in 2002 in the study by Mahmoudian *et al.* cotyledonary nodes were infiltrated with *Agrobacterium* strain GV2260. Differing from all of the preceding *Agrobacterium* mediated transformation works bacterial cells were concentrated prior to inoculation with explants. Through a mild infiltration process a significant increase in transient GUS activity was attained. The most effective factor for higher rate of transformation frequency was assigned to the use of higher concentration of *Agrobacterium* cells during inoculation. In this study pGUSINT binary plasmid which was derived from pBI121 through insertion of p35SGUSINT vector was used.

Following the works of Oktem and Mahmoudian in another study in our lab five different *Agrobacterium* strains; LBA4404, EHA105, GV2260, C58C1 and KYRT1, and two different binary plasmids; pGUSINT and pTJK136 containing *GUS* and *npt*II genes were tested for the best transformation response in lentil (Celikkol, 2002). In the study, also the factors affecting *A. tumefaciens* mediated transformation efficiency like wounding method (microcarrier and needle-mediated), effect of vacuum infiltration and cultivar difference (Firat-87 and Sultan-1 cultivars) were analyzed. By the use of best strain/plasmid couple and needle wounding, putative transgenic shoots were regenerated from cotyledonary nodes with 0.8% efficiency, micrografted to root stocks and successfully transferred to soil.

Regarding the previous studies in our lab as the basis, transformation response of another lentil tissue and micrografting based regeneration were studied in my thesis work under the heading "Optimizations of *Agrobacterium* Mediated Gene Transfer and Micrografting Systems in Lentil (*Lens culinaris* Medik) - Kamci, H. (2004) M.Sc. thesis. In the first part of the study of *Agrobacterium* mediated gene transfer to lentil embryo apex, type and intensity of injury, evacuation duration and intensity, L-cysteine induction during co-cultivation and *Agrobacterium* infection duration were among the studied parameters.

High rates of transisent GUS expressions were recovered from these studies that diminished to small number of scattered patchy GUS expressing zones during culturing under no selectional force. In the regeneration part of the study (regeneration through micrografting), factors such as grafting type, grafting stem height, and the scion health were analyzed. Higly efficient regeneration system through micrografting and plausible transient visual marker gene (GUS) expression at the apical meristem zones were the two important yields of this study.

Meanwhile in another study in our lab (Bayrac 2004), optimization of regeneration system for cotyledonary petioles of lentil was carried out. Lentil cotyledonary petiole was shown to be one of the most responsive tissues for *Agrobacterium*-mediated transformation, so an optimized tissue culture system for this tissue was foreseen to expedite the development of transgenic lentil plants. The most responsive media for shooting were MS basal medium supplied with 1 mg/L Zeatin and 1mg/L NAA, and medium supplied with 1mg/L TDZ respectively.

1.8 An Overview of Lentil Transformation and Regeneration Problems

All of the transformation studies targeting genetically engineered lentil lines were hindered with the lack of a proper regeneration system. Till to the studies that choose the cotyledonary nodal segment as the target tissue of transformation, regeneration of low number of shoots from the transformed tissues was considered as cost inefficient. Upon exploration of high regenerative capacity of the

cotyledonary nodal segments, studies for handling higher number of transformed shoots implemented usage of high concentrations of cytokinins in tissue culture media. However, during the rooting procedures it was understood that the impact of cytokinin usage was depressive to root regeneration. This issue was adequately covered in the study by Polanco and Ruiz (1997) that studied the effect of BAP on root formation from lentil shoots regenerated on BAP containing media.

This dilemma of rooting problem versus the demand for harvesting higher number of putative transgenic shoots from transformation studies of lentil were clearly resolved by Gulati et al. (2001). In their work, lentil shoots regenerated on BAP media were micrografted onto 5-6 days old rootstocks with 96% efficiency. The success of micrografting was noted to be independent of the nature and concentration of growth regulator used in shoot initiation medium and the time period for induction of shoots. In a similar work performed by Khawar and Özcan (2002) cotyledonary node explants from 21 different lentil genotypes were cultured on on MS medium containing 0.225 µM thidiazuran (TDZ). The highest shoot regeneration reported was 20.6 shoots/explants with Akm 362 genotype. The regenerated shoots were reported to be micrografted to cultivar Kayı 91 with 100% efficiency.

Although the above reports give micrografting success scores as high as 100% efficient, as a regeneration procedure micrografting is not that much easy. To maintain a success rate above 90% there should be a perfect match between the root stock and regenerated lentil shoots that are used as scion. Still there are other factors associated with micrografting can crackdown such high rates of regeneration success. Therefore regeneration success of 100% through micrografting is possible as long as you have either very low number of samples or you have achieved the perfect match between the root stock and the scion to avoid the laborious and gentle stages of micrografting. Also another important step that is strongly tied to micrografting is acclimatization, which usually introduces drastic decrements in success rates of micrograft based regeneration. Regarding the factors involved in regeneration through micrografting, type of grafting, grafting stem height, and the

scion health were analyzed in my study (Kamci 2004). Again in this study two graft types, namely cleft and whip type, were successfully adapted to lentil.

Transformation studies that yield transgenic plants in some of the legume species were shortly pointed out in previous sections. Cotyledonary section of the embryo axis in other words cotyledonary nodal segment is shown as the ultimate explant for genetic transformation either through bombardment or *Agrobacterium* mediated methods. For most of these plants transformation rate may not exceed 1%. The same case is also valid for transgenic lentils generated up to date (Gulati et al. 2002 and Akçay et al. 2009). Modifications in transformation process, strain cultivar couple, selection system for lentil should be adapted to the new advancements in transgenic technology.

Regeneration of shoots into whole plantlets through micrografting should be regarded as the optimum tool when we consider versatile tissue culture, induction and selection applications to be carried out for optimizing the ultimate transformation system to diverse lentil cultivars. But still some more advancement with our current micrografting system (which is currently on the run) is required to eliminate the highly gentle and laborious steps of grafting practice in order to handle the gentle herbaceous structure of lentil.

The regeneration protocols (leading to transformation) for many grain and forage legumes considered in preceeding sections can be simply catagorised under two main headings; first regeneration from apical or axillary meristems and second regeneration from foliage sections. Both of the regeneration protocols employ direct organogenesis but the first group maintains regeneration from already present meristematic zones that are already destined to give rise shoots. However in the second group of regeneration protocols explant is enforced to differentiate into meristematic sections that probably raise a shoot which is not normally programmed for. Also cytokinin induced shoot regeneration from the meristematic sections is shown to be confined to peripheral regions of the fully organized apical or axillary meristems. Other than soybean studies, transformation events either through

Agrobacterium or bombardment mediated methods that are usually targeting these meristem sections in most of the transgenic grain legume studies. And yileds are at most 1%. On the other hand transgenic alfalfas are routinely generated through *Agrobacterium* mediated transformation employed to the foliage tissues. As compared to soybean, alfalfa and clover transformation studies extremely low transformation rates scored for grain legumes dictate profound enhancements in transformation protocols. Although we have performed axillary meristems transformation experiments as an indispensable part of our transformation protocol as it is also stated just above it seems that either explant or its way of handling for transformation should be urgently altered for succeeding in proper rates of transformation in lentil.

1.9 Up to Date Advancements in *Agrobacterium* Mediated Gene Delivery

The starting point for an efficient *Agrobacterium* mediated transformation should be traced back to plant pathogen interaction. Dealing with the issue Kuta et. al. (2005) gives a brief review on *Agrobacterium* induced hyper sensitive response (HSR) generated during transformation. According to Kuta the HSR is correlated to explant age, pre-culture period, bacterial inoculation density and infection duration. The necrotic behavior of the HSR is mediated through accumulating antimicrobial agents (mainly phenolic compounds) at *Agrobacterium* exposed sites. Initial reaction of the tissues exposed to pathogenic determinants is described as oxidative burst (H_2O_2 release). Then signaling through H_2O_2 and pathogenesis related proteins mediates generation of reactive oxygenic species in order to kill bacteria outside the cell and damage the T-DNA released into the cytoplasmic space. Overall two solutions were given to the plant HSR; first quenching of agro induced oxidative burst and second re-programing plant cells for agro competency. Also the most effective agent for re-programing plant cells was noted to be wounding. The wound released phenolics for prevention of a probable biotic attack can be perturbed as advantage if the released phenolics (acetosyringone in this case) can be modified by *Agrobacterium* cells.

In order to touch up with the point it is quiet proper to give space to the review on plant phenolics in defense and communication (Bhattacharya et. al. 2010). The phenolics are abundant molecules in plants. And they are produced at diverse points of plant metabolism. Trauma, wounding, drought and pathogen attack are the inducers for phenolics synthesis. In the presence of H_2O_2, poly phenol oxidases activate phenols and phenol tag many proteins and enzymes which inactivates pathogens. Transcinnamic acid, coumarin benzoic acid, syringic acid are samples to phenolics that interfere with cellular enzymes. SA, IAA and GA_3 are other phenolics that down regulate *Agrobacterium* quorum sensing. Contrary to acetosyringone, SA has also antagonist effect on *vir* gene induction. Auxin is another point that *Agrobacterium* cells avoid plant pathogen response. This hormone is mediator for water deficit response in plants. Drought and cold are the two types of stresses that depress the water potential of the plant. *Agrobacterium* cells can mimic water deficit in plant and avoid HSR through synthesis of an auxin analog. The only requirement for synthesis of this auxin analog is tryptophan supplied (Escobar et. al., Article in press).

Although bacterial cells possess avoidance mechanisms against some of the plant pathogen responses a successful *Agrobacterium* mediated gene transfer usually requires external agents applied. Phenols are excreted in response to both plant-pathogen interaction and wounding. Phenol oxidation in tissue leads to necrosis and cell death. To avoid the effects of phenolics poly-phenol oxidase (PPO) inhibitors (DTT), antioxidants and phenol absorbing compounds are generally used agents. L-cysteine is among these agents that covalently bind to Fe/Cu core of the phenol oxidases (Olhoft et. al. 2001).

Besides depression of plant pathogen responses studies also consider competent plant cells concept through reprogramming the tissue for cell division or wound repair. Binns et. al. (2006) states that wounding is not a requirement for gene transfer since cell division can be triggered by other means. Also plant pathogen

response elicited during explant inoculation can be avoided through diverging gene expression induced by external means. An example can be mimicry of the wound induced signaling through application of ABA and Jasmonates.

Besides agents targeting the plant pathogen responses and hormonal reprograming of cells for induction of competence there are still some other studies that reports profound enhancements in *Agrobacterium* mediated gene transfer. Azadi et. al. (2010) reports that composition of inoculation and co-cultivation media strongly affects gene transfer rates. Researchers recorded striking rates of lilium transformation as they have added sucrose and removed KH2PO4, NH4 NO3, KNO3 and CaCl from their inoculation and co-cultivation media. It is also stated in the report that limiting PO4 concentration of the inoculation and co-cultivation media increases quorum sensing and *vir* gene inductions hence maintains *Agrobacterium* cells better attachment to plant cell wall. It was also highlighted that Ca may repress *vir* gene expression.

A 2006 review about the factors influencing efficiency of *Agrobacterium* mediated gene transfer considers almost a full vision of emerging trends in efficient gene transfer (Opabode, 2006). According to the report a pre-culture step during explant preparation in which explants are exposed to anti-necrotic agents or desiccated was noted to enhance the transformation rates. And also agents like 2,4-D, casamino acids and acetosyringone applied during inoculation and co-culture periods were noted to be among the enhancers. Decreased salt strength in inoculation and co-cultivation media was reported have enhancing effect also. Addition of surfactants, osmoprotectants and antinecrotics into inoculation media was also noted to be enhancers of gene transfer.

Above stated reviews of advancements in *Agrobacterium* mediated plant genetic transformation are schematized in figures 1.1 and 1.2.

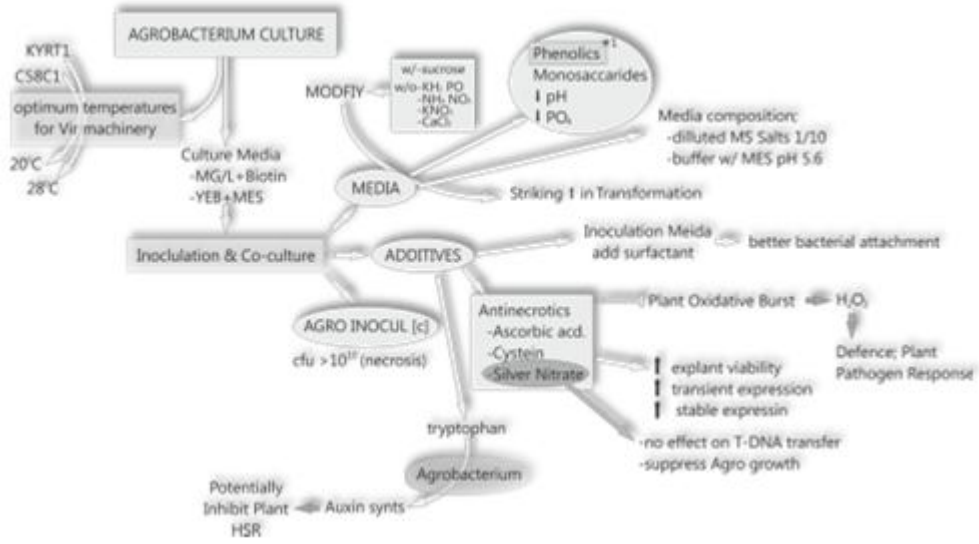

Figure 1.1: Up to date advancements in Agrobacterium culture preparation
Agrobacterium culture preparation for enhancing gene delivery to plants. Up to ted critical advancements considered.

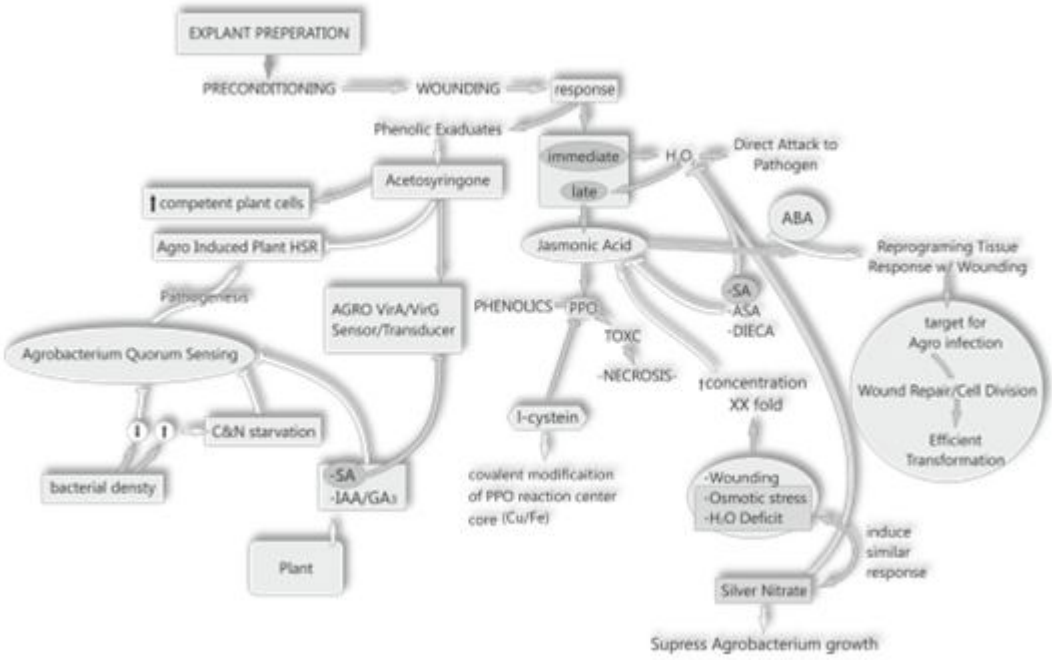

Figure 1.2: Recent considerations in explant preparation for transformation
Up to date agents and methods devised for enhancing gene delivery to plants are schematized.

1.10 MBF1c and Multifaceted Stress Tolerance

A proper transformation system that can achieve cost effective transformation rates can speed up the genetic modification studies for improving lentil. Since this crop is noted have good tolerance to cool weather conditions and show relative adaptation to different soil types (Erskine, 1984.) a strategic point in genetic improvement of lentil can be enhancement of this inborn tolerance of lentil to overcome moderate to high levels of freezing and other stress conditions. Also the poor yield due to susceptibility to insect and fungal diseases (Erskine, 1984) can be targeted in an improvement program as long as the employed transformation system compensates the costly and laborious steps of the study. In the first scene this task may be seen as multi feet transformation event for each trait we are after. But concerning this issue it is quiet suitable here to refer to the studies made on *Arabidopsis thaliana*.

Complex biological responses to en*viro*nmental stimuli within the boundaries of tissues, organs and organism require concerted playing from the notes of genomic information. During recovery of DNA information in the form of RNA the basic transcription machinery should be interacting with transcription factors docked on promoter-regulator elements on DNA. It is revealed that in yeast and humans this interaction is maintained through transcriptional co-activators that maintains the true active transcription (Kwok et al. 1994. Ge and Roeder 1994. Knaus et al. 1996). The plant homologs of these transcriptional co-activators were also long been expected to be revealed.

There are two classes of transcriptional co-activators. The first class of co-activators shows enzymatic activities that modify chromatin structure and alter availability of transcripts. The second class which we are interested in does not have enzymatic activity but regulates the docking of the basal transcription machinery with the transcription factors (Näär et al., 2001, Sugikawa et al. 2005).

A transcriptional co-activator denoted as multiprotein bridging factor 1 (MBF1), which was first extracted from silk glands of the silk worm, (*Bombyx mon*) was shown

to be also present in yeast (Takemaru et al. 1998), human (Kabe et al, 1999) and Drosophila (Liu et al. 2003). It mediates the connection between activator and TATA-box binding protein (TBP).

Identification and studies with other MBF1 (tomato (Zegzouti et al. 1999), potato (Godoy et al. 2001) and tobacco (Matsushita et al. 2002)) also showed that MBF1s are functioning in defense related responses. Recently three MBF1 subtypes were isolated from *Arabidopsis thaliana* (AtMBF1a-c). AtMBF1a, b expressions were seen throughout the plant almost constitutively suggesting that AtMBF1a, b expressions are regulated in the same manner, whereas AtMBF1c expression is not. The functions of the all three AtMBF1s are confirmed through partial recovery of yeast *mbf1* mutant phenotype. With this study also the co-activator function of MBF1s were confirmed. And in *Arabidopsis* all three MBF1s are shown to have differential tissue specific expression of in response to various effectors. Specifically ABA application was shown to increase AtMBF1c expression significantly supporting that AtMBF1c promoter domain shares similarity with ABA-responsive element (ABRE) (Giraudat et al. 1994, Hobo et al. 1999, Shen and Ho 1995, Shen et al. 1996). Further evaluation of the AtMBF1c (At3g24500) transcription from the ATH1 gene chips revealed that MBF1c expression is elevated in response to drought, heat, salinity, hydrogen peroxide and pathogen infection (Rizhsky et al., 2004b; Tsuda and Yamazaki, 2004).

Finally Mitler et al. (2005) reported that constitutive expression of AtMBF1c in *Arabidopsis* maintains stress tolerance against a number of abiotic and biotic stress factors and some combinations of abiotic stresses. That is AtMBF1c can be used for genetic engineering of plants for multi stress tolerance.

1.11 Aim of the Study

World wide spread and cultivation of lentil revealed the actual potential of lentil races for their tolerances to a number of multiple stress factors. Due to its nutritional value and use as food lentil has been receiving increasing attention through the globe. We expect that in near future with advent of an efficient transformation method in lentil, the piles of information on transgenics and conventional breeding may boost lentil transgenic studies.

Regarding the current transformation protocols in legumes and advancements in lentil transgenics and as long as transient expressions rates are considered (Kamçı 2004) stable transformation rates in lentil might be regarded as very low.

Aim of this study is to bring the rate of stable transformation in lentil to approximately 10-15%. And generate transgenic lentil lines expressing AtMBF1c.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Cloning Related Materials

2.1.1.1 Vectors, Coding Sequences and Primers

The vectors used in this study are as follows: IV 1.1, pBlueScriptSK+, pCambia 1305.1, pCR8 (linear), pEarleyGate vector series, pPMI-GFP, pPZP101, pTJK136, pUNI51-MBF1c. Among these vectors IV 1.1, pBlueScriptSK+, pCambia1305.1, pPZP101 are commercially available vector backbones available among the lab sources. The permission letter and graphical map for use of pTJK136 is given in appendix b and c respectively. The pPMI-GFP vector was kindly provided by Prof. Dr. Ekrem Gürel. The pEarleyGate vector series (100-104) and pUNI51-MBF1c vectors were purchased from Arabidopsis Tair site and retrieved from the Arabidopsis Biological Research Cencter (ABRC) with the enclosed letters of limited use label license (appendix c).

Primers designs were made with Primer3 (Rozen and Skaletsky, 2000) software. Primers were purchased from either IDT or Alpha DNA. See appendix b for a full complement of primer sets.

2.1.1.2 Molecular Cloning Kits and Restriction Endonucleases

For elution of agarose gel electrophoresed DNA fragments and for cleaning of either PCR amplified DNA fragments or restriction endonuclease digested DNA molecules GeneMark gel elution kit was used.

Plasmid preparations were made with Fermentas plasmid miniprep kits.

Restriction endonucleases were purchased mainly from Fermentas, but some restriction enzymes were also purchased from NEB.

Three brand T4-DNA Ligase kits were used throughout the study. These were Fermentas, Roche and NEB T4-DNA ligase kits.

For DNA blunting Fermentas T4-DNA Polymerase and NEB DNA Blunting Kit were used.

For routine PCR amplifications other than cloning Fermentas Taq-DNA polymerase was used. For cloning practices Agilent brand Herculase II Fusion DNA Polymerase was used.

2.1.1.3 Tools, *E. coli* Strains and Services Used in Molecular Works

BioRad brand mini and midi sub gel electrophoresis and midi electrophoresis power supply instruments were used for DNA purity analysis and fragment separation.

Escherichia coli DH5a and TOP10 competent strains were used for transformation of plasmids and recombinant molecules and -80^{o}C stock preparations of plasmid and recombinant DNA molecules.

Agarose gel imaging and analyses were made with UV-P gel imaging and documentation system.

Two sequencing services were employed for analysis of the clones generated. First one is sequencing service of Iontek which is a local company and the second one is McLab is an U.S company.

2.1.2 Bacterial Media Used for *E. coli*

For *E. coli* either liquid LB or solidified (with 1.5% agar) LB supplied with appropriate antibiotics was used as culture media and -80°C stock preparations. For cloning practices, transformed competent *E. coli* cells were recovered in SOC medium supplied with final 20 mM Glucose.

2.1.3 Agrobacterium Strains and Plant Transformation Vectors

pPZP101-MBF1c/ManA and pPZP101-MBF1c/GUSint/ManA are the two plant transformation vectors that are generated in this study. Full sequence information and annotations of the two vectors and plasmids and sub-clones generated are given in appendix b.

The two *Agrobacterium tumefaciens* strains used in this study; KYRT1 (with pTJK136 binary plant transformation vector) and C58C1 were retrieved from the lab stocks.

2.1.4 Bacterial Media Used for *A. tumefaciens*

For conventional culturing, growth and for -80°C stock preparations of *A. tumefaciens* strains, YEB media supplemented with appropriate antibiotics was used. For recovery of electroporated *Agrobacterium* cells either liquid LB media or SOC media supplemented with 20mM glucose were used.

During plant transformation experiments culturing of *Agrobacterium* cells were made initially with YEB+MES media but later MG/L media was used instead of YEB+MES media.

During re-suspension of the *Agrobacterium* cells cultured for plant transformation a newly formulated inoculation media, which is also used as co-cultivation media after solidification was used.

2.1.5 Plant Materials, Plant Tissue Culture and Transformation Media

Lens culinaris subsp. *orientalis* cv.Sultan-1 was used as plant material throughout the thesis study. Seeds were provided by Eskişehir Anadolu Agricultural Research Institute.

The main germination media for the seeds were water-agar (0.6%) plates, but occasionally full strength MS (Murashige and Skoog 1962) macro and micro nutrients supplemented with MS vitamins, 3% sucrose and 0.6% agar were used.

Agrobacterium co-cultivation media for the transformed explants is solidified inoculation media which is reformulated according to the recent advances in *Agrobacterium* mediated transformation to plants.

Following co-cultivation transformed explants are monthly sub cultured on selective media formulated as MS macro and micronutrients with MS vitamins, 3% mannose in place of sucrose, and 0.6% agar, 2.9 gr/l MES 250 mg/l Augmentin or Timentin and 1 mg/l benzylaminopurine (BAP). Whenever agrobacterium contamination is seen in sub-cultures sub-culture media is supplemented with 0.05% plant preservation media (PPM).

Regenerated shoots harvested from each sub-culture of in selection media are grafted in 3-5 days old lentil seedling. These grafts are then maintained either with half strength Hoaglands` E medium or half strength MS macro and micro nutrients with MS vitamins in perlite within a closed chamber till recovery.

2.2 Methods

2.2.1 Methods for Molecular Cloning

Recombination and handling the intended DNA molecule in intended orientation in a plasmid DNA is not an easy task if conventional cloning practices are considered. The recently developed recombinant DNA technologies-GateWay cloning system is rather simple and do not require extensive planning and confirmation tests as

compared to conventional cloning. But as compared to GateWay system conventional cloning provides huge flexibility during practice and also adaptation of any vector to GateWay system requires conventional cloning.

The events that are followed for conventional cloning steps can be listed as follows. First amplified DNA molecules are digested at compatible specific restriction endonuclease sites. If there are not adequate compatible restriction sites then restriction endonuclease sites are either introduced through PCR or restriction sites of cloning vectors are utilized else digested DNA fragment are blunt prepared for ligation. Then prepared DNA fragments are ligated and transformed into competent *E. coli* cells. Single colonies produced from these transformed *E. coli* cells are analyzed to detect the intended clones.

Methods used for molecular cloning from plasmid preparation to ligation are given in logical order in the sequence of cloning format in the sequential sections below.

2.2.1.1 Plasmid Preparations

Initial start point of cloning is obviously the plasmid DNA preparation step. Care is given to this step owing to the limitations of commercial plasmid mini-prep kits. Bacterial cultures older then overnight growths are not used for plasmid prep. Plasmids of *E. coli* DH5a and TOP10 cultures are isolated with Fermentas plasmid mini-prep kit according to the manual. Plasmids from *Agrobacterium* cultures were also prepared but half the recommended culture volume (1 ml) was used in this case.

2.2.1.2 Restriction Endonuclease Digest

The second point of cloning practices is preparation of DNA fragments. And the initial step is generally restriction digest. The restriction digest of plasmid DNA were done with the recommended restrictions digest buffer.

Although restriction digestion is a straight forward process, it requires special attention and planning as the digestion reaction gets complex if sequential or

double restriction digests are required. Also if further processes required for recovery or modification of the intended DNA fragments, restriction digestion that might be coupled directly to the downstream processes should also be planned carefully.

Pin points of restriction digestion are summarized as follows. The initial and most critical step of restriction digest is purity of the plasmid prep. For this purpose commercial plasmid min-prep kits were used routinely. The volume of the DNA molecule to be restricted may not exceed the 3X volume of the total restriction digest reaction volume. Total volume of the restriction enzyme(s) stock (in 50% glycerol) also may not exceed 10X volume of the total restriction digest reaction mix volume. Digestion period should be carefully planned in order to maintain complete digestion of the DNA with the restriction enzyme amount used. During preparation of restriction enzyme mix contamination with nonspecific exonucleases must be avoided. Restriction digest is carried out at its specified temperature overnight.

The starting measures for planning the digest is the amount of DNA to be digested and activity of the restriction enzyme in prepared buffer. Minimum restriction digest reaction volume is chosen as 20X of the restriction enzyme volume. And for extra µl of enzyme added (restriction enzymes or other enzymes within 50% glycerol stock) to the reaction mix total volume is increased by 10 µl. Otherwise glycerol amount exceeding 10% in the restriction digest mixture will result in nonspecific enzyme activities. As the total volume of the reaction mixture is concluded, first DNA is added to the reaction tube, and then restriction digest buffer is added. Finally the enzyme(s) is (are) added to the mixture and mixture volume is brought to total volume with sdH_2O. The last step is mixing the contents through pippeting up and down half the volume of the reaction mix and overnight incubation of the contents at optimum temperature for the restriction enzyme used (usually 37^{o}C). Formulation of the restriction digest reaction is tabulated below (table 2.1)

Table 2.1: Restriction digest reaction mixture calculation table

Restriction Digest Mixture Concentration Considerations	
Components	**Volumes in µl**
DNA	V
Buffer (10X)	1+(V1+2+4)
RE enzyme 1	V1
RE enzyme 2	V2
BSA (10X)	V3
Alkaline Phospahtase	V4
H_2O	TV-(1+V+V3+2X(V1+V2+V4))
Total Volume (TV)	(1+(V1+2+4))X10
note that BSA is only added if NEB enzymes are inclued into the estriction digest	

2.2.1.3 Blunting Reactions

For blunting reaction the NEB blunting kit or Fermentas T4-DNA polymerase enzyme were used. Blunting reactions were carried out within the restriction digest mix after completion of digest. The same volumetric concerns for restriction enzyme digest preparation are valid for blunting reaction also. Both of the brands provide flexible buffer range for T4-DNA polymerase activity including the restriction enzyme buffers. The critical point for both of the brands is the minimal dNTP concentration (100µM). First dNTP mix (final 100µM) is added to the restriction digest mix then 1µl of blunting enzyme mix (NEB) or T4 DNA Polymerase along with 10µl of sdH_2O (10µl for each µl of enzyme added) is added to the mix. Blunting reactions are carried out in thermo-cycler for 20 minutes at 42°C. Blunted DNA fragments are then separated through gel electroporation. Fragments are excised from the gel under UV illuminator with 365nm UV-C range lamps and then purified with Gel-purification kit.

2.2.1.4 Conventional PCR and TD-PCR

If restriction digest and blunting reactions do not let proper handling the fragments then specific restriction recognition sites are integrated through PCR. The two methods used for this purpose are conventional PCR and touch down (TD) PCR

methods. While conventional PCR provides single or gradient Tm points for primer annealing, TD-PCR maintains a range of Tm values that maintains stringency in Tm and amplification of the initial templates. We have made better use of TD-PCR during amplification of fragments for cloning.

2.2.1.5 DNA Fragment Elution and PCR Cleaning

The very critical part of cloning is perhaps the final preparation step of DNA fragments that are recovered either from PCR amplification or restriction digestion and agarose gel electroporation step. As long as not required, gel separation of DNA fragments and further recovery of them within the gel under UV illumination is not performed. If required DNA fragments are excised from the gel under UV-C spectrum with 365 nm UV lamps.

Both gel elution and PCR cleaning were done with GeneMark gel elution kit. Also restriction digest mixes are cleaned with the same kit. During practice gel elution and PCR cleaning were done according to the recommendations of the manufacturer.

2.2.1.6 Ligation Reaction

Fermentas, NEB and Roche T4 DNA ligase kits were used throughout the study. *Vir*tually all of the brands recommends calculation of free DNA ends and restricts the total DNA amount to 100 ng for a successful ligation reaction. Other than blunt end ligation reactions which were found to be non-specific and inefficient, in all of the sticky end ligation reactions no free DNA end calculations were made and 100 ng maximum DNA amount rule is not strictly obeyed. Instead full attention was given to proper preparation of DNA fragments and planning of the ligation reaction.

If the ligation is made with T4-DNA ligase kit the ligation reaction is carried out overnight at 16^{o}C in thermo-cycler. If on the other hand fast ligation kit is used ligation is carried out at ambient room temperature for five.

Generally total ligation volume is prepared as 10µl. But if ligation of multiple fragments is considered total ligation volume can be calculated as 15-20 µl incase amount of DNA fragmnets might be scarce.

2.2.1.7 Preparation of Competent *E. coli* and Transformation

Initial works of cloning and vector construction were preparation of *E. coli* -80°C stocks of the DNA materials used as source. Either electroporation or chemical transformation was utilized for introducing plasmid material into competent *E. coli* cells (DH5a or TOP-10 strains). The same methods were also used for transforming the ligation products into *E. coli* cells. Mainly Ru-Cl method with TOP-10 cells was preferred.

The rubidium chloride method described here is adopted from "Standard Molecular Plant Biology Protocols" at Arabidopsis Tair web site. The main steps and media compositions are not changed, but minor modifications were made on handling the materials.

2.2.1.7.1 Competent *E. coli* Preparation

A single bacterial colony recovered from a fresh spread or streak plate is the start point. Generally streaking from the older competent or -80° C stock suffice for handling single colony in an overnight duration

Single colony taken from the plate is inoculated either directly to batch culture (100 ml medium in minimum 250 ml flask) or to a starter culture (5 ml medium in 50 ml falcon) at 37°C and 180 rpm. Original protocol describes SOB as the culture medium, but LB also works fine. If starter culture is omitted, growth of batch culture is maintained till OD_{600}=0.2 or 0.4-0.6 as long as SOB medium or LB is used respectively. If starter culture is prepared, following overnight growth full content is transferred into batch culture with the conditions just described.

Then the batch culture yield is harvested with centrifugation at 4500 rpm for 15 min in a pre-chilled rotor at 4°C. Sterile 50 ml falcon tubes are used generally for spinning down the bacteria at 4500 rpm.

The supernatant is discarded and the bacterial pellet is re-suspended in 25 ml pre-chilled RF1 buffer (1/4 of the batch culture volume). Re-suspended bacteria is left in ice for 15 minutes and then centrifuged at 4500 rpm in Sigma 3K30 centrifuge at 4°C for 15 minutes. Supernatant is discarded and bacterial pellet is re-suspended in pre-chilled 4 ml RF2 buffer (1/25 of the batch culture volume). Buffer compositions are given in table 2.2 below.

The re-suspended bacteria in RF2 buffer is immediately separated to 100 or 50 µl aliquots into sterile liquid nitrogen (-80°C) frozen eppendorf tubes in racks and immediately sorted into -80°C. For testing the competence of the bacteria prepared, 10 ng of plasmid prep is transformed.

Table 2.2: Rubidium chloride buffers compositions; RF1 and RF2

RF1 buffer	100 ml	RF2 Buffer	50 ml
RbCl	1.2 g	RbCl	60 mg
$MnCl_4H_2O$	0.99 g	MOPS*2	1 ml
KOAc*1	3 ml	$CaCl_2\ 2H_2O$	0.55 g
$CaCl_2\ 2H_2O$	15 g	Glycerol	7.5 g
(*1) 1M pH7.5 (adjust w/ KOH).			
(*2) 0.5M pH6.8 (adjust w/ NaOH).			
Adjust the buffer pH values to 6.8, filter sterilize and store at room temperature.			

2.2.1.7.2 Transformation of Competent *E. coli* Cells

Recover 100 µl of competent cells from -80°C and thaw on ice and mix with inversions prior to use. If ligation product is to be transformed whole ligation mixture (maximum 20 µl) is added to 100 µl competent cells and mixed thoroughly with the aid of pipette tip. If intact plasmid is to be transformed 50 µl of competent cells can be mixed with 3-5 µl of plasmid prep. Then competent cell and DNA mix

are left on ice for 30 minutes. Following ice incubation transformation mix is heat shocked for 15 seconds at 42°C, returned to ice and incubated for one minute. Finally transformation mix is transferred to 500 µl SOC medium and conditioned in orbital shaker at 180 rpm and 37°C for one hour. Then 100 µl of the conditioned transformation mixture is spread on to LB-agar media prepared with appropriate antibiotic(s).

2.2.1.8 Electrotransformation of *E. coli*

Electrotransformation method described here is rather simple and used during initial works for preparation of bacterial stocks with intact plasmids. For this protocol, competent strain spread as single colonies on LB agar, 1mm electroporation cuvettes, electroporator (Biorad Gene Pulser I or II or Biorad MicroPulser Electroporator), sterile distilled water (sdH_2O), Tris-EDTA (10mM) or sdH_2O eluted plasmid DNA, SOC medium, vortex mixer and microfuge are required.

Efficiency of transformation is directly correlated to culture age. One months old plate generally yields the worst results. Single colony of competent *E. coli* strain (TOP10 or DH5A) is recovered from the surface of the agar with the aid of sterile pipette tip and inoculated into the sterile 1.5 ml eppendorf tube. Bacteria are suspended in 400 µl sdH_2O with the aid of vortex mixer and then spun down at 12 krpm in microfuge for 30 seconds. Following precipitation H_2O phase was pipetted out, remaining the bacterial pellet. Second round of re-suspension was made with 200 µl sdH_2O and bacteria are spun down again for 30 seconds at 12 krpm. The H_2O phase is again pipetted out and final re-suspension is made with 100 µl sdH_2O. Then bacterial suspension is sorted in ice for transformation.

100 µl of re-suspended bacteria is loaded into pre-cooled electroporation cuvette in ice. 5-10 µl of plasmid DNA (50 to 100 ng/µl [DNA]) is added into the bacterial suspension and electroporated with 100 ohm 1.8 kV and 25 µF rating if Biorad Gene Pulser I or II is used. If Biorad Micro-Pulser is used electroporation is done with the *E. coli* program loaded.

Immediately after electroporation bacterial suspension is recovered from the cuvette and diluted with 800 µl SOC medium. Then bacterial suspension is left for recovery in rotary shaker incubator at 37°C for one hour. Finally 100 to 150 µl of recovered bacteria is spreaded on agar solidified LB with appropriate antibiotics. With this method a number of positive bacterial clones containing the plasmid can be recovered after overnight incubation at 37°C.

2.2.1.9 Molecular Analysis of Recombinant Clones

Initial analysis of the colonies and detection of the clones was performed either through colony-PCR or through plasmid size detection where appropriate. As long as the candidate clones were detected, mini-preps of the candidate colonies were prepared. Then plasmids were digested with restriction enzymes that were utilized during cloning in order to reveal if there is any unintended fragment inserted through those restriction sites. There may be a need for analysis of directional orientation of the insert in the plasmid in the case of bidirectional cloning where single restriction enzyme site was utilized for cloning. This problem is resolved in two ways. First way is colony-PCR where primer set spans a short sequence covering both vector and the insert. Second way is generally proceed when there is no sequence information but a simple restriction map is available. In this condition a crucial restriction enzyme site is preferred that digests the vector and the insert at an asymmetric position. The resulting fragments are then separated with simple gel electrophoresis to detect the direction of the insert in that clone.

2.2.1.9.1 Rapid Size Check of Recombinant Plasmids

Generally the initial method for diagnosis of the recombinant clones is fast clone detection based on screening of plasmids on size basis to differentiating the plasmids with inserts (Crickmore, N. 1997).

In this method bacteria are lysed and total lysis mixture is agarose gel electrophoresed with respect to intact plasmid (without insert). The clones with insert are detected in the gel with respect to the intact plasmid on size basis.

2X lysis buffer is prepared as mixture of 20% Sucrose w/v, 200 mM NaOH, 120 mM KCl, 10 mM EDTA, 0.5% SDS and 0.1% Bromophenol Blue. The buffer is stored at -20°C till used. The dye usually fades with subsequent freeze thaw cycles then small amounts of dye is added in to the buffer

Lysis buffer is heated on hot block to 45°C. Transformants colonies are recovered from the agar surface and inoculated into 20µl eppendorf tubes for re-suspension in 20 µl sdH_2O. Fifteen µl of re-suspended colony is mixed with 15 µl of 2X lysis buffer and incubated in hot block at 45°C for five minutes. The rest of the bacterial suspension will be used as reference for bacterial growth upon detection of the putative clones. Five minutes of heat lyssis is followed by for 5 minutes o five incubation. Then lysis mixture is spinned down 10 minutes at max speed. Finally 15 µl of the lysate upper phase is loaded to 0.8% agarose gel. As a reference 2-3 µl of intact plasmid DNA with 1X buffer is also loaded and contents are electrophoresed. The higher molecular weight bands with respect the reference (intact plasmid) are cultured for further analysis.

This method is easy to apply, convenient and cost little as compared to colony PCR. But for total sizes of 5000 bps and higher, stringency of differentiating control and plasmids with insert is lost significantly for insert/ plasmid ratios of 1/10 and lower.

2.2.1.9.2 Colony PCR

Routine PCR analysis adapted to colony screening is the basis of colony PCR methods applied in this thesis. For the recombinant DNA molecules that cannot be detected through rapid size checking or for the insert containing plasmids that have complement of primers spanning the insert and plasmid

The critical part of the PCR mixture preparation for colony PCR is the template since intact bacterial cells are inoculated in to the PCR mixture as template source. Colonies recovered from the agar surface are inoculated into 200µl eppendorf tubes and re-suspended with 50µl sterile distilled water. One to three µl of the bacterial

suspension is used as template source. Master mix for PCR is mixed with template just before the start of PCR. The critical point about the PCR is cycle number to be set to 35-40 cycles.

At the end of PCR whole PCR yield is loaded to agarose gel and electrophoresed for analysis of the recombinant clones. Bacterial suspensions are referred for colonies containing the recombinant clones and cultured in LB with appropriate antibiotics for plasmid prep and -80°C stock preparation.

2.2.1.9.3 Restriction Digest Analysis

The final analysis of the recombinant plasmids is restriction digestion. Restriction digestion is performed in order to check the integrity of the restriction sites through which the insert is combined with the plasmid DNA, and to check the orientation of the insert in plasmid if ligation is made through a single restriction site. If colony PCR is an available tool for insert analysis then only restriction site integrity can be performed to validate that insertion is clean and devoid of any unintended fragment. The same concerns of restriction digest preparations (preceding section 2.2.1.2) also holds for restriction digest analysis of the plasmids with insert.

2.2.1.10 *E. coli* -80°C Stocks for Plasmids and Recombinant Molecules

For introducing recombinant DNA molecules and plasmids into competent *E. coli* cells electrotransformation and chemical transformation methods were used. After analysis and characterization of recombinant molecules and plasmid DNA -80°C glycerol stocks were prepared for long term storage. Overnight cultured bacterial clones are mixed with equal volumes of sterile 50% glycerol and directly transferred to -80°C as glycerol stocks.

2.2.1.11 TOPO® Cloning

Topo® cloning was made for insertion of MBF1c into the entry vector pCR8. No special modifications were made during practice. Cloning was done according to the recommendations of producer.

2.2.2 Methods for *Agrobacterium* Culture and Maintenance

2.2.2.1 *Agrobacterium* Electrotransformation

Electroporation of *Agrobacterium* with the prepared transformation vector is based on Cold Spring Harbor protocol for *Agrobacterium* electroporation (Cold Spring Harb. Protoc.; 2006 pdb.prot4665).

Agrobacterium stock cells are cultured overnight in 5 ml LB with appropriate antibiotics at 28°C and 180 rpm as starter culture. Then 500 ml of LB in 1 lt flask is inoculated with this starter culture. When the cells have reached log phase (OD_{600}=0.5-0.7), the whole batch culture is chilled in ice. The entire steps following are carried out at 4ºC. Bacteria are pelleted in centrifuge (Sigma 3K30) at 4000g for 10 minutes at 4ºC in a pre-chilled rotor. Then supernatant is discarded and bacterial pellet is suspended with 5-10 ml of ice-cold H_2O with a wide-bore pipette until no clumps remain. The final volume of bacterial suspension is brought to 500 ml with ice-cold H_2O. and re-pelleted at 4000g for 10 minutes at 4ºC. The re-suspension and centrifugation steps are carried twice with re-suspension volumes of 250 and finally 50 ml. Another round of centrifugation and re-suspension is made for collecting the total bacterial yield in 5ml 10% (v/v) ice-cold, sterile glycerol. Finally *Agrobacterium* cells are separated into sterile eppendorf tubes as 50 µl aliquots and snap frozen in eppendorf racks immersed in liquid nitrogen and stored at -80°C

Electro-competent *Agrobacterium* cells recovered from -80°C are thawed in ice. Standard mini-prep eluted (10mM Tris-EDTA) or sdH_2O eluted plasmid DNA can be used for electroporation. The thawed competent cells and 1-2 µl of plasmid DNA are gently mixed with the pippet tip and left in ice for five minutes. Then the mix is loaded into pre-chilled 1 mm electroporation cuvette. If the electroporator automated (Bio-Rad MicroPulser) electroporation is made with the preset program build-in otherwise electroporator (Bio-Rad Gene Pulser II) is manually calibrated to 25 µF, 2.4 kV and 200 ohm and electroporation is made in 5 msec pulse length. Immediately electroporated bacteria are recovered from the cuvette and suspended

in 800 µl SOC medium for recovery for four hour at 28°C and 180 rpm. Finally 100 µl of transformed bacteria are spread on LB-agar plates with appropriate antibiotics. It is better to spread plate the transformed *Agrobacterium* with serial dilutions. Spreaded plates are then left for growth at 28°C for two days. The emerging colonies are analyzed for the transformed plasmid.

2.2.2.2 -80ºC *Agrobacterium* Stock Preparations

The same method of -80ºC stock preparation for *E. coli* is also employed for *Agrobacterium* long term storage stock preparation. Completely the same procedure is applied for *Agrobacterium* cells that are stated in section 2.2.1.10.

2.2.2.3 Colony PCR for *Agrobacteria*

Colony PCR analysis of Agrobacteria are the same with that of colony PCR for *E. coli* refer to the section 2.2.1.9.2 for details.

2.2.3 Methods Used for Plant Tissue Culture and Transformation

2.2.3.1.1 Lentil Seed Surface Sterilization

Lentil seed surface sterilization was standardized in master thesis study on Sultan-1 cv., (Kamçı 2004). Starting from the bleaching step surface sterilization was carried out in laminar flow cabinet under aseptic conditions. During surface sterilization first seeds are rinsed with liquid hand soap under running tap water. Then seeds are bleached approximately 10 minutes with 5% NaOCl solution. The solution is decanted and bleached seeds are immediately and momentarily dehydrated with 70% ethanol. Following ethanol is decanted and healthy, intact seeds are separated on a sterile filter paper rinsed with sterile distilled water at least two times and directly sown*in vitro* for germination.

2.2.3.1.2 Lentil Explant Preparation

Three days old lentil seedling germinated on water agar plates were used as explant source. Seedlings are handled aseptically during explant preparation steps. Target tissue is cotyledonary nodal segment. *De novo* explant isolation methods were devised in order to increase the accessibility to the meristematic zones that are engraved within the cotyledonary petioles and to simplify explant preparation steps.

Tree different variations of cotyledonary node isolation are devised. These are schematized in figure 2.1 and named as conventional isolation (1) epicotyl excised (2) and cotyledons stripped (3). The first isolation method is the standard cotyledonary node isolation in which the cotyledons and embryo axis are separated through an excision at the cotyledonary petioles. At the end small portion of cotyledons linked to cotyledonary petioles are left on the embryo axis and cotyledonary meristems are engraved at the junction point. The second isolation method is devised in order to reveal the cotyledonary meristems during explant isolation and even introduce injury during the process. So as to maintain the injury the final incision (figure 2.1/ 2b) is targeted directly in to the meristems. In the third explant isolation method cotyledons are stripped down through the hypocotyl along the embryo axis. Injury is expected as the tissue is torn down at the cotyledonary petiole-embryo axis junction.

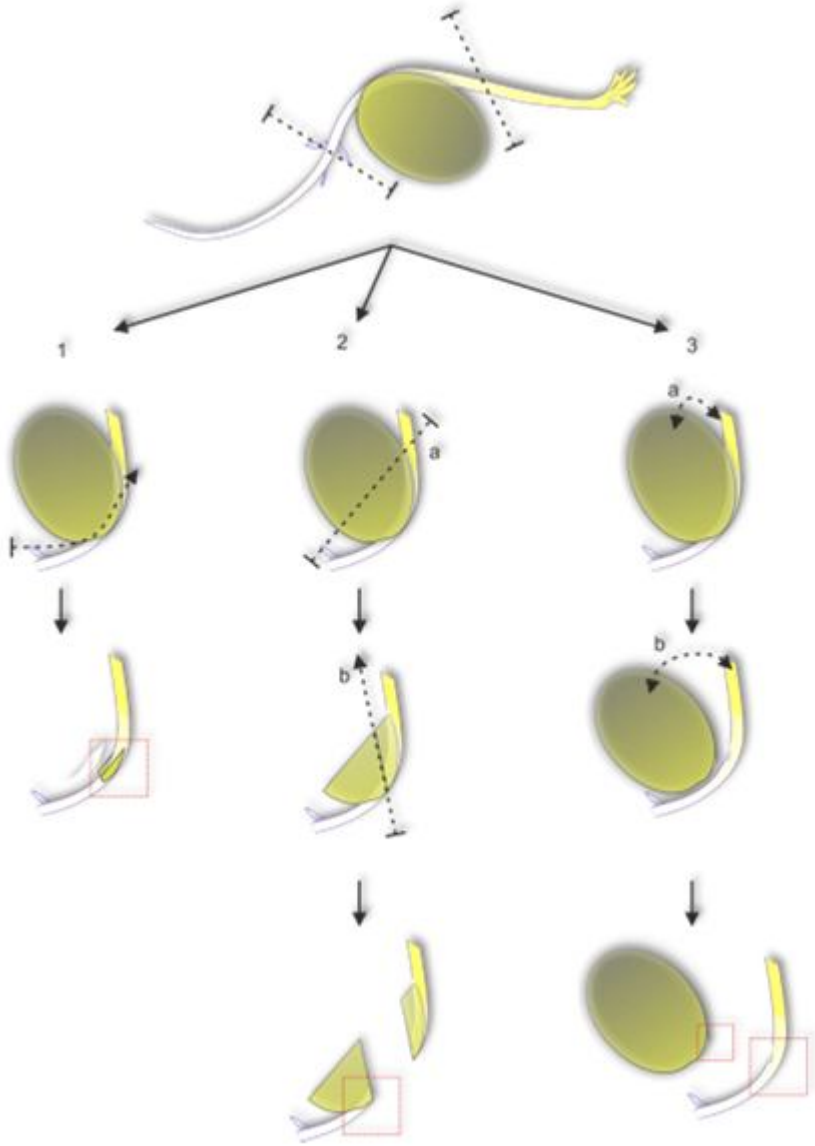

Figure 2.1: Modified cotyledonary node isolation methods
The three different explant isolation methods used during the study are shown. Both epicotyl and hypocotyl segments of the embryo axis are trimmed initially during conventional cotyledonary node isolation (1). Then cotyledons and embryo axis are separated through an incision into the cotyledonary petioles in hypocotyl to epicotyl direction. In the second method (2) first both of the cotyledons are trimmed along the embryo axis (a). Then a second incision is made through the epicotyl-hypocotyl juncture directly into the cotyledonary nodal meristems such that epicotyl is excised while meristems are injured. In the cotyledons stripped method (3) cotyledons and embryo axis pulled apart for separation while the tissues are injured. Red boxes are showing where the meristems tissues reside.

2.2.3.2 Pre-culture of Explants Prior to Transformation

Pre-culturing experiments are considered in results and discussion section. The co-cultivation media prepared either with TDZ or BA is used as pre-culturing media.

2.2.3.3 Agrobcaterium Preparations

2.2.3.3.1 *Agrobacterium* Inoculation and Co-cultivation Media Formulation

Agrobacterium inoculation media reformulation is tabulated below (table 2.3). Macro and micronutrients with MES and sucrose is considered as base media. pH of the base media is titrated to 5.6, autoclaved and stored at 4ºC till use. The rest of the components are added just prior to use. The same formulation without sucrose and with agar (0.6%) is called as co-cultivation media.

Table 2.3: Inoculation media formulation

Inoculation Media		
	Per Liter	**Preperation Precautions**
Macro Nutrients		
$MgSO_4.7H_2O$	0.5 ml	Autoclave
$FeCl_3.6H_2O$ & EDTA	5 ml	Filter Sterilise
Micro Nutrients		
H_3BO_3	0.25 ml	
$MnCl_2.4H_2O$	0.25 ml	
$ZnSO_4.7H_2O$	0.25 ml	Autoclave
$Na_2MoO_4.2H_2O$	0.25 ml	
$CuSO_4.5H_2O$	0.25 ml	
Additives		
MES	3.9 gr	Autoclave
Sucrose	3%	
Thiamin	10 mg	Filter Sterilise
Myo-Inositol	100 mg	
Tryptophan	10 mg	
Ascorbic Acid	100 mg (200 uM)	Filter Sterilise
Silver Nitrate	2 mg (0.01 uM)	
Acetosyringone	200 uM	Prepare Fresh
L-cystein	40 mg (0.33 uM)	
Macronutrients and Micronutrients are from Hoaglands' E Medium		

2.2.3.4 Transformation of explants

Transformation of explants prepared was done through inoculation of re-suspended and acetosyringone activated (1h) agrobacteria cells to injured and pre-cultured explants for 90 minutes. For this purpose overnight grown *Agrobacterium* (*A. tumefaciens* C58C1 or KYRT1 strains) batch cultures in MG/L media supplemented with appropriate antibiotics and 20µM acetosyringone are harvested at OD_{600}=0.8 and spun down in Sigma 3K30 centrifuge at 4500 rpm for 15 minutes. For re-suspending each 100ml of bacterial pellet precipitated through centrifuging, 5 ml of inoculation media is used. These re-suspended bacterial cells are induced with 200µM acetosyringone for one hour and immediately used for explant inoculation. Following inoculation, excess agrobacterial suspension is washed thoroughly with sterile distilled water and dry blotted onto sterile filter paper prior to transfer into co-cultivation media.

2.2.3.5 Co-culture Selection and Sub-culture

Co-cultivation of transformed explants was made on 0.6% agar solidified inoculation media which is named as co-cultivation media. Following co-cultivation, explants are sub-cultured in selective media (section 2.2.3.3.1) under fluorescent bulbs with 18h light and 6 hours dark photoperiod at 25ºC temperature in tissue culture room. Sub-culturing is made on monthly basis and at the start of each sub-culture necrotic sections of explants are removed and regenerating shoots are excised from their base tissue and separately inserted into the selective media.

2.2.3.6 Micro-Grafting

Healthy shoots recovered from the selective media at the end of each fourth and fifth sub-culture were grafted on three to five days old lentil seedlings. The basis of grafting roots back to the micro-grafting based regeneration optimization studies by Kamçı (2004). Important advancements were integrated to this basis resulted in more efficient and less labor intensive method of micro-grafting in lentil that can be applied to other annual species with slender stem segments.

A linear segment close to the cotyledons on the root stock is selected as the graft site. An angular slash that transverse the stem girth is introduced into the stem axis. Then to the cotyledonary distal site of this slash an incision (that does not exceed the boundaries of the stem axis) is introduced along the way of stem axis. The slash and incision introduced sequentially in to the stem axis seems like stretched uppercase letter "L". A scion that is prepared with an angular excision into its stem axis. This prepared scion is then inserted in to the root stocks` stem while its cut site is facing down the cotyledons such that root stock stem vascular structure is facing the vascular structure of the scion stem. Details of the progress are given in section 3.4.8. Whole grafting event is practiced under dissecting microscope in sterile distilled water aseptically. Then grafts are planted into perlite into humidity chamber and watered with either half strength Hoagland's` solution or half strength liquid MS media. Three grafts planted in each humidity chamber that is composed of two cups closed on top of each other and the upper one is transparent for maintaining illumination. During recovery of the grafts secondary shoot growth emerging from the axillary meristems of the root stock is routinely examined and excised. Recovery of the grafts ranges between 7-14 days. Elongation scions are watched as they approach to the rim of the upper transparent cover. Upon their touch to the cover a hole is made at the rim in order to acclimatize the scion as it emerges out of the cover.

Approximately at the end of the second week recovered and acclimatized grafts are potted in left in greenhouse for growth.

2.2.4 Molecular Analysis of Putative Transgenics

Molecular analysis of the putative transgenics was only performed through leaf PCR. For this purpose an array of plant genomic DNA isolation methods were analyzed, but none of them were considered to be fast and efficient in practice. For this reason a more fast plant genomic DNA isolation method is devised that is yet to be diagnosed further.

2.2.4.1 Plant Genomic DNA Isolation

Wang et al., (1993) describe a NaOH based template preparation from leaf, callus or cotyledon explants through grounding with 0.5N NaOH (10 µl for every mg of tissue). Following grounding, suspension is immediately diluted (1/100) into 100mM Tris buffer (pH 8.0). Then 1µl of the final sample is used as PCR template at least in 30-40µl total PCR volume. Dilution of template in final PCR volume approaches to 3/10000 with this method.

In a more optimized template preparation procedure by Hosaka (2004) cross contamination and grounding takes more attention. Besides an elution buffer formulation (100mM Tris-HCl pH 8.0; 50 mM EDTA pH 8.0; 500mM NaCl; 1.25% SDS; 0.2% 2-mercaptoethanol) used during grounding is considered. Grounding of leaf material is done in plastic zipper bag with 500µl elution buffer and 100µl of the final suspension is neutralized with 32µl of (5M)potassium acetate. Then a 10 second maximum speed centrifugation step is followed by dilution of 10 µl of supernatant in 490µl sterile distilled water. From this final mixture 2µl is used as template for PCR amplification. Final dilution of template in total 10µl of PCR volume becomes 3/10000 with this procedure.

A relatively simple template preparation method is studied by Berendzen et al., (2005). Researchers describe their method as sucrose prep due to high sucrose content of the grounding buffer (50mM Tris-HCl pH7.5; 300mM NaCl; 300mM Sucrose). In this method 10mg of leaf tissue is ground in 100µl grinding buffer and then another 100µl buffer is added prior to boiling. Then a final step of centrifugation (at maximum speed) is performed prior to PCR reaction and 50-100µl of supernatant is spared as PCR template. 1-2 µl of template is used in a 50µl PCR reaction. Dilution of template in final PCR volume is 1/50 at most.

The final protocol of template preparation can be termed as boiling prep that also concentrates on grinding of the explants (Wang et al., 2009). For this purpose

researchers devised PCR tube attached to a 1 ml pipette tip as pestle. Grinding is done in 1.5 ml eppendorf tube with 60µl, 0.25M NaOH. Then ground explants are boiled for 30 seconds. Afterwards 240 µl of boiled buffer (composed of 100mM Tris-HCl pH7.6 and 5 mg/ml polyvinylpayrollidone (PVP)) is added to the ground mixture, boiled again for 2 minutes and centrifuged for 5 minutes at 10 krpm. Supernatant collected as template source is stored either at 4ºC or -20ºC. 2µlof supernatant is used for a PCR amplification volume of 25 µl. Final dilution of template in 25µl of PCR volume is approximately 1/10 for this protocol.

Two important points that holds for the protocols reviewed above are grounding step and template dilution in total PCR volume. Other than these two points volumetric considerations and buffer compositions might be regarded important.

As applied in our laboratory none of the grinding methods of the protocols listed above was regarded as efficient. For grinding all of the protocols use various aqueous solutions that are buffered or not. In our protocol grinding is made with ordinary 1 ml pipette tip inside 1.5 ml eppendorf tubes with the aid of sucrose crystals that immediately absorbs whole aqueous content of the explant. And template dilution is omitted with final ethanol precipitation if preferred. Our newly devised protocol is as follows.

One to two leaf pieces of fresh leaves, approximately $1cm^2$ are ground in 100mg sucrose. Then 50 µl of 0.5M NaOH is added to the ground explants and boiled for 1 minute on hot block. Then 200µl of elution buffer (100mM Tris-HCl pH 7.5; 300mM NaCl; 300mM Sucrose; 1.25% SDS; 0.2% 2-mercoptoethanol) is added to the bleaching solution and left to boiling 2 minutes more. Whole suspension is then left in ice for cooling down. Then freshly prepared 150 µl 5M ammonium acetate is added to the suspension for neutralization and mixed well through inversions. Final step of template isolation is centrifugation for 5 minutes at maximum speed.

Supernatant covered with a waxy film at the top is punctured with a pipette tip through bubbling into the solution and 250 to 300µl volume is taken carefully without disturbing the pellet and pipetting waxy cover.

Supernatant is precipitated with 2V 99% ethanol through centrifugation at maximum speed for 10 minutes. Supernatant is decanted carefully without disturbing the pellet and the pellet is washed once with 70% ethanol. Finally DNA pellet is dried and suspended in 50µl 10mM Tris-HCl (pH7.4) or elution buffers of plasmid mini-prep kit.

Although clear bands of genomic DNA is observed during agarose gel electrophoresis of the preparation made with our protocol, it should further be tested in order to reach to a clear conclusion.

2.2.4.2 Leaf Genomic DNA PCR Analysis

PCR from the prepared leaf genomic DNA 3 µl was used as template for amplification of fragments from the delivered T-DNA. No special precautions were considered during design of PCR reaction.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Plant Transformation Vector Construction

The heaviest effort of this thesis work was undertaken for cloning and vector construction part. The headings listed below as a picture of this effort and it is also a vision of the self-progress from zero to transformation vector construction. For a brief view of what was done, a scheme is given below in figure 3.1 demonstrating the progress of a plan till the target (transformation vector) is reached. The scheme given is in logical order rather than chronological order since nearly all of the work was carried out with alternative plans where it ended up with the most plausible product. According to the logical order cloning work was divided into three main parts; first part dealing with selection and marker genes, second part MBF1c cloning and third part transformation vector selection and construction.

First part named "Handling Visual Markers and Selection Markers" deals with the *nptII* and *GUSint* cassettes from pTJK136 transformation vector as the initial selection and visual marker genes respectively. Than another selective marker gene; the phospho-mannose isomerase (PMI) was considered as an alternative to neomycin phospho-transferase II (nptII) gene. This selection gene was recovered from pPMI-GFP binary vector as a cassette with maize ubiquitin 1 promoter and 35S terminator (M.Ubi1-ManA-T35S). This ManA cassette along with the MBF1c cassette generated in IV 1.1 through restriction and ligation processes were sub-cloned into pPZP101 but both due to the maize promoter of ManA cassette and due to the concerns noted in section 3.1.5.3 about MBF1c cassette this constructed vector was omitted from the study. Due to the concerns on maize promoter also, the PMI coding

sequence was later cloned under CaMV35SS promoter (P35S-ManA-TNOS). Along with this P35S-ManA-TNOS cassette and also MBF1c cassette cloned under 35S promoter (P35S-MBF1c-TNOS; section 3.1.5.4) the target transformation vector was constructed in pPZP101 (pPZP101 ManA-MBF1c; section 3.1.3). Final afford undertaken for the first part was regeneration of sequence information of the GUSint cassette (*in-silico*) from patent information and reference materials.

The second part named "MBF1c Cloning and Cassette Construction" deals with the cloning attempts of the functional gene; the multi-protein bridging factor 1c. It is noted as modulator protein that takes effect in stress response-strength and initiation speed of the stress response itself. In the initial works the target vector for cassette construction was impact vector 1.1 harboring the chrysanthemum rubisco subunit 1 promoter. It was thought that the rubisco subunit 1 promoter was perfectly suiting our work since MBF1c expression could be regulated by light under this promoter. The very first efforts undertaken for this purpose were recovery of MBF1c coding sequence from its donor vector that it was sent within (pUNI51-MBF1c) and sub-cloning into the intermediate cloning vector (pBlueScriptSK+) and then sub-cloning into IV 1.1. These works covered the initial unplanned efforts for PRbcS1-MBF1c-TRbcS1 cassette construction in chronological order and the results were rather cumbersome. First problem raised with PRbcS1-MBF1c-TRbcS1 cassette was an in-frame double start codon, one from the vector (not taken into account) and the other from the coding sequence. This was eliminated with emptying the first one. Another problem raised with the cassette was observed at the final step of sub-cloning into transformation vector pPZP101. Scarcity of the compatible restriction sites for sub-cloning MBF1c cassette from IV 1.1 into transformation vector enforced recombination of the pPZP101 and IV 1.1-MBF1c. The IV 1.1 backbone was to be removed from the resulted hybrid vector, leaving the cassette in pPZP101. However an XhoI restriction site at 3' end of the MBF1c cds hindered emptying IV 1.1 backbone. This problem was eliminated at one step back through a second round of emptying work for IV 1.1-MBF1c. At this point *in-silico* analysis of the IV 1.1-MBF1c cassette left a doubt on the removed double start codon. Shortly stated, the

ambiguity was on the first start codon removed that it might be regenerated after blunting step when ligation is done. All of these problems forced the way of PCR

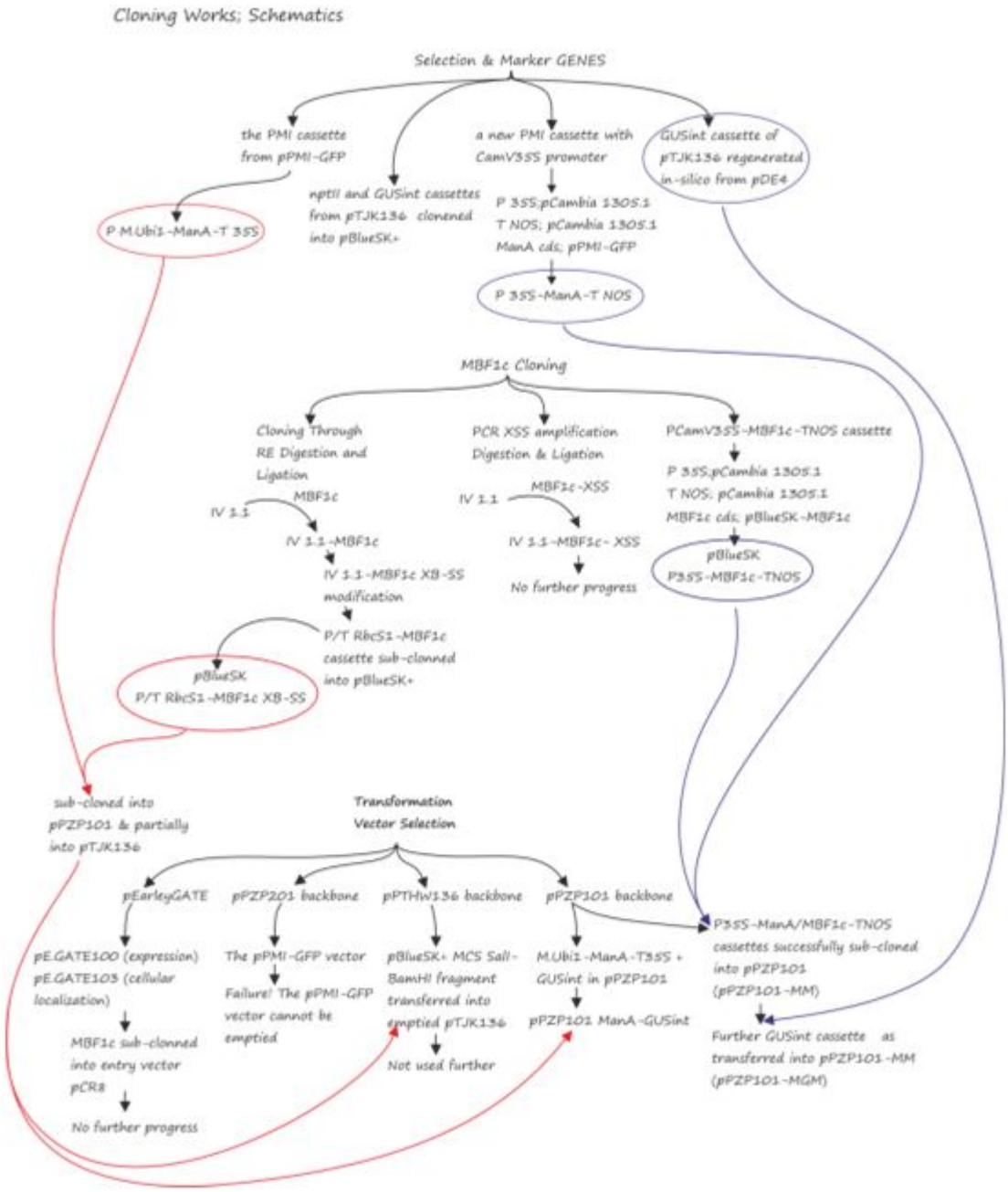

Figure 3.1: Schematic view of cloning works undertaken

based cloning plan that may eliminate these unintended outcomes. Primers designed for amplification were flanking MBF1c with XbaI and SalI-SacI restriction sites. The MBF1c amplicon was to be digested with XbaI and SacI restriction

enzymes and then ligated into IV 1.1. As an alternative plan the same apmlicon was to be digested with XbaI and SalI restriction enzymes for cloning between CaMV35SS promoter and NOS poly-A sequence yielding the P35S-MBF1c-TNOS cassette which was used later in third step for final transformation vector construction; the pPZP101 ManA-MBF1c.

The third part named "Selection and Construction of Transformation Vector" deal with the alternative binary vectors that were considered as the candidate binary transformation vectors. The initial vector taken into account was pTJK136. However as the incomplete restriction map and cassettes recovered from the vector along with the unknown sequence data considered, pTJK136 was omitted after emptying and regenerating multiple cloning site. Along with pTJK136 the pPMI-GFP vector was taken into account since it was constructed on pPZP201 backbone and already had PMI selection cassette (PM.Ubi1-ManA-T35S). This vector was also omitted since it was not possible to empty the GFP cassette. Next an alternative plan with two options was developed. First one was based on pPZP101 as the ultimate transformation vector with conventional cloning practice and the second one was based on pEarleyGate vectors with completely alternative cloning practice; the Gateway Technology. Both of the alternative options were carried out concomitantly.

At the end it was possible to generate a transformation vector based on pPZP101 containing the P35S-ManA-TNOS and P35S-MBF1c-TNOS cassettes (pPZP101 ManA-MBF1c). *Agrobacterium* mediated transformation was started with C58C1 strain loaded with this transformation vector. Initial transformation events resulted in total loss of the explants. Later transformation works also resulted in loss of the explants to a great extent. In order to make diagnosis of what may be cause, the visual marker GUSint (sequence regenerated *in-silico* in the section 3.1.4) was sub-cloned into the pPZP101 ManA-MBF1c construct (resulting vector; pPZP101 ManA-GUSint-MBF1c) and used in later transformation works.

3.1.1 Handling Visual Markers and Selection Markers

3.1.1.1 Sub-Cloning nptII and GUSint Cassettes

Purpose of cloning GUSint and nptII cassettes into pBlueScriptSK+ was to handle both of them in pBlueScriptSK+ as reference clones and also to provide unique restriction sites flanking these cassettes in pBlueScriptSK+ whenever needed for sub-cloning. Both the GUSint and nptII cassettes were recovered from pTJK136 and inserted into pBlueScriptSK+ through SalI and BamHI restriction sites respectively.

3.1.1.1.1 Generating *E. coli* DH5a Clones of pTJK136 and pBlueScriptSK+

In order to handle pTJK136 and pBlueScriptSK+ in adequate quality and quantity *E. coli* DH5a clones of the vectors were prepared through chemical transformation. For this purpose miniprep of pTJK136 was prepared from KYRT1. pBlueScriptSK+ and DH5a were available from the lab sources. New stocks of competent DH5a cells were prepared with Rubidium Chloride method.

Following transformation into DH5a and selection in ampicillin (100µg/l) and streptomycin (300µg/l) plates, respectively two randomly selected colonies for pBlueScriptSK+ and pTJK136 were further cultured for plasmid analysis. Then size and purity checks for both vector was performed in agarose gel electrophoresis. Finally -80^{o}C stocks of the DH5a clones were prepared.

3.1.1.1.2 Sub-Cloning GUSint and nptII Cassettes into pBlueScriptSK+

It was not possible to identify the GUSint and nptII fragments correctly in agarose gel electrophoresis due to in-adequate information that will be pointed out in the following sections. In separate restriction reactions pTJK136 was digested with SalI and BamHI almost to completion for GUSint cassette and nptII cassette release respectively. Approximately 3000 and 2000 bp fragments for GUS and nptII cassettes were separated respectively in agarose gel electrophoresis. Meanwhile pBlueScriptSK+ was also prepared through SalI and BamHI digestions in two separate reactions (see appendix d, table D.1 for digestion mix details).

Following gel extraction of GUSint and nptII cassettes, GUSint and nptII fragments were combined with their respective pBlueScriptSK+ digests (SalI for GUSint and BamHI for nptII respectively) for co-elution from the same colon. At the final step, fast ligation reaction (Fermentas NEB or Roche fast ligation kits) was carried out for each elute. Concentrations of vector and insert were not taken into account during ligation (see appendix d, table D.2 for compositions of the ligation reaction mix).

Whole ligation reactions were then transformed into competent *E. coli* (DH5a) cells. And spread separately to amp LB agar plates. Following overnight incubation at 37°C emerging colonies were examined for the right clone, for insert, the direction of the insert and for restriction sites used in cloning.

3.1.1.1.3 pBlueScriptSK+GUSint Analysis

Among the putative pBlueSK+GUSint clones cultured on media (LB-Agar with antibiotics) four colonies were randomly selected and cultures overnight in liquid LB with ampicillin. First analyses of the putative clones were made on the size basis. Clones that show approximately 6 kbp fragment sizes were further analyzed through restriction digests for both revealing the GUSint cassette insert integrity and direction of GUSint cassette (figure 3.2). Throughout these analysis bidirectional pBlueSK+GUSint clones were recovered among the selected colonies.

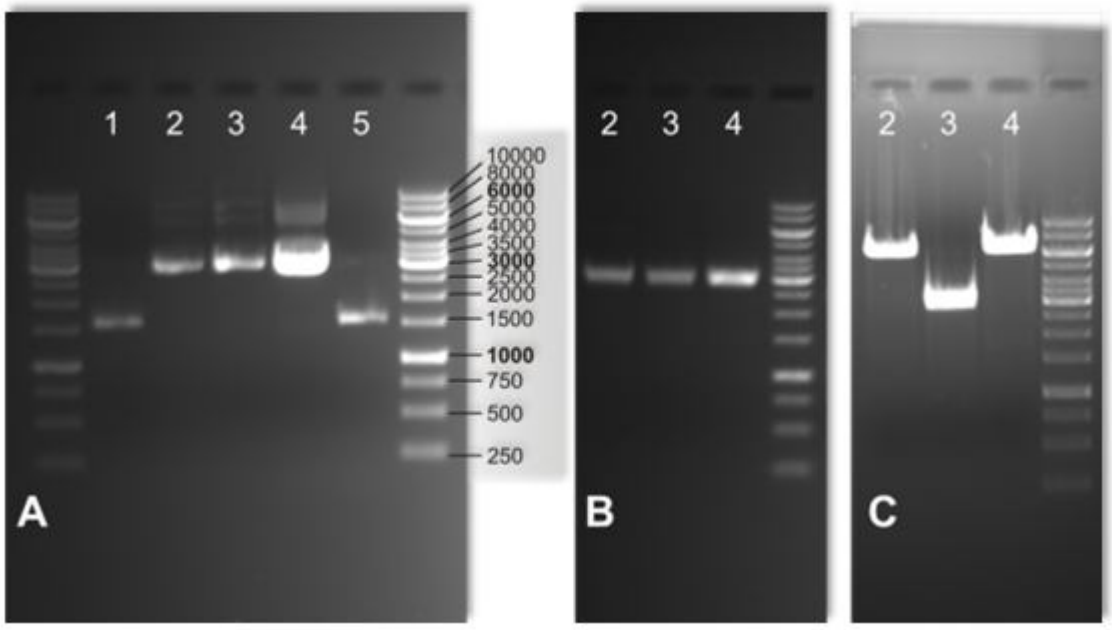

Figure 3.2: Analysis of putative pBlueSK+GUSint clones
(A) Plasmid size analysis from randomly selected 5 colonies showing that clones 2-4 (lanes 2-4) contains putative GUSint cassette inserts in pBlueScriptSK+. Lane 5 control; intact pBlueSCriptSK+. (B) Restriction analysis of the clones 2-4 for integrity of the SalI restriction sites for the putative pBlueScriptSK+/GUSint clones. No extra bands were seen. Sizes of the GUSint cassette and pBlueScriptSK+ are nearly the same (approximately 3 kbp) so single band is observed in the gel image. (C) PstI digestion of the same clones (2-4) to determine GUSint insert orientation in pBlueScriptSK+. 5′ end of the promoter region of CaMV35SS-GUSint-TNOS cassette contains a PstI site. Another PstI site resides in multiple cloning site of the pBlueScriptSK+. So PstI digestion reveals the orientation of the cassette for the clones 2-4. As the fragment sizes indicated clones 2 and 4 contains GUSint cassette in SK direction whereas it is in KS direction in clone 3.

3.1.1.1.4 pBlueScriptSK+nptII Analysis

The same route of clone analysis was performed also for pBlueSK+nptII putative clones. Analysis of the randomly selected colonies revealed both of the bidirectional integration of nptII in pBlueSK+ at BamHI restriction sites (figure 3.3).

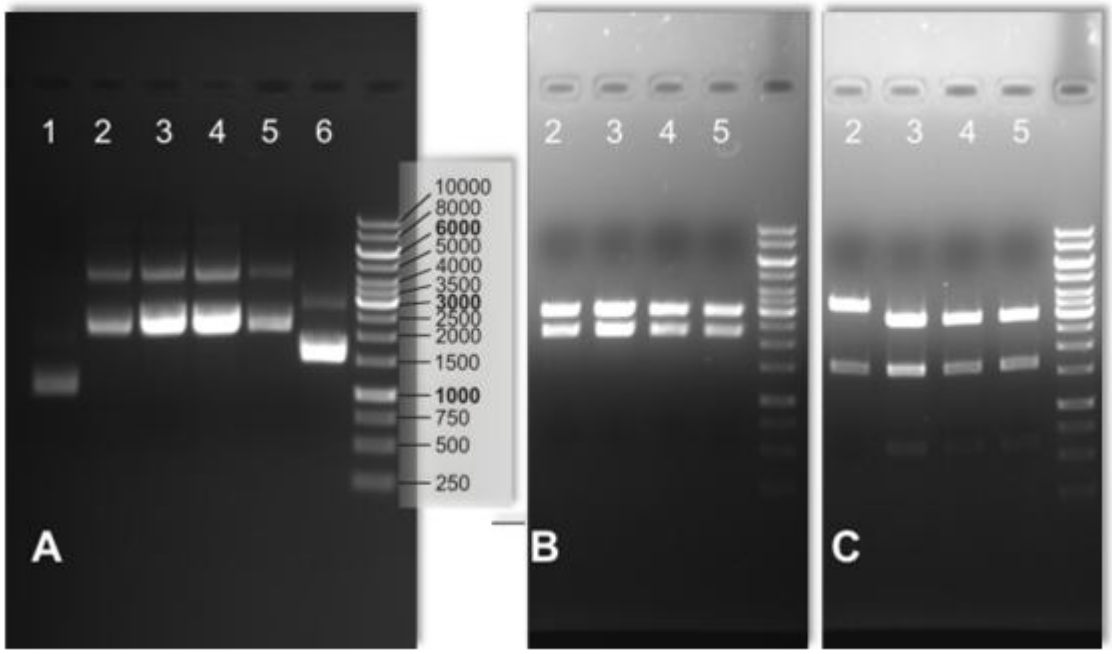

Figure 3.3: Analysis of putative pBlueSK+nptII
(A) Plasmid size analysis for detection of the putative pBlueScriptSK+/nptII clone. Lanes 1-5 are plasmid mini-preps from randomly selected putative clones for nptII insert; lane 6 is intact pBlueScriptSK+. Clones 2-5 that gave approximately 5 kbp size (middle bands) were further analyzed. (B) BamHI digestion of the putative pBlueScriptSK+ clones (2-5 from (A).) Empty plasmids and nptII cassette bands have expected band sizes (approximately 3000bps and 2300bps respectively.) (C) PstI restriction analysis of the prospective pBlueScriptSK+/nptII clones 2-5. Sequence information revealed a PstI restriction site at the 5′ end of the nptII coding sequence. Another PstI restriction site resides in multiple cloning site of the vector. As a consequence PstI can be used to detect the insert orientation. Clone number 2 digestion with PstI yielded two bands with approximate sizes of 3500 and 1700 bps. On the other hand clones 3-5 with the same digestion yielded three bands with approximately 3000, 1700 and 600 bps sizes. We may state that the two band yielding clone number 2 possibly has the nptII cassette inserted in SK direction whereas the three band yielding clones numbered 3-5 has the insert in KS direction. Another possibility that might be revealed is there may exist another PstI restriction site prior to BamHI at the 3′ end of the nptII cassette. As we consider the data recovered from restriction analysis of the pTJK136 vector and with the information just stated above (presence of another PstI restriction site at the end of nptII cassette) another ambiguity about the pTJK136 arises that nptII cassette orientation may not be as it is pictured in the map but in reverse orientation.

3.1.2 An Alternative Selection Marker; PMI

Phospho-mannose isomerase is bacterially encoded enzyme (*E. coli ManA* gene) that undertakes isomerization of phospho-mannose to phospho-fructose. This enzyme is not encoded in plants and culturing in mannose media results in accumulation of phospho-mannose to toxic levels. So this enzyme is being used in transgenic plant technology as selective marker.

One of the binary vector that harbors PMI as selective marker is the pPMI-GFP used in bentgrass transformation (figure 3.4). The PMI cassette from this vector was taken into account as the selective marker for our transformation studies. In the pPMI-GFP vector PMI is flanked by maize ubiquitin 1 promoter (M Ubi1) and 35S 3'terminator sequence.

In order to utilize a range of alternative restriction sites for subsequent cloning practices also the PMI cassette was sub-cloned into pBlueScriptSK+ at HindIII restriction sites.

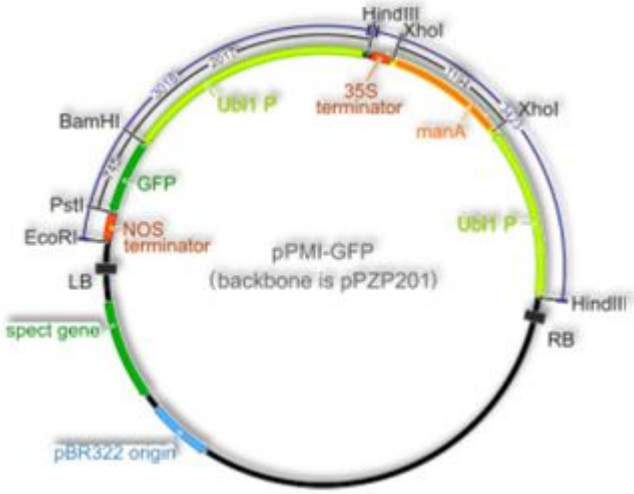

Figure 3.4: Simple graphic map of pPMI-GFP binary vector

3.1.2.1 Recovery of PMI Cassette from pPMI-GFP and Ligation to pBlueSK+

Both pBlueSK+ and pPMI-GFP plasmids were HindIII digested (see appendix d, table D.3 for restriction digest mix details). Only one restriction site (HindIII) was used for sub-cloning PMI cassette into pBlueSK+. So in order to avoid self-ligation of HindIII digested pBlueSK+, fast alkaline phosphatase (FAP) was also added to the reaction mix. The PMI cassette liberated from the pPMI-GFP vector was separated through gel electrophoresis.

The HindIII digested and FAP treated pBlueSK+ was cleaned with PCR cleaning kit and PMI cassette from gel electrophoresis of pPMI-GFP digest was recovered with gel elution kit. Then rapid ligation reaction was proceeded with T4 DNA Ligase (see appendix d, table D.4 for ligation mix details).

Following ligation, transformation of ligation mix and overnight culture on antibiotic LB agar plate was performed. The emerging colonies were analyzed for the pBlueSK+ManA cassette insert.

3.1.2.2 Analysis of the pBlueSK+PMI Cassette clones

Analysis of PMI inserts was initially performed through size detection since pBlueSK+ with and without PMI cassette insert (2958 and 6381 respectively) is quite distinct on size basis. Also since ManA is of *E. coli* origin colony PCR detection of PMI insert will require primers that should span the coding sequence (cds) and plasmid for correct detection. Else colony-PCR detection of ManA cds will result full positive.

Randomly selected 13 colonies scanned for pBlueSK+/PMI cassette with fast clone detection method that utilize hot lysis and direct gel profiling for high copy number plasmids size analysis (figure 3.5). Among the 13 only five of the colonies gave the expected ~6000 bps bands. The corresponding colonies were cultured overnight for plasmid isolation and plasmids were further digested with restriction enzymes for insert size (HindIII digest) and direction (XhoI digest) analysis.

The insert size and insertion site was confirmed with HindIII digest of the putative clones. The 3423 and 2958 bps fragments of the PMI cassette and pBlueSK+ vector backbone gave adequate resolution in agarose gel electrophoresis confirming the insert and insert site was ok (figure 3.6/ H). Further analysis was performed for insert orientation confirmation.

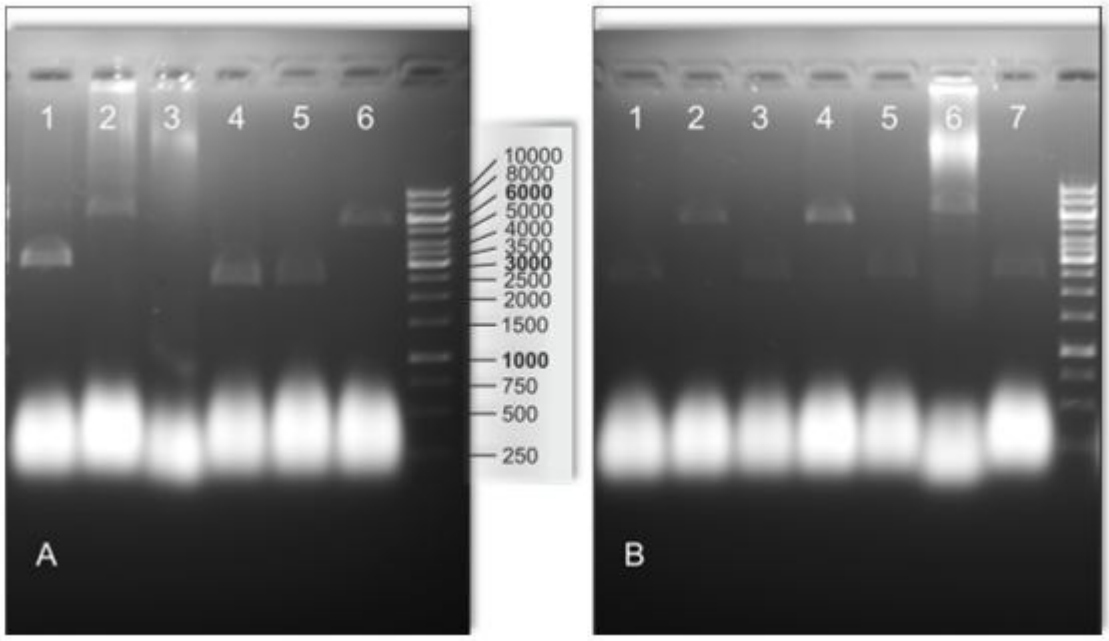

Figure 3.5: Fast clone detection for pBlueSK-PMI construct
Lanes A2, A6, B2, B4 and B6 are the putative clones giving linear bands aligned with 6000 bps fragment of the ladder.

It was implied in the original map of pPMI-GFP (figure 3.7) that XhoI cuts PMI cassette at asymmetric points and XhoI might be used for PMI insert direction analysis. However during *in-silico* reconstruction of PMI and GFP cassettes in pPZP201 another XhoI restriction site was revealed inside the double Ubi1 promoter. Furthermore XhoI digests of the putative pBlueSK+PMI gave extra bands other than expected (figure 3.6/ X). According to the *in-silico* evaluations of the artificial sequence pBlueSK+PMI cassette it was calculated that PMI cassette insertion in SK orientation in pBlueSK+ would give 3638, 1292, 1188 and 244 bps fragments upon XhoI digestion. Among these 3638 and 244 are strictly specific to PMI insert in SK orientation. Also if the insertion occurred in KS direction XhoI fragment lengths were

calculated as 3160, 1298, 1188 and 722 bps where at this time direction specific bands were 3160 and 722 bps (figure 3.7).

As a result of all of the calculations restriction analysis and artificial sequences generated we can say that clones A2, A6 and B6 have PMI insert in KS orientation in pBlueSK+ and B2, B4 have the insert in SK orientation (figure 3.6/ X).

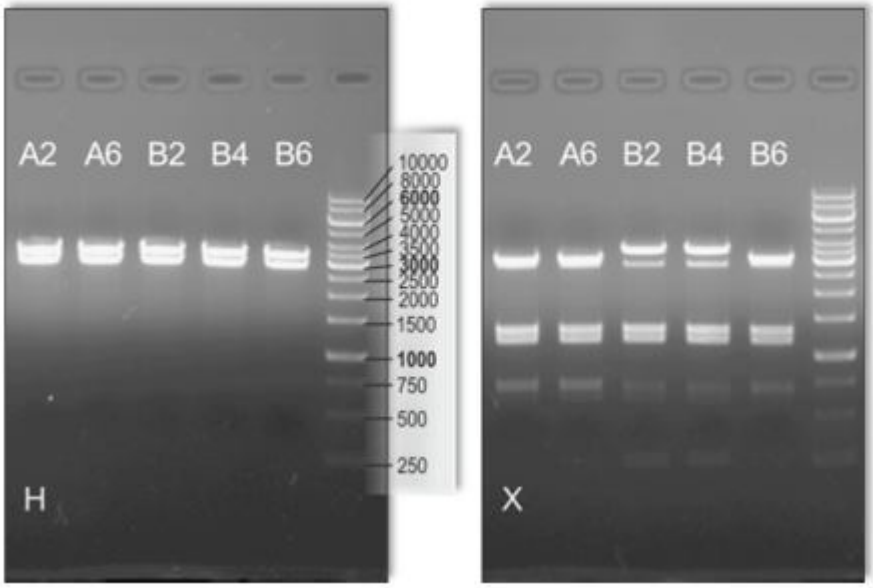

Figure 3.6: HindIII and XhoI restriction analysis of pBluSK-PMI cassette
(H) Clones numbered A2, A6, B2, B4 and B6 were further analyzed for insertion sites and size (H; HindIII digestion). All of the clones were insert positive, insert was unique and had expected size. (X; XhoI digestion) The same clones were also digested with XhoI for direction analysis. Clones B2 and B4 have PMI in SK orientation evident with 3600 and 250 bps fragments.

3.1.1 PMI cassette Construction with P CaMV35SS

As it was stated in the section 3.1, the ManA cassette from the pPMI-GFP vector contains maize ubiquitin one promoter. Due to this fact its use in lentil transformation was omitted. In place of the PMI cassette from pPMI-GFP a CaMV35SS driven ManA cassette construction was planned.

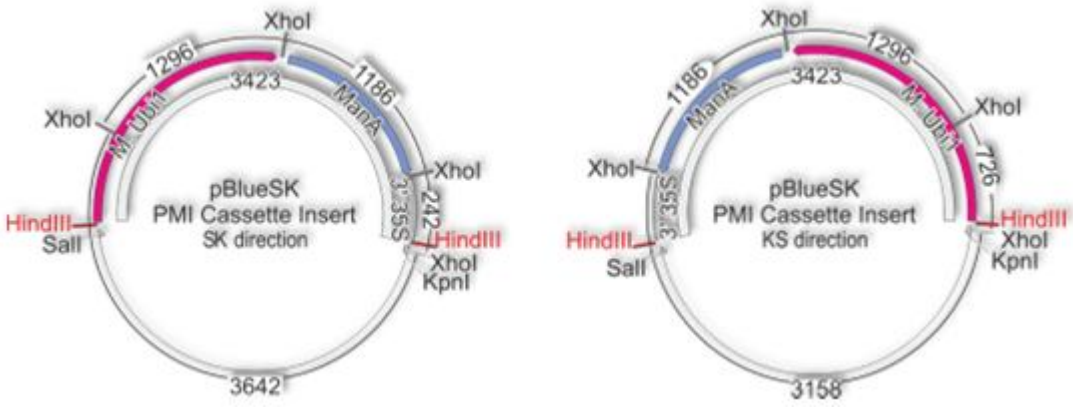

Figure 3.7: pBlueSK+PMI forward and reverse clones
The size of the XhoI fragments generated due to the PMI cassette orientation in pBlueSK+. The 242 bps 3'35s sequence and 3642 bps vector backbone plus first segment of ubiquitin promoter are the determinant fragments of PMI cassette in SK direction. The 3158 bps vector backbone plus the 3'35S sequence and 726 bps of the initial segment of the ubiquitin promoter are the determinant fragments for PMI cassette in KS direction.

The promoter and terminator sequences were CaMV35Ss and NOS poly-A sequences respectively and recovered from pCambia1305.1. ManA coding sequence was available as the PMI cassette in pPMI-GFP. It was not possible to recover every fragment with suitable restriction sites and recombine them in specific order for cassette construction. In order to overcome this obstacle and to leap over successive steps of restriction digestion and ligation events directional cloning was considered where the required restriction sites may be integrated through PCR. Specifically the directional cloning system of IGEM was inspired for recombination between the promoter, coding sequence and terminator fragments. Unique compatible restriction enzyme pairs were chosen between the promoter-gene and gene-terminator couples that disappear upon ligation. These were SpeI-XbaI and SalI-XhoI for PCaMV35SS-ManA and ManA-TNOS pairs respectively. As long as the cassette is generated its recovery and sub-cloning into transformation vector should require still other restriction enzyme sites at 5' and 3' ends of the cassette. Commonly used but unique restriction sites were chosen for this purpose. These were BamHI-XmaI-EcoRI for 5' end and HindIII-XmaI for the 3' end of the cassette. Main scheme of the cloning practice was given in the figure 3.8 below. The same concerns of issues are valid for PCaMV35SS-MBF1c-TNOS cassette construction.

3.1.1.1 Primer Design for CaMV35Ss, ManA and TNOS

Primer designs for CaMV35SS promoter, ManA coding sequence and NOS terminator were done with Primer3 (Rozen and Skaletsky, 2000) primer design software. Detailed information about the primers is given in figure 3.9. Primer designs of CaMV35SS promoter and NOS terminator were done on the available sequence data of pCambia-1305.1 vector. Restriction enzyme recognition sites at 5′ and 3′ flanking regions of each fragment were integrated into the primer tails with base mismatches. No mismatch pairs where integrated into the sequence of the fragments where possible. For the ManA coding sequence since 5′ and 3′ flanking sequences were not known and since only the coding sequence was available (retrieved from NCBI) primer design was performed depending on this sequence only. In order to maintain non-pairing between the primer pairs to be designed, mismatches were integrated into the body of coding sequence as a last option. That is, reverse primer contained 3 base mismatches at 3′ end of the ManA coding sequence without any change in codon meaning.

3.1.1.2 PCR amplifications for CaMV35Ss, ManA and TNOS

For PCR amplification of the CaMV35SS promoter, ManA coding sequence and NOS terminator approximately -5^{o}C of the Tm values was taken into account and maximum Tm was arbitrarily taken as 65^{o}C. Fragments were amplified in single Touch-Down PCR reaction with Herculase-II polymerase. The PCR reaction mixtures and TD-PCR program are tabulated in tables D.5 and D.6 respectively in appendix d.

3.1.1.3 Recombination of CaMV35SS, ManA and TNOS Fragments

For recombination of the fragments into CaMV35SS-ManA-TNOS cassette in pBlueSK+ first the TD-PCR amplified fragments were analyzed for their purity and size with gel electrophoresis (figure 3.10). Then restriction digest plan and restriction digest was performed prior to ligation. Finally ligation mixture was transformed into *E. coli* and spreaded on solid media for colony growth. Clone analysis from the

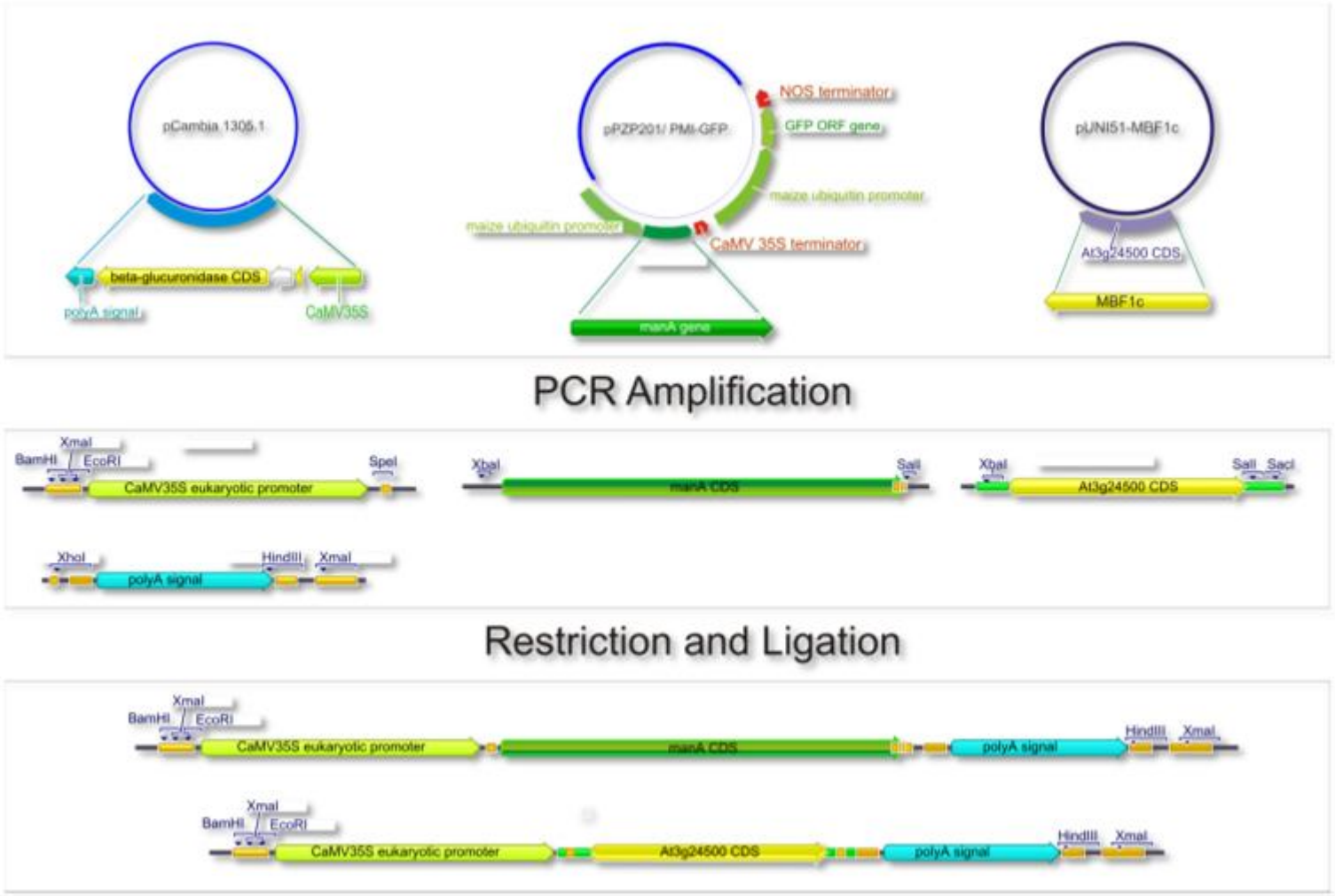

Figure 3.8: Schematic view of CaMV35SS-MBF1c/ManA-NOS cassette construction

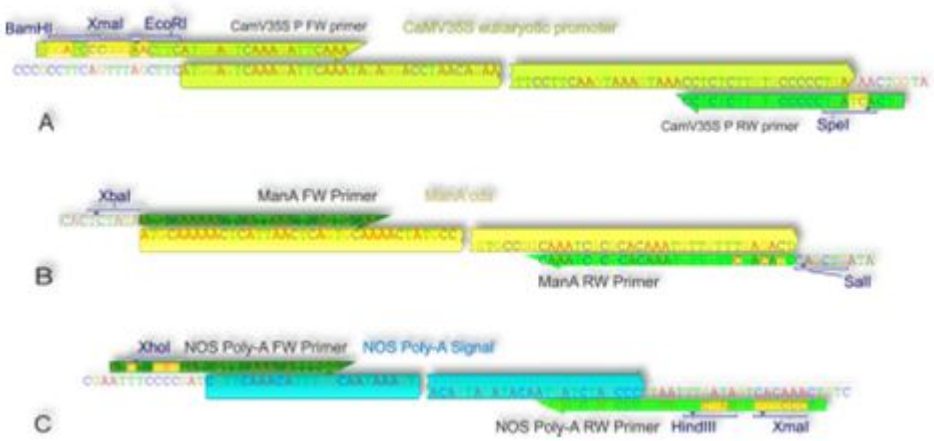

Figure 3.9: Primers for CaMV35SS-ManA-TNOS cassette construction
Bases noted in yellow boxes on primer arrows show the mismatch sites. Base mismatches on primer tails of 35S promoter and NOS terminator were located on the flanking sequences of the fragments since the sequence data was available (A and C). For ManA on the other hand since only the coding sequence was exactly known, flanking sequences spanned with the primer tails were directly assigned with the restriction enzyme recognition sequences. Also in order to find strictly non pairing primer pairs 3' end of the ManA coding sequence was accommodated with 3 base mismatches within the boundaries of codon usage (B). Also the 5' ends of each primer were extended 2 to 3 bases beyond the location of the neighboring restriction enzyme according to the recommendations of Fermentas. These 2 to 3 base extensions were addressed for full activity (100%) of respective enzyme during digestion following PCR amplification of the fragments. Following primer designs in Primer3 (Rozen and Skaletsky, 2000) software the Tm values of each pair were calculated in IDT's oligo analyzer service. Conditions for the analysis were as follows: dNTPmix: 0,2mM MgCl2: 2mM Primer: 0.25µM Na: 0. The calculated Tm values are as follows: CaMV35SS promoter 71.6ºC and 68.3 ºC; ManA coding sequence 68.9 ºC and 71.3ºC; NOS terminator 67.2ºC 72.1ºC for forward and reverse primer pairs respectively

emerging colonies were performed on three basis; size analysis or colony-PCR and sequencing. Confirmed clones were further used in final transformation vector construction.

3.1.1.3.1 Restriction Digestion of the Fragments and pBlueSK+

Current ligation kits by Fermentas or NEB uses T4-DNA ligase enzyme and kit manual strictly restricts the total amount of DNA to 100ng in a single ligation reaction. The ligation reaction is catalyzed in expense of ATP and rate is strictly

affected by relative ATP/ADP concentration. At the start of the ligation reaction ATP/ADP ratio is at maximum and catalysis rate is termed as burst ligation where approximately 90% of the ligation takes place. At this step if there occurs nonspecific and unwanted fragments and if the total amount of DNA in ligation mixture exceeds the recommended (100ng) amount, ligation will shortly proceed to steady state low rate phase without adequate quantity of prospected ligation product. PCR amplification circumvents presence of nonspecific fragments but generates high copy number of each fragment that is to be prepared through restriction digestion. Otherwise ATP might be used up through ligation of any of the nucleic acid component in the reaction mixture.

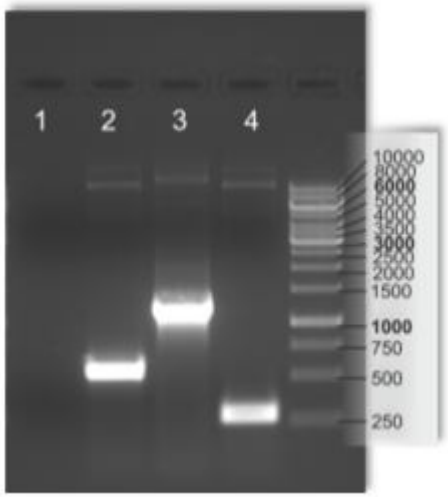

Figure 3.10: Fragment analysis of CaMV35SS, ManA and NOS amplicons
Fragment analysis for TD-PCR amplification of (2) CaMV35SS promoter, (3) ManA coding sequence and (4) NOs terminator sequences. Lane 1 is negative control. Fragments are aligned with prospected of the ladder (SM0311-Fermentas). Upper faint bands in each lane are the templates.

For ManA cassette construction it was previously stated that different restriction enzyme recognition sites were integrated to the 5′ and 3′ ends of each fragment. That means double digests are to be performed in order to generate complementing sticky ends. Here the point of concern is the highest activity of two different enzymes in one unique buffer. Since fragments amplified with PCR are in large quantities their digest till completion is very critical in order to maintain adequate concentration and quality of sticky ends that are fed to ligation step.

The double digest plan for the cloning vector and fragments are as follows: pBluescriptSK+ digest with BamH-XmaI, CaMV35SS promoter digest with BamHI-BcuI(SpeI), ManA coding sequence digest with XbaI-SalI and NOS terminator digest with XhoI-XmaI. Selections of buffers for these double digests are critical for maintaining highest percent activity of each enzyme in the same reaction mixture. So buffers of both NEB and Fermentas were utilized. Overnight restriction digestion of the vector and the fragments were carried out at 37°C. The selected buffers and activities of enzymes in these respective buffers are listed in appendix d, table D.7 and restriction digestion reaction mixtures are also given in appendix d, table D.8.

3.1.1.3.2 Ligation of the Fragments and pBlueSK+

Following overnight digestion, the whole restriction digest mixes were collected and cleaned with gel purification kit and eluted into the same tube to a volume of 25 µl. Then from this mixture of fragments ligation was performed. Ligation reaction mix is given in appendix d, table D.9. Fast ligation was performed with Fermentas fast ligation kit. Then total ligation mixture was transformed into competent Top-10 cells. Following overnight incubation at 37°C emerging colonies were analyzed for the putative clones.

3.1.1.4 Analysis of CaMV35SS-ManA-TNOS Cassette

Ligation products transformed into *E. coli* TOP-10 competent cells are not suitable for colony PCR check of the ManA gene, since the gene is already bacterial origin. Also analysis of the cassette insert through size check is very efficient and rapid procedure. So first size analysis of the pBlueSK+ clones were done for CaMV35SS-ManA-TNOS cassette integration. Then plasmid mini-preps of the prospective clones were done for further PCR analysis of the cassette constructed. Finally sequencing info was utilized to confirm the cassette.

3.1.1.4.1 Size Check CaMV35SS-ManA-TNOS Cassette

Size analysis of the pBlueSK+ 35SManANOS clones were performed with fast clone detection method. The gel image of the randomly selected 14 colonies is given in

figure 3.11. Among the 14 colonies only three of them gave larger fragment size with respect to the pBlueSK+ intact vector (Figure 3.11). There were middle sized clones (7 and 9) that were taken into account during PCR analysis as internal check.

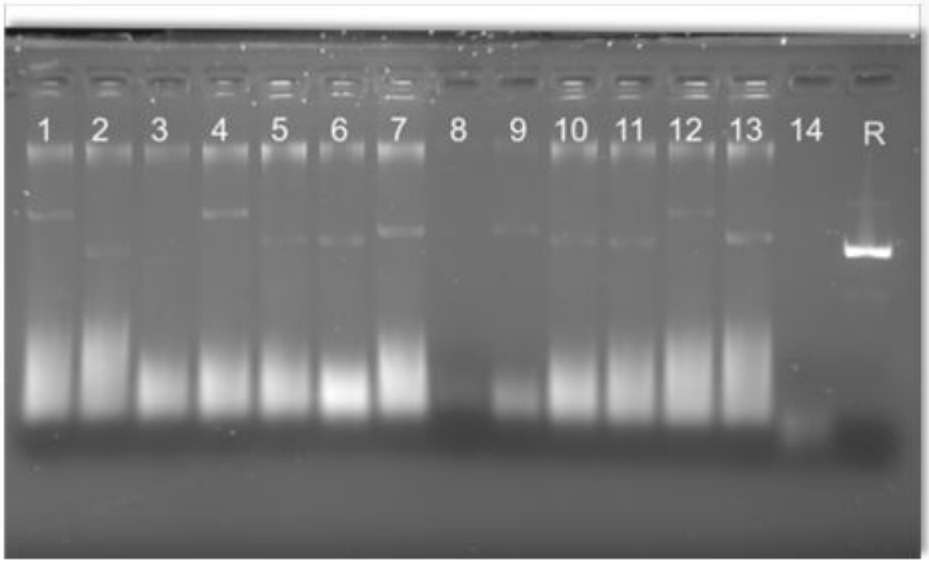

Figure 3.11: Fast clone detection for CaMV35SS-ManA-NOS cassette
Fast clone detection for CaMV35SS-ManA-NOS cassette from randomly selected 14 colonies. Colonies 1, 4 and 12 were selected for their size that these may have PCaMV35SS-ManA-TNOS cassette inserts when compared to the (R) intact pBlueSK+ plasmid as a reference. One of the other clones (9) that show a middle band and hence size was selected as an internal control. These selected clones were further PCR checked for revealing the expected cassette size.

3.1.1.4.2 PCR Check of CaMV35SS-ManA-TNOS Cassette

PCR analysis of the ManA cassette generated was performed with CaMV35SS forward primer and NOS reverse primer. PCR reaction composition and PCR program was tabulated in appendix d (tables D.10 and D.11 respectively). The gel electrophoresis of the PCR reaction result was given in figure 3.12. As expected clones 1, 4 and 12 gave the expected fragment aligned at 2000 bp fragment of the ladder. Two of the clones were selected and sent sequencing for detailed analysis of the cassette.

3.1.1.4.3 Sequencing of CaMV35SS-ManA-TNOS Cassette

For sequencing, the generated clones were sent to MacLab. The M13 forward and reverse primer sets were addressed for sequencing the prospective cassette in pBlueSK+. *İn-silico* generated sequence of the whole cassette in pBlueSK+ was used for contig-assembly with the four sequences the two independent clones retrieved from MacLab. At least 25 base pairs were trimmed from the 5' ends of the sequences retrieved. Also approximately 170 base pairs were trimmed from the 3' ends of the seqeunces for contig assembly. The contig assembly was schematized in figure 3.13 below.

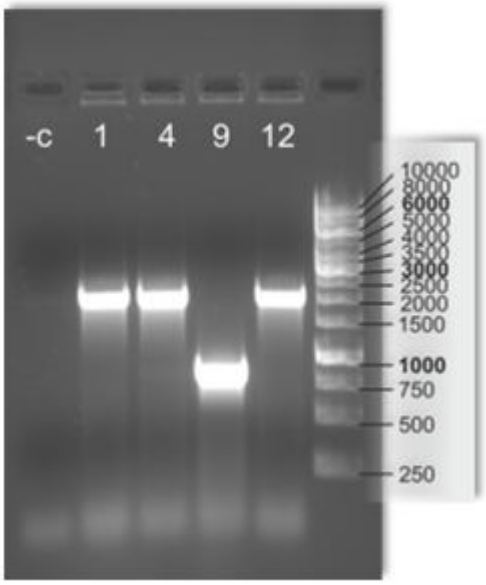

Figure 3.12: PCR check of the selected pBlueSK+P35SManATNOS clones
PCR check of the selected clones numbered 1, 3, 9 and 12. –C stands for negative control. Clones 1, 4 and 12 gave the expected fragment size of approximately 2000 bp (calculated; 2012 bp) length.

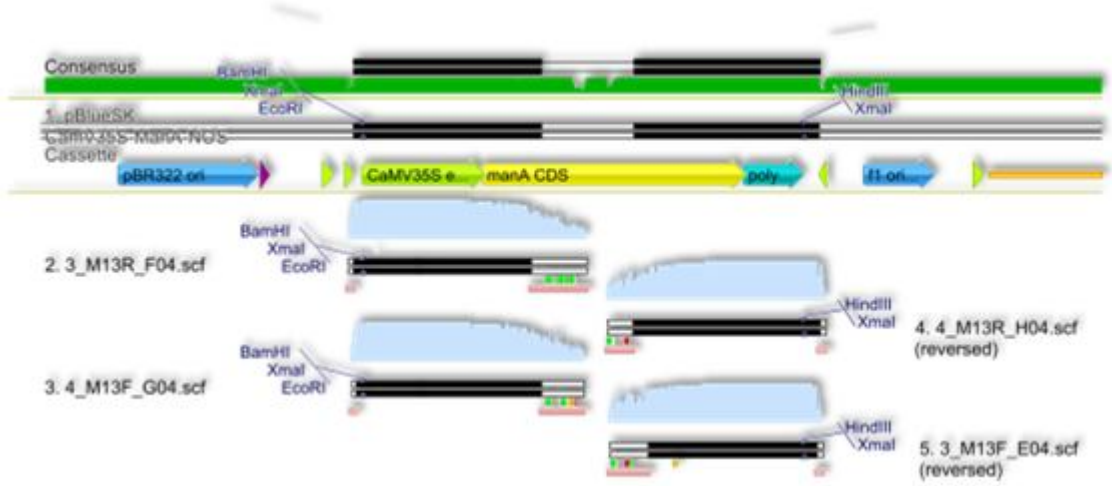

Figure 3.13: Contig assembly for CaMV35SS-ManA-TNOS sequence reads
Contig assembly of the sequence data retrieved from the MacLab and *İn-silico* generated CaMV35SS-ManA-NOS sequence in pBleSK+. Standard sequencing service of the MacLab was purchased; generating approximately 700 bp read did not reveal the whole coding sequence for ManA gene.

3.1.2 *In-silico* Reconstruction of GUSint

With the CaMV35SS-ManA-NOS and CaMV35SS-MBF1c-NOS cassettes generated transformation vector construction was committed to finalization as the two cassettes were sub cloned into pPZP101 (given in section 3.1.6.3.2). Lentil transformation experiments were started with this transformation vector (pPZP101 ManA-MBF1c). But first two experiments were failed due to loss of all of the transformed explants. In order to observe what may be happened it was quiet plausible to accommodate the vector with a visual marker gene. Hand in we had GUSint cassette from pTJK136 but the only available restriction site was SalI since sequence data was not available. We had quite a lot of reference material for pTHW136 and pTJK136 vectors. Re-evaluation of these materials revealed that we may regenerate the whole GUSint cassette sequence *in-silico*. Missing parts of the puzzle were the pDE4 vector that contains an intron-less GUS gene and GUSint sequence generated through insertion of potato light sensitive gene-1 second intron. The pDE4 sequence was retrieved from patent information and GUSint sequence was regenerated *in-silico* from the GUS coding sequence and ST-L1 intron 2 sequences. Schematic view of the processes of *in-silico* regeneration of GUSint

cassette is given in figure 3.14. The sequence regenerated for GUSint, pBluescriptSK+ clone of the CaMV35SS-GUSint-NOS cassette and pDE4 vector are given in appendix b.

3.1.3 MBF1c Cloning and Cassette Construction

3.1.3.1 Sub-cloning MBF1c from pUNI52 to IV 1.1

Along with the cloning works that were undertaken as noted in preceding sections, analysis of the received MBF1c coding sequence (from Arabidopsis TAIR) and its further cloning works were carried out also.

At first place pUNI51-MBF1c clone received was analyzed for the insert as it is recommended in the documentation sent with MBF1c stock. Then MBF1c coding sequence is sub-cloned into pBlueScriptSK+ for utilizing unique restriction sites of the pBlueScript vector. Finally MBF1c was sub-cloned into IV 1.1 under rubisco subunit 1 promoter (RbcS1.) The Rubisco S1 promoter was addressed as its high level light inducible expression.

3.1.3.1.1 Confirmation of MBF1c Coding Sequence Insert in pUNI51 Clone

Purpose was confirmation of *Arabidopsis thaliana* At3g24500 gene (coding for ethylene-responsive transcriptional co-activator protein MBF1c), clone was sent in pUNI51 with U23216 stock number from Arabidopsis Biological Resource Center (ABRC.)

The method of clone construction was stated as recovery of the coding sequence by reverse transcription from mRNA and ligation of SfiI adaptor sequences and finally ligation into pUNI51 at SfiI restriction site. pUNI51 belongs to the uni-vector system developed for Arabidopsis based genetic studies. pUNI51 vector can only be propagated in Escherichia coli PIR1 host strain. DH5a strain is not suitable for replication of this vector because of origin of replication concerns.

3.1.3.1.2 Restriction Analysis of MBF1c Insert

The U23216 numbered of *Escherichia coli* PIR1 host strain clone containing pUNI51-MBF1c was cultured in LB+ kanamycin (25µg/l) overnight at 37°C. Then the pUNI51-MBF1c plasmid was isolated with mini-prep.

For analysis of the insert either PCR amplification or EcoRI- HindIII double digestion was recommended in the reference information enclosed with the stock sent. However detailed analysis of both clone and vector revealed more plausible restriction sites (figure 3.15). A HincII restriction site was located on both MBF1c cds and multiple cloning site of the vector. Also it was revealed that the first HincII site is at position 103 from the 5′ end of the MBF1c cds (MBF1c cds is 447 bps long and thus HincII is at an asymmetric position.) Besides HincII, two other restriction sites flanking the insert (EcoRI and NotI) were determined inside the pUNI51 multiple cloning site. These two sites were utilized for full length recovery of MBF1c coding sequence (see appendix d, table D.12 for pUNI51-MBF1c clone analysis and recovery of MBF1c cds).

The gel electrophoresis image shown in figure 3.16 confirms presence of MBF1c cds insert in pUNI51 and demonstrates that the direction of the insert is as it is depicted in figure 3.15.

The sequence data for pUNI51-MBF1c clone was not received. So it was generated from sequence data of both pUNI51 vector and MBF1c cds according to the information enclosed with the clone received. As a result the 5′ and 3′ flanking sequences around the MBF1c cds is not exact.

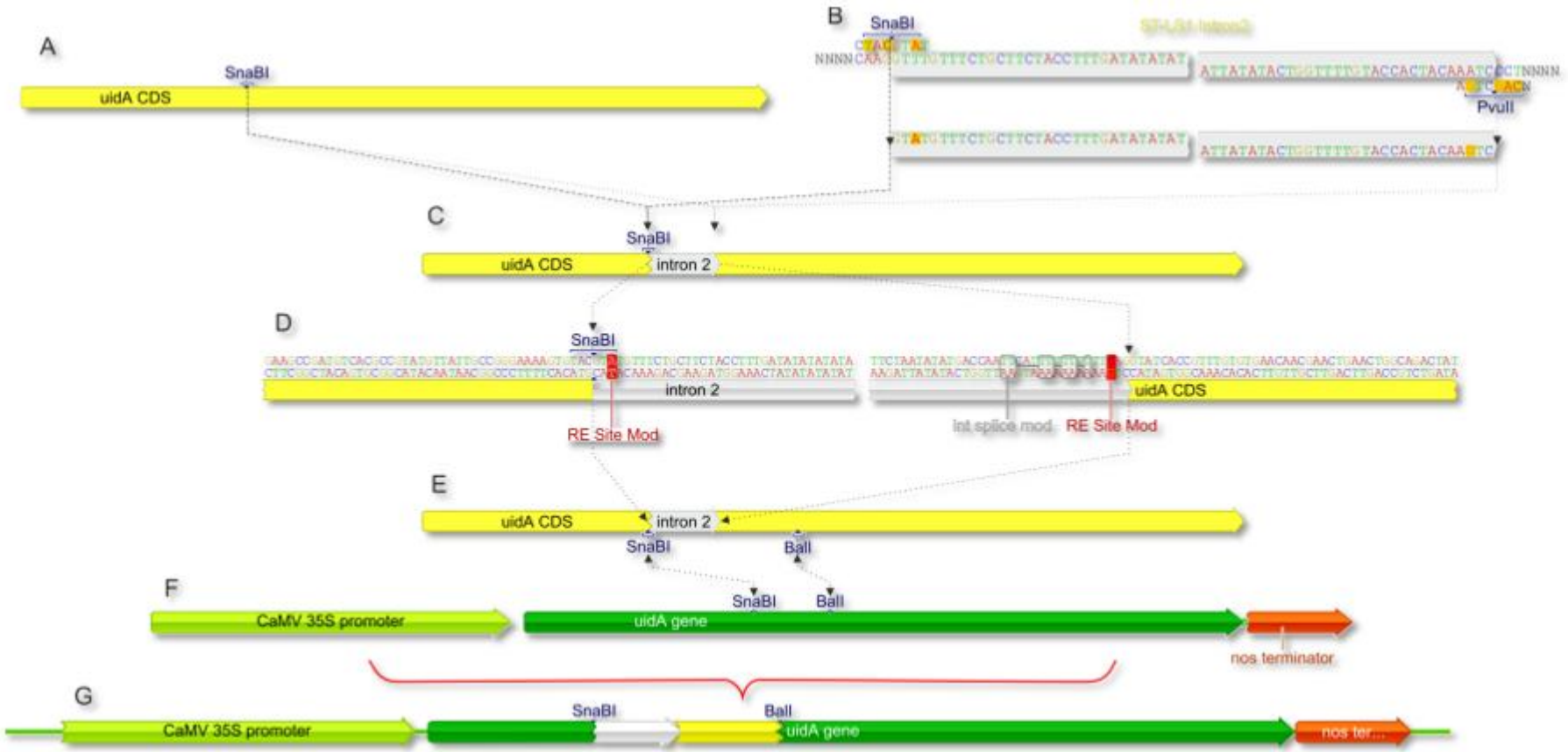

Figure 3.14: Schematic view for regeneration of the CaMV35SS-GUSint-NOS cassette

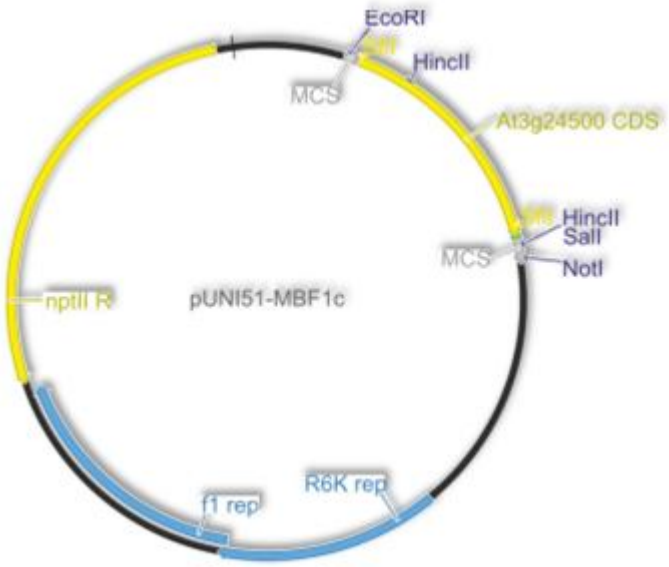

Figure 3.15: Graphical map of pUNI51-MBF1c
pUNI51-MBF1c clone was regenerated with the information cited in the written material sent with clone and sequence data for pUNI51 and MBF1c cds (At3g24500 gene, GI; 28466837.) Note for HincII, EcoRI and NotI restriction sites.

3.1.3.1.3 Cloning MBF1c cds into pBlueScriptSK+

Multi protein bridging factor 1c (MBF1c) was noted to be received in pUNI51 cloned between two SfiI sites. The only available restriction sites for intact recovery of the MBF1c cds were EcoRI at 5' end and SalI at 3' end. But there were no compatible restriction sites complementing these EcoRI and SalI restriction sites in the target plasmid, IV 1.1. As a result, in order to carry out cassette construction, IV 1.1 compatible restriction sites of pBlueScriptSK+ were utilized.

The EcoRI and SalI restriction enzyme couple was used for recovery of MBF1c from pUNI51-MBF1c (figure 3.15) and hence pBlueScriptSK+ was prepared with the same restriction enzyme couple (figure 3.35) and. Restriction digestions were followed by agarose gel electrophoresis separation of the fragments. Then the linearized pBlueScriptSK+ and MBF1c cds were extracted from the gel with Qiagen gel purification kit and proceeded to ligation. The restriction digest composition and ligation reaction mixture are given in appendix d, table D.13 and D.14, respectively.

The whole ligation product was used in transformation to *E. coli* DH5a competent cells. After overnight incubation at 37oC in ampicillin plate some of the emerging colonies were analyzed for MBF1c insert.

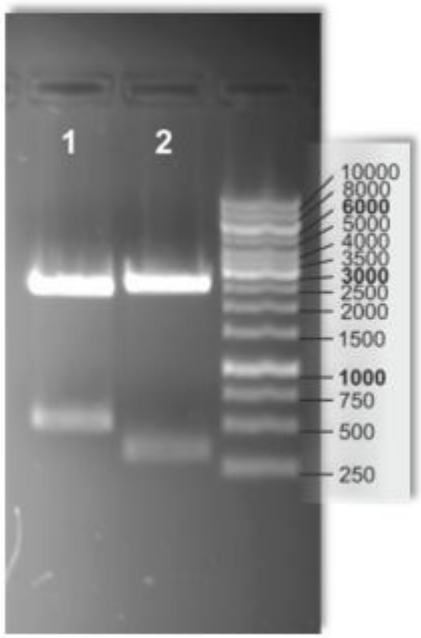

Figure 3.16: Restriction analysis of pUNI51-MBF1c clone
Restriction analysis of pUNI51-MBF1c clone. Lane 1; EcoRI-NotI double digest yielding approximately a 500 bp and a 2500 bp fragments. Lane 2; HincII digest resulting approximately 360 bp and 2700 bp fragments.

3.1.3.1.4 Analysis of the Putative pBlueSK-MBF1c Clones

Randomly selected 6 colonies were analyzed on size basis for the presence of insert. A total of 3410 bp was calculated as the predicted size of pBlueSK-MBF1c clone (Figure 3.17).

Initial size check of the plasmids from the six colonies revealed only four of them having the expected size of 3410 bp (Figure 3.18/ A wells 1, 2, 5, 6). For the four candidate clones further analysis was carried out in order to confirm the confirmation of insert, insert restriction sites, and its direction (Figure 3.18/ B and C.)

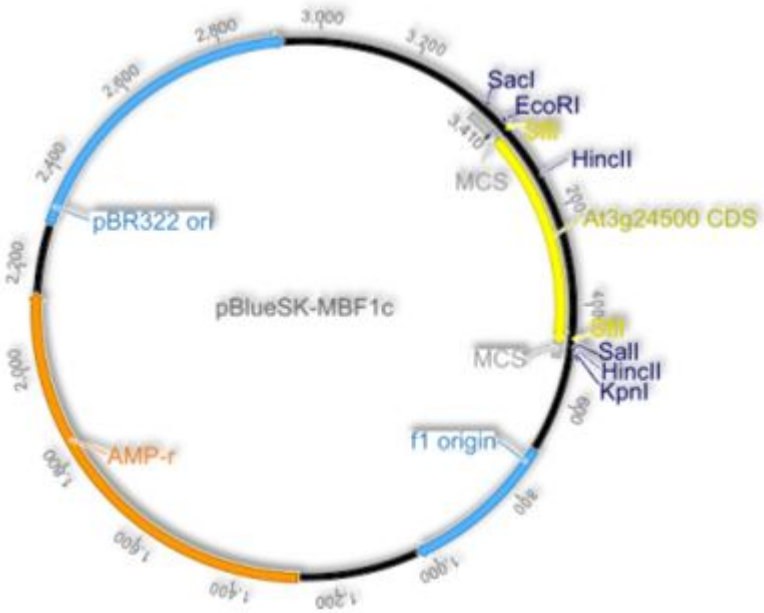

Figure 3.17: Graphical map of generated pBlueSK+MBF1c clone
Map generated for pBlueSK-MBF1c clone. Consider the restriction sites depicted, size (base numbers) of the insert and whole plasmid.

3.1.3.2 Cloning MBF1c cds into IV 1.1

Directional cloning was planned for sub-cloning MBF1c cds under rubisco promoter into IV 1.1. Detailed analysis of the MBF1c flanking sequences (pBlueSK-MBF1c) and Impact Vector 1.1 cloning sites revealed only single compatible restriction sites available for cloning. Eco52I site was at 5′ end of the MBF1c cds in pBlueScript and NotI was between Rubisco promoter and terminator in IV 1.1. Recognition sequences for these restriction enzymes are as follows; 5′-GC^GGCCGC-3′ and 5′-C^GGCCG-3′ for Not I and Eco52I respectively. The two other restriction enzyme sites were XhoI and BglII (from pBlueSK-MBF1c and IV 1.1 respectively) for blunt ligation of the 3′ end of the MBF1c cds to 5′ end of the Impact Vector Rubisco terminator. See figure 3.19 for details of restriction enzyme positions utilized for sub-cloning.

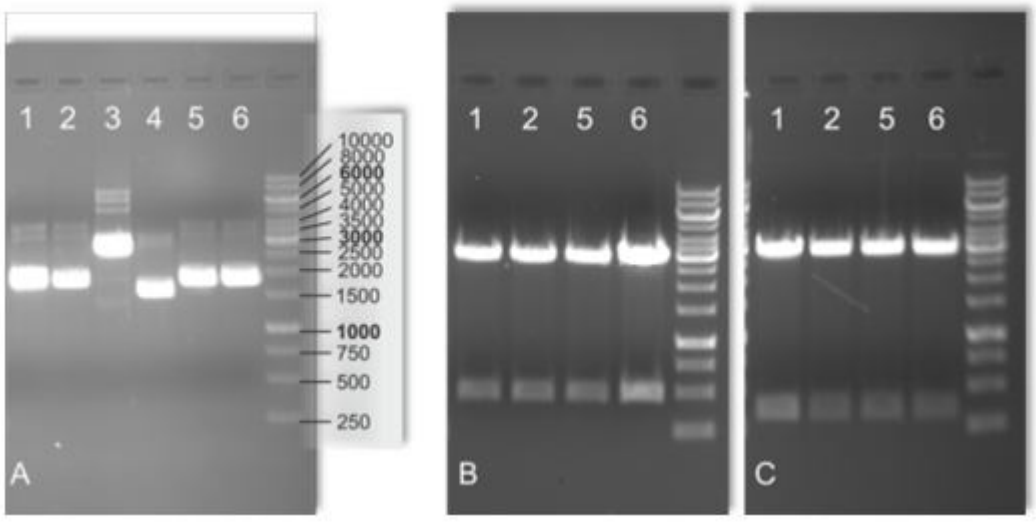

Figure 3.18: Analysis of pBlueSK+MBF1c clones
(A) Plasmid size analysis of randomly selected 6 colonies for putative pBlueSK-MBF1c clones. Candidate plasmids were electrophoresed as intact, without any restriction digestion. Electrophoresis conditions were 7V/cm, in 0.7% Agarose gel with 1X TAE buffer. In this electrophoresis condition the middle bands in each well are the linearized plasmid sections of minipreps. According to the middle band positions well numbers 1, 2, 5, 6 may contain expected size (~3410bp.) So the candidate clones are 1, 2, 5 and 6. (B) EcoRI-SalI double digest of the selected clones 1, 2, 5 and 6. EcoRI and SalI sites remained intact and insertion is clean. (C) HincII digestion of the same clones (1, 2, 5 and 6) confirmed the insert direction in SK orientation (SalI-KpnI direction).

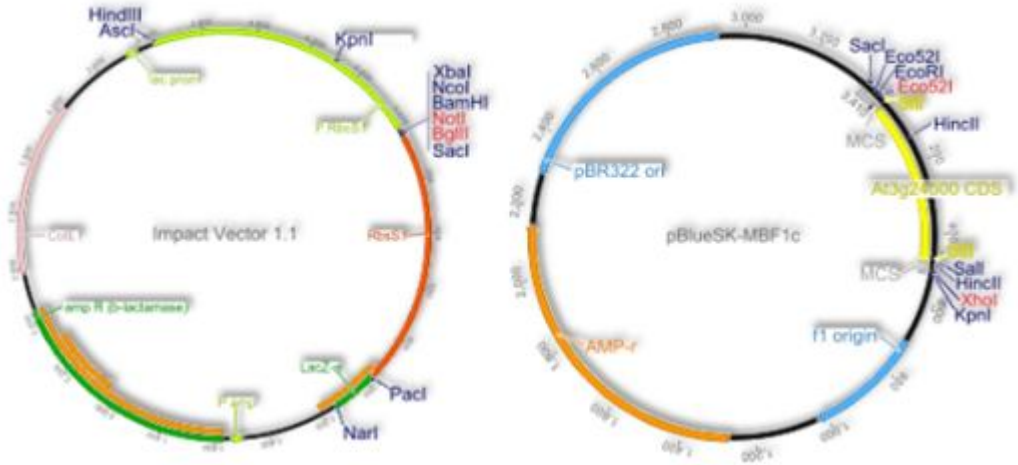

Figure 3.19: Restriction site analysis for MBF1c cloning from pBlueSK+ to IV 1.1
Available restriction sites for MBF1c sub-cloning into Impact Vector 1.1. Consider NotI and BglII restriction sites from IV 1.1 and Eco52I and XhoI sites from pBlueSK-MBF1c. Also note that compatible NotI, Eco52I sites and in compatible (blunt ligated) BglII and XhoI sites were utilized in order to maintain directional cloning.

3.1.3.2.1 XhoI, BglII Restriction Digests and Blunting Reactions

Preparation of both vector and insert was done with two initial restriction digests interfered with a blunting reaction.

The blunted restriction sites were digested first, namely XhoI and BglII for pBlueScriptSK+ and Impact Vector 1.1 respectively. The first restriction digestion reaction is tabulated in appendix d, table D.15.

T4 DNA Polymerase (Fermentas) was used during blunting reaction and blunting reaction was performed without cleaning the restriction enzymes directly after overnight digestion. For the reaction only T4 DNA Polymerase and dNTP was added into the restriction mixture. Blunting reactions were carried out at 11°C for 20 minutes (see appendix d, table D.16 for blunting reaction mix).

3.1.3.2.2 Eco52I, NotI Restriction Digests and Ligation Reactions

The second restriction digestions were made after cleaning the first digestion and blunting mixture with PCR Cleaning Kit (Fermentas). Each of the cleaned mix was eluted to 30µl sterile distilled water for restriction digestion preparation (see appendix d, table D.17 for restriction digest mix).

The final steps of preparations before ligation were recovery of MBF1c fragment and cleaning of IV 1.1 reaction mix with. The 481 bp fragment of MBF1c coding sequence was gel extracted after separation in agarose gel electrophoresis while IV 1.1 backbone was purified with PCR cleaning kit. Then ligation reaction was proceeded with eluted MBF1c fragment and prepared Impact Vector 1.1. Refer to appendix d, table D.18 for ligation reaction mix.

Whole ligation mixture was used for transformation. Transformed bacteria were selected on Ampicillin (100mg/l) LB-agar plates.

3.1.3.2.3 Analysis of the Putative IV 1.1-MBF1c Clones

Since the sequence information for both IV 1.1 and MBF1c cloned in pBlueScriptSK+ were available a hypothetical (*in-silico*) map was generated for use in restriction analysis. Depending on this map the same size analysis and insert orientation analysis was also performed for IV 1.1-MBF1c clone

Total cell lysis was performed from five randomly selected colonies of the overnight cultured transformants. Size resolution of the IV 1.1 (4500 bps) and IV 1.1-MBF1c clones (5000 bps) was not quiet discriminative in 0.7% agarose gel electrophoresis. So for comparison wells containing the total cell lysis of the putative clones were flanked with plasmid DNA of IV 1.1 (well numbers marked with * in figure 3.20). Size analysis showed four out of five clones with greater band sizes with respect to intact IV 1.1 bands. These four clones were further analyzed for plasmid, insert size and orientation.

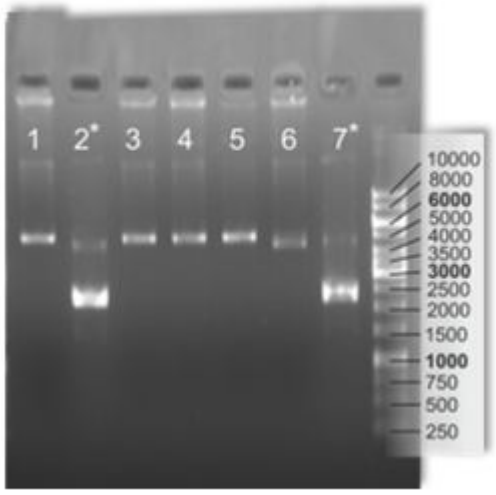

Figure 3.20: Size analysis of generated IV 1.1-MBF1c clones
Size analysis of randomly selected five colonies for IV 1.1-MBF1c clone. Well numbers 1, 3-6 are randomly selected putative clones whereas well numbers 2* and 7* are intact IV 1.1 without insert. Note that there is a slight band shift between the intact plasmid and the first four of the putative clones; this is due to the size difference between the intact plasmid (IV 1.1; 4630 bps) and plasmid with the insert(IV 1.1-MBF1c; 5107 bps). Also note the Ladder separation pattern; in particular 4000 and 5000 bps. According to the gel image the putative clones are the colonies numbered 1, 3-5.

Analysis of the IV 1.1-MBF1c map generated (figure 3.21) revealed that both of the blunted XhoI and BglII sites originating from pBlueScript and IV 1.1 plasmids respectively were regenerated after ligation event. Linearization of the putative IV 1.1-MBF1c clones were made through this XhoI site (Figure 3.22/ A). Also a HincII site at 3' end of the MBF1c cds originating from pBlueScript was used for direction analysis where HincII digestion recovers a 361 bps fragment of MBF1c (Figure 3.22/ B). In HincII digestion results an unexpected band of approximately 100 bps was observed (Figure 3.22/ B). This might be an artifact of blunt end ligaton performed at 3' end of the MBF1c and 5' end of the IV 1.1 Rubisco terminator sequences. One probable scenario about the artifact might be of the 3' to 5' insertion of another MBF1c to this site. With this ambiguity revealed that might be resulted from blunt end ligation at one end of the ligation reaction we may omit using this P RbcS1-MBF1c-T RbcS1 cassette in next stages. Instead directional and PCR cloning could be utilized.

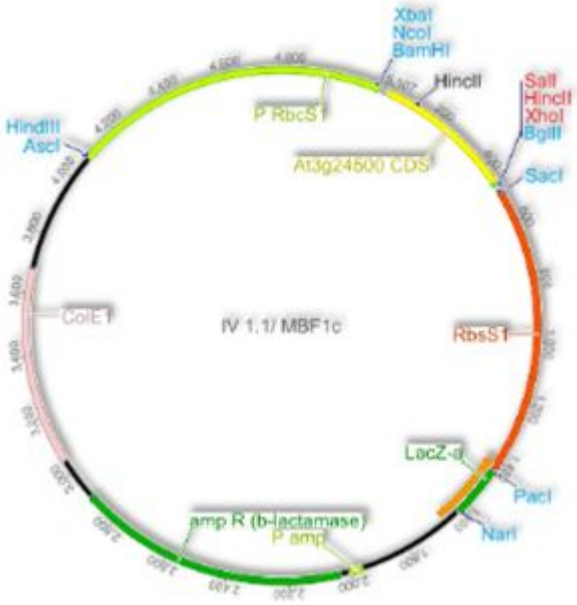

Figure 3.21: Graphical map of IV 1.1-MBF1c
Simple hypothetical restriction map generated for IV 1.1-MBF1c. Blue typed restriction sites are original to IV 1.1. Red typed restriction sites are originating from pBlueScriptSK+. Note that the NotI and Eco52I compatible restriction sites from IV 1.1 and pBlueScript respectively were diminished upon ligation while XhoI and BglII sites were regenerated upon blunt end ligation.

3.1.3.3 Direct Cloning MBF1c into IV 1.1 through PCR Amplification

Sub-cloning of MBF1c from pUNI51 to pBlueSK+ and then to IV 1.1 through restriction digestion steps resulted in accumulation of additional restriction sites flanking the MBF1c coding sequence (figure 3.23). Also scarcity of the restriction sites flanking the whole RbcS1-MBF1c cassette leaded us recombination of whole IV 1.1-MBF1c with the target transformation vector, pPZP101. But these accumulated restriction sites flanking the MBF1c coding sequence hampered securely removal the IV 1.1 backbone.

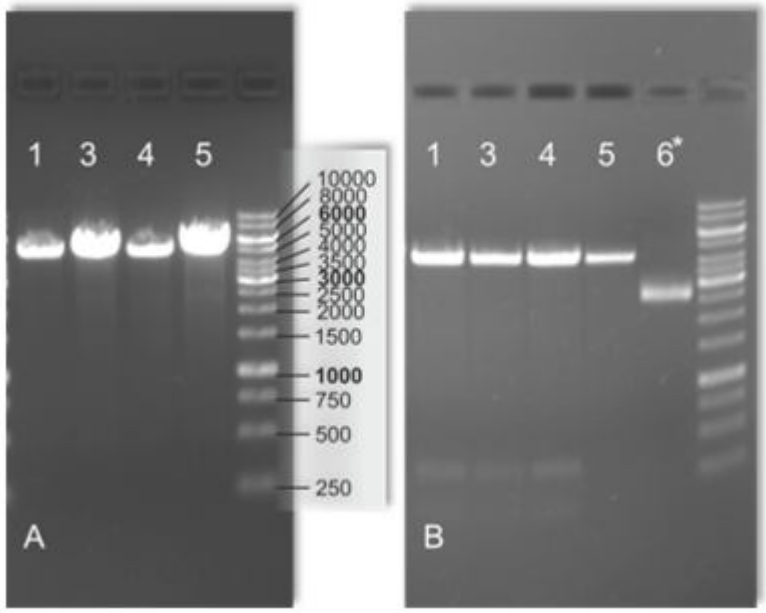

Figure 3.22: Size and direction analysis of IV 1.1-MBF1c clones
The putative clones addressed in size analysis in figure 3.20 were analyzed further with XhoI digestion (A) in order to reveal the linear sizes of the putative clones with respect to the linear ladder (SM0311-Fermentas). Sizes were around the expected 5000 bps band aligned with the ladder. Further restriction digestion analysis was performed with HincII (B) for confirmation of the directional cloning. Wells numbered 1, 3-5 are the putative clones whereas 6* is the intact plasmid. HincII digestion of the clones numbered 1, 3 and 4 showed two lover bands around 250 bps. This banding pattern for HincII digestion was unexpected. This may result as a consequence of impure clones or insertion of unintended fragments during blunt end ligation.

Attempts for emptying resulted in loss of fragments from RbcS1-MBF1c cassette. In order to overcome this hurdle although emptying these restriction sites was taken into account the core solution was addressed as a clean cloning through PCR

amplification of MBF1c coding sequence with suitable flanking restriction sites and insertion into IV 1.1 through single restriction digestion and ligation process.

Figure 3.23: Restriction sites accumulated at MBF1c flanking regions
Some of the restriction sites accumulated at MBF1c flanking regions during sub-cloning.

For this purpose two plans were taken into account; first one is amplification of the MBF1c through asymmetric PCR with two primer sets and hybridization of the amplicons and direct ligation into digested IV 1.1, second one is amplification of the MBF1c with primers that integrate restriction sites at 5′ and 3′ flanking sites of the coding sequence and sub-cloning into IV 1.1 with single restriction digestion. The next two sections pointed out these two plans.

3.1.3.3.1 Asymmetric PCR Based Cloning of MBF1c into IV 1.1.

The first PCR based cloning experiment undertaken for sub-cloning MBF1c into IV 1.1 was asymmetric PCR. Aim was to clone MBF1c directly into the restricted IV 1.1 following hybridization of two amplicons generating 5′ and 3′ sticky end MBF1c amplicon (figure 3.24).

PCR amplification of MBF1c was done with two different primer pairs, namely long and short. For the PCR reaction made with long primers the forward primer was in excess and the reverse primer was in excess for the reaction made with short primers (Asymmetric PCR). The two PCR products were then mixed and left to hybridization for overnight at 94°C. Hybridization was followed by ligation of MBF1c in to NcoI-SacI digested IV 1.1.

It was possible to generate single stranded fragment but cloning into IV 1.1 was unsuccessful. Hence standard PCR cloning experiments were devised thereafter.

3.1.3.3.2 PCR Amplification, Digestion and Cloning of MBF1c in IV 1.1

In order to maintain a clean cassette construction in IV 1.1 the bordering restriction sites (XbaI and SacI) of the multiple cloning site of the IV 1.1 were targeted. For this purpose first primers were designed then PCR amplification and restriction digests were performed eventually ligation transformation and analysis of the clones were carried out. Detailed information about the processes are given in the following sections.

3.1.3.3.2.1 Primer Design and PCR Amplification of MBF1c

XbaI and SacI are the two restriction sites bordering the multiple cloning site of the IV 1.1 that is between RubiscoS1 promoter and terminator (Figure 3.21). For cloning MBF1c with this two restriction sites, primer designs were made on the artificially generated pBlueSK-MBF1c vector sequence. For this purpose approximately 10 bp upstream and downstream sequences flanking MBF1c (in pBlueScript) were spanned for primer design. The tails of forward and reverse primers contained XbaI and SalI-SacI restriction sites for integration respectively (Figure 3.25).

For comparison of the Primer-3 generated Tm values, IDT Tm calculations were also made with the following conditions: dNTPmix; 0.2 mM $MgCl_2$; 2 mM Primer; 0.25 µM and no Na. With this component mix IDT Tm values calculated were 65.3°C for Fw primer and 68.4 ºC for Rw primers. The Primer-3 generated Tm values, Herculase Taq-DNA Polymerase and IDT tested PCR compositions were used for MBF1c amplification through TD-PCR (see appendix d, table D.19 for PCR reaction mix). Also the Touch Down-PCR program generated for MBF1c amplification is listed in appendix d, table D.20. And gel electrophoresis check of the TD-PCR amplification of MBF1c with XSS primers is shown in figure 3.26.

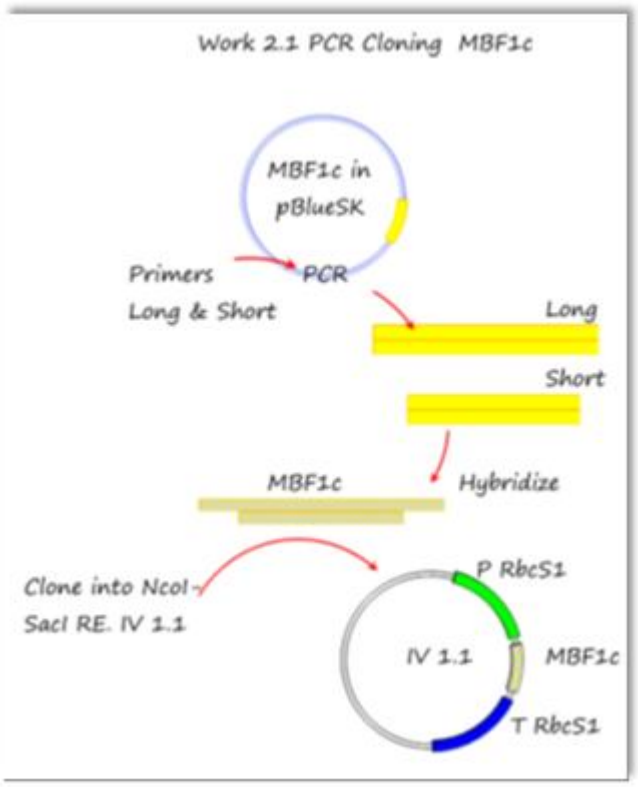

Figure 3.24: Asymmetric PCR based cloning of MBF1c into IV 1.1

Figure 3.25: MBF1c XSS primer set

MBF1c XSS primer set for cloning was designed with Primer 3 (Rozen and Skaletsky, 2000). Note the 5′ mismatch sequences of the primer pair for addition of XbaI and SalI-SacI restriction sites through PCR amplification. The primer sequences are 5′-ATA TCT AGA CAT GCC GAG CAG ATA CC-3′ for forward primer and 5′-GGA GCT CGT CGA CTC ATT TCC CAA TTT TAC-3′for reverse primer. Primer-3 Tm values are 55.85°C for Fw and 59.94°C for Rw primers. Amplification of MBF1c was directly performed with Touch Down (TD) PCR without any optimization step.

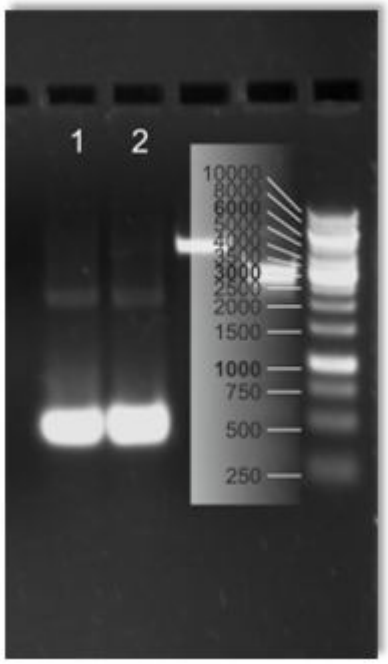

Figure 3.26: Gel image of TD-PCR for MBF1c with XSS primers
Lanes 1 and 2 are showing MBF1c amplification. Consider the nonspecific bands around 2500bps with respect to the 476 bps MBF1c lining around 500 bp fragment of the ladder SM0311.

3.1.3.3.2.2 Restriction Digestion of MBF1c XSS and Cloning into IV 1.1

XbaI-SacI restriction digests of PCR amplified MBF1c and IV 1.1 was preferably made through double digest. Fermentas buffer system was evaluated to be improper for double digest, so NEB buffer system was considered. NEB 4 buffer was found to be 100% efficient for both of the enzymes in the same reaction mix (see appendix d, table D.21 for details of restriction digest mix).

Prior to ligation double digests were cleaned with PCR purification kit. Ligation was performed with fast ligation kit. Ligation reaction mix composition is given in appendix d, table D.22. Then whole ligation product was transformed into competent *E. coli* and spread plated on LB Agar with ampicillin (100mg/l). Following overnight 37^{o}C incubation colonies were analyzed for insert.

3.1.3.3.2.3 Analysis of IV 1.1-MBF1c XSS Clones

Initial analysis of IV 1.1-MBF1c XSS clones were done on size basis. Initially total cell lysis of randomly selected five colonies was compared with intact plasmid on

agarose gel electrophoresis (Figure 3.27/ A). 0.7% agarose gel in 1X TAE buffer and 8V/cm electric field rating for 30 minutes did not produce adequate resolution for discrimination of intact IV 1.1 and putative IV 1.1-MBF1c XSS. So the same five colonies are cultured overnight for mini-prep. Then intact plasmid DNAs of the five colonies were electrophoresed for a period of 50 minutes (figure 3.27/ B) with respect to IV 1.1. At this time it was possible to discriminate the intact IV 1.1 and IV 1.1 with inserts but it was not quite clear if the linear middle bands of the plasmid preps were aligned with 5000 bps ladder marker in 1X TAE gel electrophoresis buffer.

For further analysis colony PCR was made for MBF1c XSS insert from another set of randomly selected colonies (see appendix d, table D.23 and D.24 for details of colony PCR mix and program). Colonies showing positive signals were cultured overnight and plasmid preps were prepared. Gel electrophoresis of the plasmids gave clear alignments of middle (linear plasmid) bands with 5000 bps ladder fragment in 1X TAE (figure 3.28)

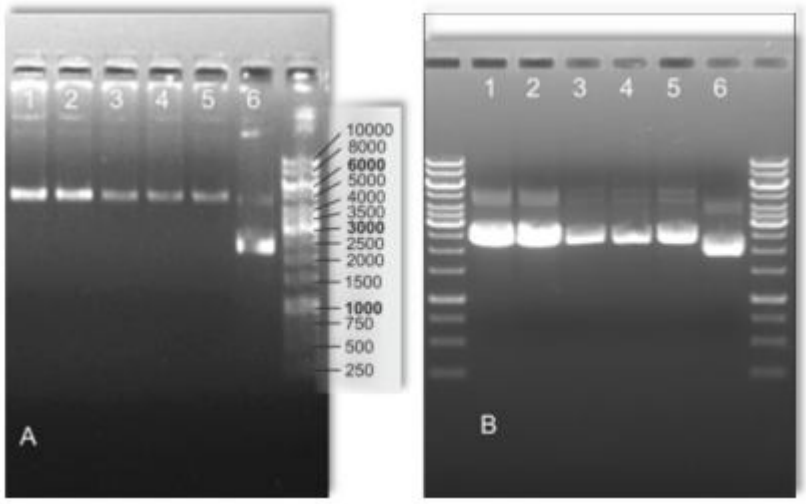

Figure 3.27: Size analysis of the IV 1.1 MBF1c XSS clones
(A) Gel electrophoresis of randomly selected five colonies from putative IV 1.1-MBF1c clones (lanes 1-5) and intact IV 1.1 plasmid (Lane 6). Alignment of the bands with respect to the ladder (SM0311-Fermentas) and intact plasmid is not quite clear. (B) Gel electrophoresis of plasmid mini-preps of the same five colonies and intact IV 1.1. Clear discrimination between the intact plasmid and putative clones are prominent but proper alignment of the linear plasmid fractions with 5000 bps fragment of ladder is not clear.

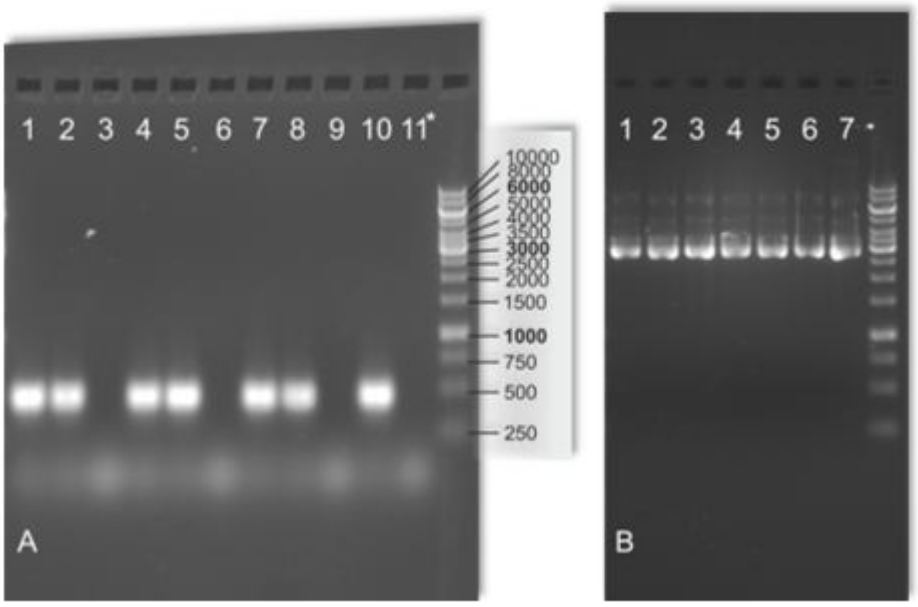

Figure 3.28: Colony PCR and size check for the IV 1.1 MBF1c clones
(A) Colony PCR results of randomly selected putative IV 1.1-MBF1c XSS clones (lanes 1-10, lane 11 is negative control) were compared to (B) plasmid mini-preps of the colony PCR positive clones in section A. Linear fraction of the plasmids (the middle bands) are clearly aligned with 5000 bps fragment of the ladder.

3.1.3.4 MBF1c Cassette Construction with P CaMV35SS

Due to the problems with MBF1c sub-cloning into IV 1.1 an alternative cassette construction was devised. This new cassette construction was based on the same method that generated ManA cassette. That is MBF1c was to be cloned under CaMV35SS promoter with NOS terminator. So the same procedures for 35S-ManA-NOS cassette construction works were proceeded for 35S-MBF1c-NOS cassette construction. Only difference was use of MBF1c XSS amplicon from the section 3.1.5.3.2.1 was used instead of ManA amplicon. The steps for CaMV35SS-MBF1c-NOS cassette construction are as follows.

The MBF1c XSS Amplicon generated (section 3.1.5.3.2.2) was first cleaned with PCR cleaning kit and eluted to 40µl of sterile distilled water. Then restriction digestion was performed with XbaI-SalI enzymes (see appendix d, table D.25 for restriction digest mix). The same procedures for ligation of CaMV35SS-ManA-NOS cassette were used for CaMV35SS-MBF1c-NOS cassette construction. The ligation reaction

mixture is given in appendix d, table D.26. Following ligation and transformation events CaMV35SS-MBF1c-NOS Cassette analysis were performed for choosing the correct clones.

3.1.3.4.1 Analysis of CaMV35SS-MBF1c-NOS Cassette

Analysis of the CaMV35SS-MBF1c-NOS cassette was carried out on two bases. First is detection of MBF1c containing clones through colony PCR method and evaluation of the plasmid sizes that the clones contain then sequencing the candidate clones for complete check of the cassette sequence. The sequencing showed that there is complete consensus between the *in-silico* generated cassette and the experimentally generated cassette.

3.1.3.4.1.1 Colony PCR for CaMV35SS-MBF1c-NOS Cassette

Colony-PCR check for CaMV35SS-MBF1c-NOS cassette was performed with MBF1c XSS primer set. Randomly selected 11 colonies were analyzed for MBF1c coding sequence. Among the 11 colonies only 7 of them gave positive band for MBF1c amplification (figure 3.29/ A) and between these 7 colonies 6 of them were further analyzed for the plasmid size (figure 3.29/ B). Size check was done with the plasmids prepared as mini-preps. Colonies numbered 5, 9, 10 and 11 gave linear plasmid bands (middle bands of the lanes numbered 5, 9, 10 and 11) aligned approximately over the 4000 bp fragment of the ladder. The CaMV35SS-MBF1c-NOS cassette length is around 1200 and within the plasmid total size reached to 4200 bps. Among these colonies two of them were sent to sequencing. Colony-PCR reaction mixtures and PCR program are given in appendix d, table D.27 and D.28 respectively.

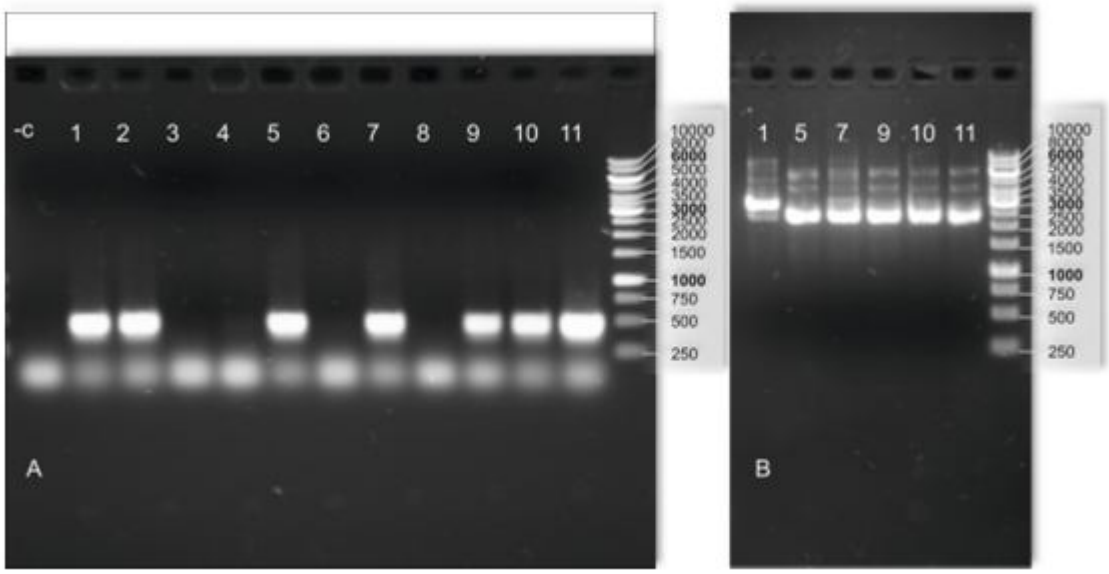

Figure 3.29: Colony PCR and size check for 35S-MBF1c-NOS clones
Colony-PCR results of putative 11 colonies (A) and size check (B) of six colonies selected among the positive clones in A. Colony-PCR analysis result 7 positive among the randomly selected 11 colonies. From these 7 colonies six of them checked for their size revealed that clones 5, 9, 10 and 11 are the candidate clones for sequencing.

3.1.3.4.1.2 Sequence Information of Candidate CaMV35SS-MBF1c-NOS Clones.

For sequencing, two of the four candidate colonies were sent to MacLab. Sequences generated with M13 forward and reverse primers were assembled into contigs with *in-silico* generated pBlueSK+ CaMV35SS-MBF1c-NOS clone. At least 25 base pairs were trimmed from the 5′ ends of the sequences retrieved. Also 40-160 base pairs were trimmed from the 3′ ends of the sequences for contig assembly. The contig assembly was schematized in figure 3.30 below.

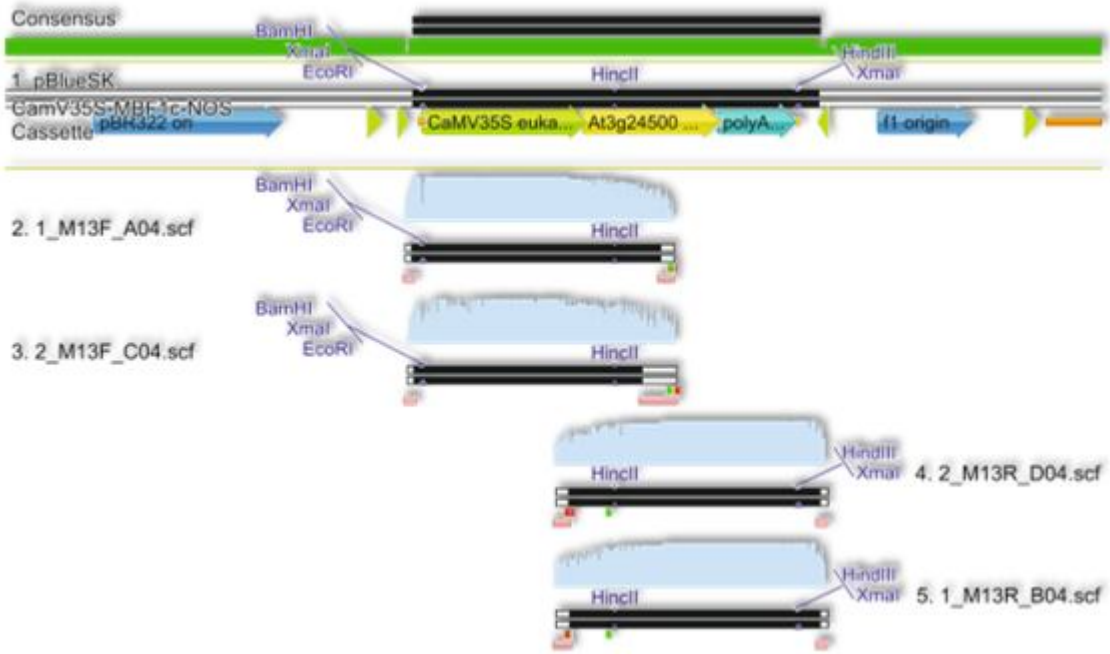

Figure 3.30: Contig assembly of 35S-MBF1c-NOS sequence reads
Contig assembly of the sequence data retrieved from the MacLab and *in-silico* generated CaMV35SS-MBF1c-NOS sequence in pBleSK+. Standard sequencing service of the MacLab was purchased; generating approximately 700 bp read spanned the whole 1250 bp sequence of CaMV35SS-MBF1c-NOS cassette.

3.1.4 Selection of Plant Transformation Vector Backbone

3.1.4.1 pTJK136 or pPMI-GFP as Binary Transformation Vectors

Quiet number of literature information can be retrieved about utilizing pTJK136 as binary vector during *Agrobacterium* mediated gene transfer to plants. Also lentil transformation experiments conducted in our lab used mainly this vector during studies. As a result the same vector was initially addressed as the sole option in this thesis studies. Using this vector for transformation studies in my thesis required additional information concerning the T-DNA and vector backbone map or sequence. Patent information and literature searches extended the map of the T-DNA region only. In depth analysis of the restriction map generated with the information gathered did not verify this map, but introduced more ambiguity. A small section of restriction analysis is shown in figure 3.31 below. It is noted in the

GUSint cassette restriction that the cassette contains an internal PstI site. However contrary to this information restriction digests cannot prove this internal PstI site. Current restriction map of the T-DNA region is given in figure 3.32.

Due to lack of sequence information and as a consequence, non-reproducible restriction map of the T-DNA region along with limited amount of literature data and patent information it was not possible to handle pTJK136 or its T-DNA region. The restriction map information gathered and restriction analysis only permitted intact recovery of both GUS and NPTII cassettes pointed out in preceding sections. All of these findings suggest that restriction of pTJK136 and manipulation of any cassette for cloning seem problematic. So it is likely that pTK136 will not be the choice of transformation vector from this point on. As an alternative pPMI-GFP vector was considered since it is based on pPZP201 and whole sequence of the vector backbone is known.

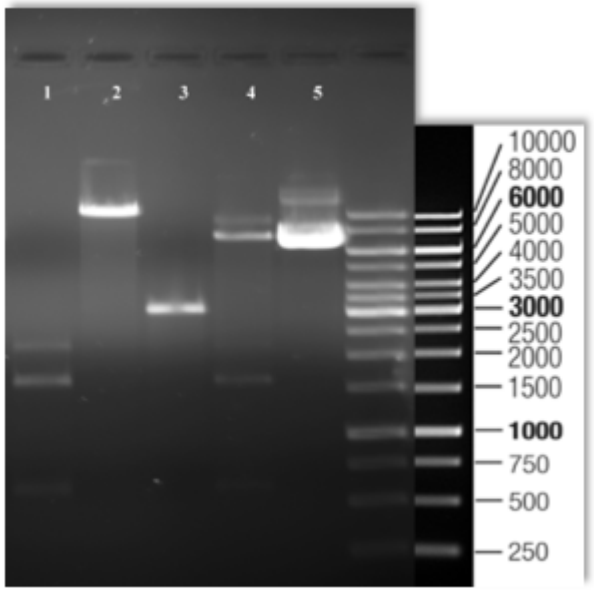

Figure 3.31: Restriction analysis of pTJK136
Restriction map of pTJK136. Lane 1 is PstI digest of SalI fragment (GUSint cassette) from pTJK136, GUSint cassette contains two PstI restriction sites yielding three fragments which are approximately 2.25, 1.75, 0.6 kbp. The same bands recovered in lane number 4 when pTJK136 without nptII cassette was restricted with PstI again. Upper two bands may be resulting from incomplete digestion. Both nptII cassette (lane3) and pTJK136 without GUSint cassette (lane2) did not produce any extra bands with PstI restriction. Lane 5 is intact plasmid.

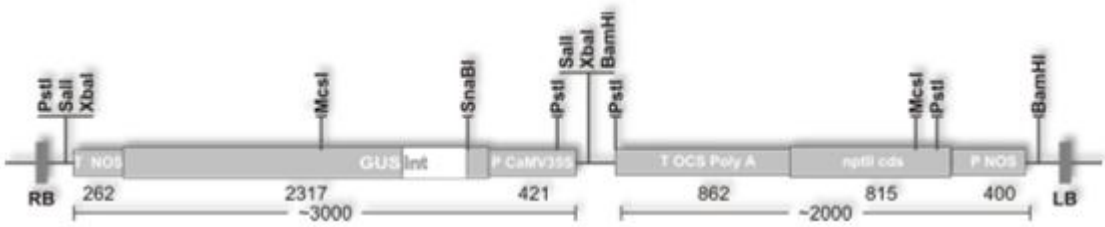

Figure 3.32: Current hypothetical restriction map for pTJK136 T-DNA
Current restriction map of T-DNA region for pTJK136 is given. Map was generated depending on patent and literature information gathered.

The known sequence of the vector backbone and presence of PMI selection cassette were the two causes that put pPMI-GFP vector among the candidate binary vectors. The pPMI-GFP vector is constructed on pPZP201 and the sizes of the vectors are 13573 and 7132 bps respectively. However emptying pPMI-GFP through EcoRI and HindIII restriction sites gave approximately 8500 bp vector backbone (gel data not shown; see figure 3.33). That is one of the restriction sites noted in the map is not actually present. The problematic restriction site should be the EcoRI site since we showed that intact PMI cassette can be recovered through HindIII digestion.

As a consequence since pPMI-GFP vector cannot be emptied this vector was also eliminated

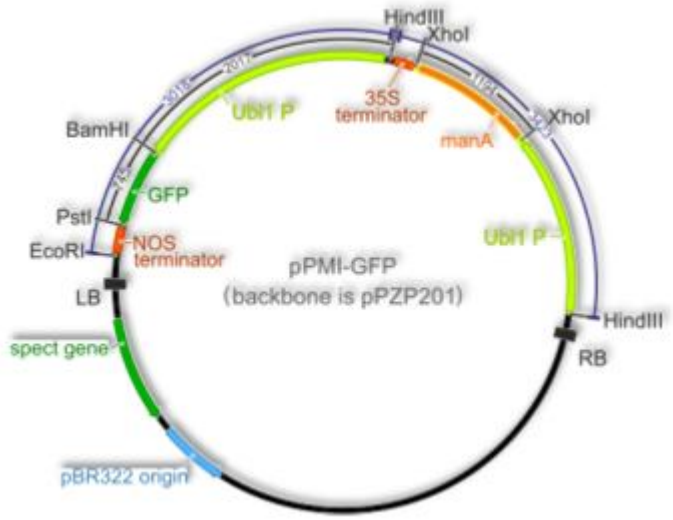

Figure 3.33: Simple graphical map of pPMI-GFP vector

3.1.4.2 Regeneration of pTHW for Cloning

Since it was not possible to figure out what will be the transformation efficiency as we may select one of the pPZP vectors as the binary vector for lentil transformation, it was plausible to prepare the pTJK136 backbone (pTHW) as the backup as the second binary vector.

In the original figure for pTJK136 the vector backbone plus the nptII cassette was annotated as pTHW136 (figure 3.34). So the vector backbone without any cassette integrated is annotated as pTHW in this thesis. Steps for emptying pTJK136 are as follows. First pTJK136 was SalI emptied and pBlueScriptSK+ (see figure 3.35 for simple view of pBlueScriptSK+ graphical map) was ligated to the emptied vector at SalI restriction site. The generated pTJK-nptII/pBlueScriptSK+ hybrid vector was selected for both spectinomycin and ampicillin resistance. Then these spectinomycin and ampicillin positive clones were further digested with BamHI restriction enzyme. At the end of BamHI digestion the right clone yielded approximately 7kb emptied pTHW vector backbone while reverse clone continued to have pBlueScriptSK+ with 10 kb approximate size. Right clone is then self-ligated to yield the empty pTHW vector. The process is schematised in figure 3.36.

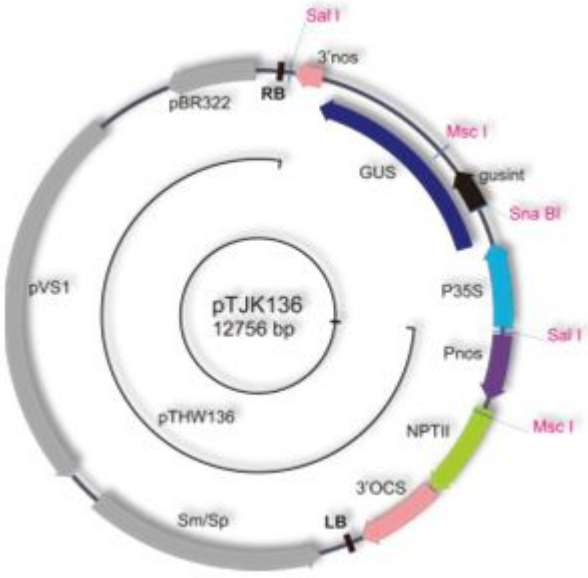

Figure 3.34: Regenerated graphical map of pTJK136

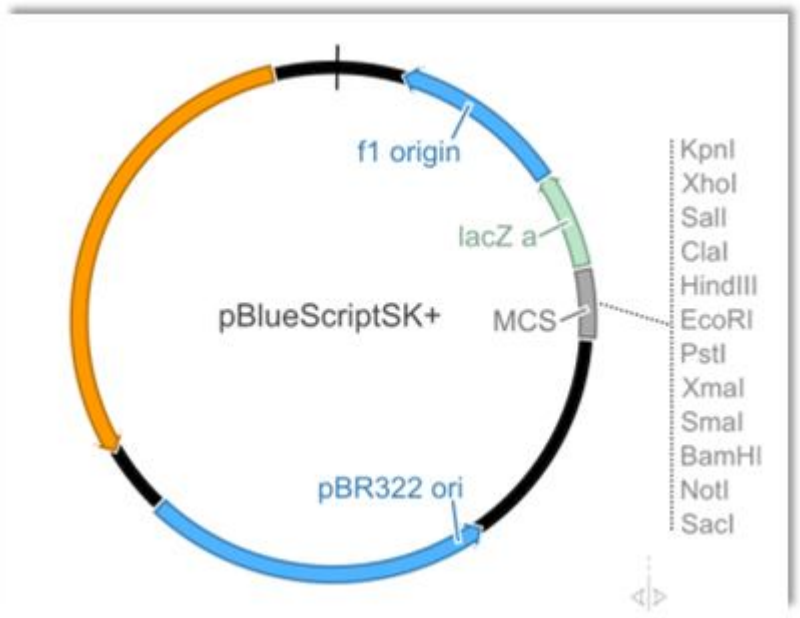

Figure 3.35: Graphical map of pBlueScriptSK+
Graphic map of pBlueScriptSK+. This vector is used as the intermediate vector throughout the cloning practices where necessary. Note that the multiple cloning site which starts with SacI and ends with KpnI is abbreviated as SK also denoted in the name of the vector (pBluescriptSK+). The same abbreviation is used in the following chapters to denote the direction of the insert cloned into the vector.

3.1.4.3 Alternative to pTHW; pPZP101

pPZP101 was chosen as an alternative of pTJK136 and pPMI-GFP. The three very important point about choosing pPZP101 was its availability its sequence data and similarity to pTJK136 backbone and its availability among the lab stocks. The pBR322 and pSV1replication origins and stability, mobility domains common in binary transformation vectors are also shown in pTJK136 and pPZP101 in figure 3.37.

3.1.4.3.1 Strategy of Generating pPZP101-MBF1c Transformation Vector

Both GUS-PMI construct in pBlueScriptSK+ and MBF1c-PMI construct in IV 1.1 were generated beforehand for blunt end ligation into pPZP101. Many prospective attempts for cloning PRbcS1MBF1cTRbcS1-PMI cassettes from IV 1.1 to pPZ101 were failed. In these attempts mainly blunt end ligation of 5867 bps PRbcS1MBF1cTRbcS1-PMI cassettes and 7027 bps pPZP101 were considered. The

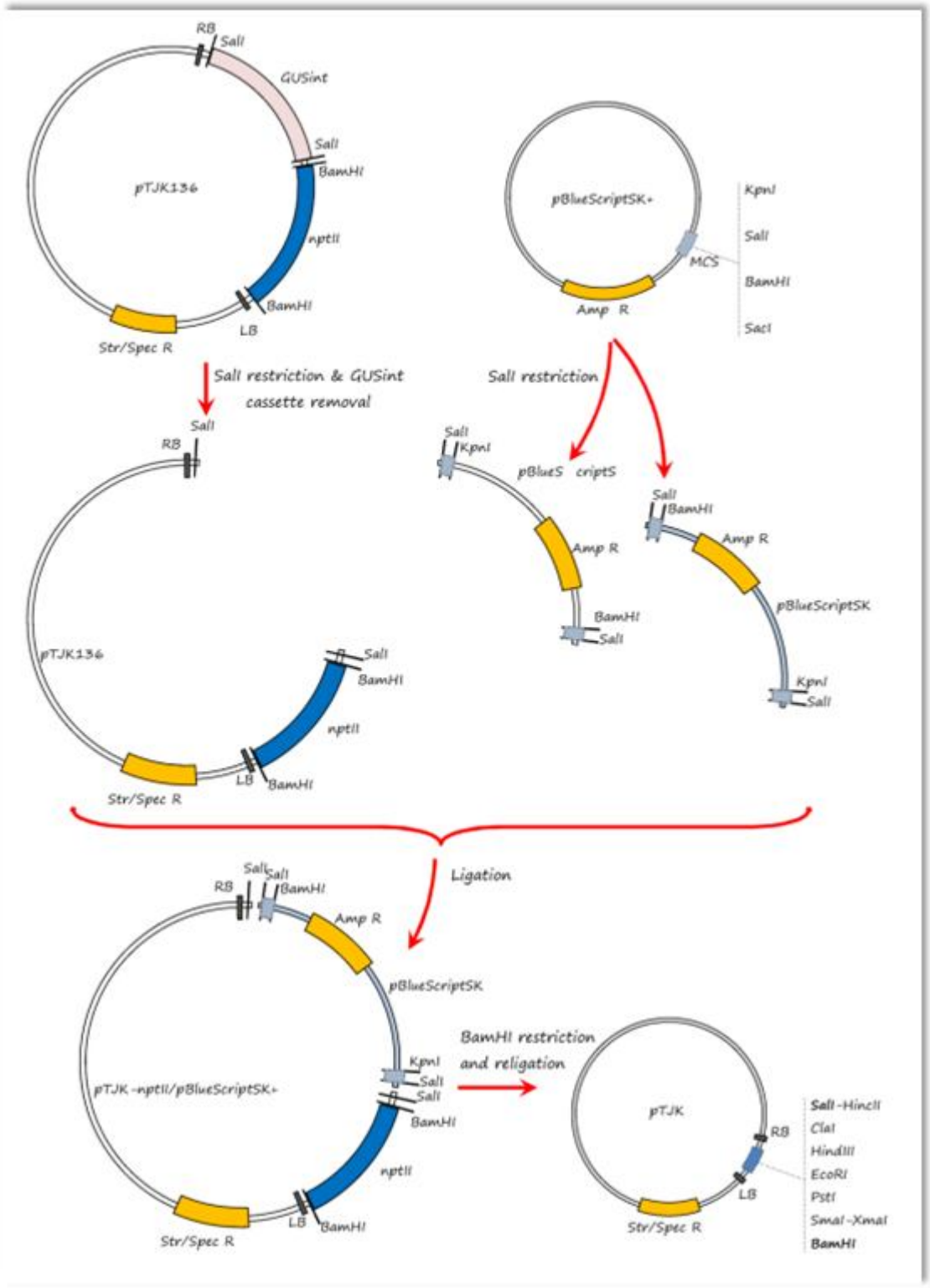

Figure 3.36: Regeneration of pTJK from pBlueScriptSK+ and pTJK136

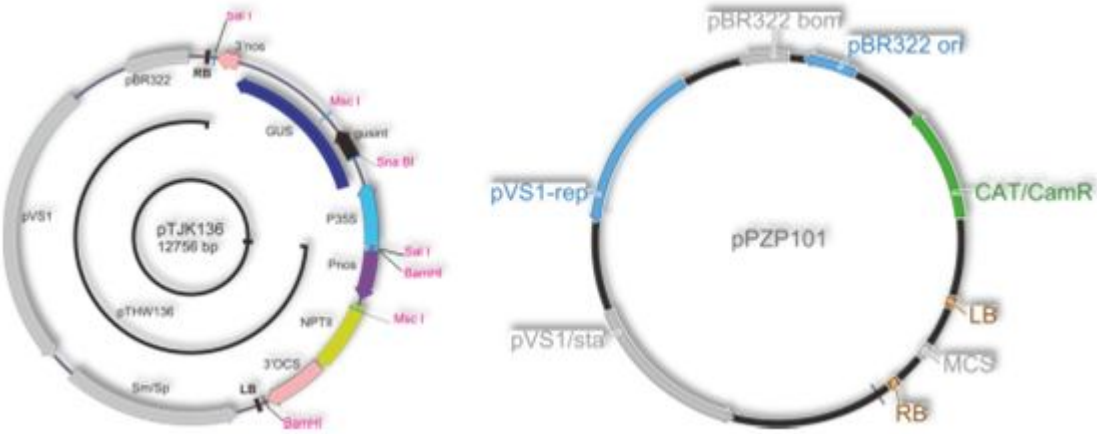

Figure 3.37: Comparison of pTJK136 and pPZP101 vectors
pTJK136 and its alternative pPZP101. Consider vector backbones. Both of the vectors contain pBR322 domains for replication and conjugation in *E. coli* strains. Also a pSV1 domain (for stability and replication in *Agrobacterium* species) is shared in these vectors.

causes for failure were addressed as to the large fragment sizes along with lover efficiency of blunt end ligation. From this point on sticky end ligation is preferred against blunt end ligation. For this purpose again the pBlueSK+ vector was used as the intermediate cloning vector for transfer of the PRbcS1 MBF1c TRbcS1 cassette into pPZP101.

3.1.4.3.1.1 *Cloning P MBF1c Cassette from IV 1.1 to pBlueScriptSK+

Transfer of RbcS1-MBF1c cassette from IV 1.1 to pBlueSK+ was resolved with HindIII-NarI and HindIII-ClaI restriction enzyme pairs respective. NarI and ClaI generate compatible ends. Restriction site positions for both of the vectors are given in figured 3.38.

Following sub cloning of RbcS1-MBF1c cassette into pBlueSK+ available restriction sites are shown in figure 3.39. Compatible restriction site search for final cloning of the cassette from IV 1.1 to pPZP101 revealed HindIII-XhoI sites and HindIII-SalI sites for pBlueSK+ RbcS1-MBF1c and pPZP101 vectors respectively. A major problem with this plan was fragmented recovery of the RbcS1-MBF1c cassette through HindIII-XhoI digestion. Consider the red highlighted XhoI restriction sites in figure 3.39.

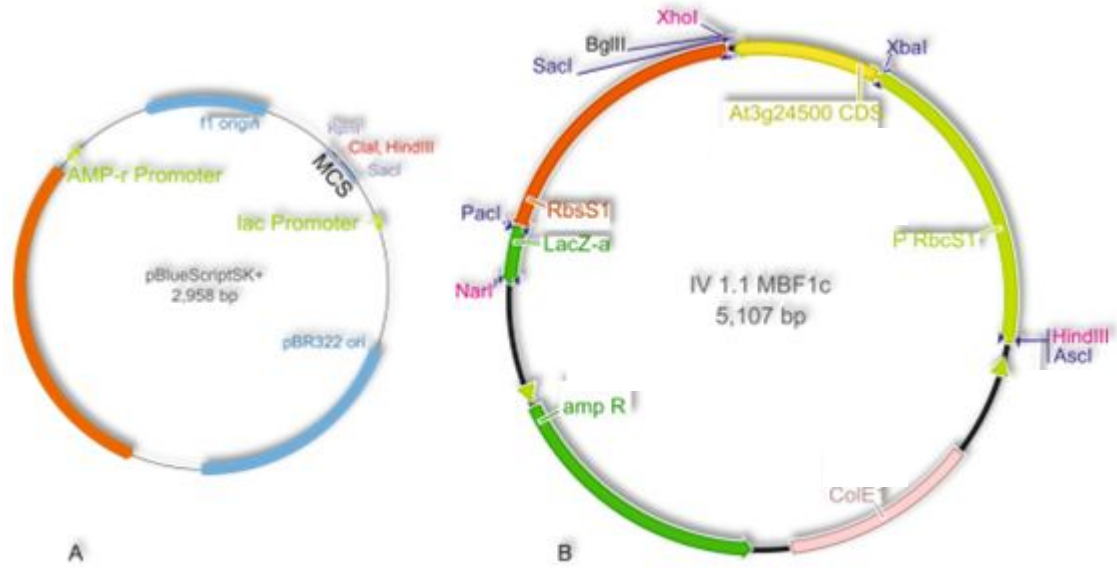

Figure 3.38: Compatible restriction sites of pBlueSK+ and IV 1.1-MBF1c
Compatible restriction sites of pBlueSK+ (A) and IV 1.1 RbcS1-MBF1c clone (B) are shown that are used for sub-cloning RbcS1-MBF1c cassette into pBlueSK+.

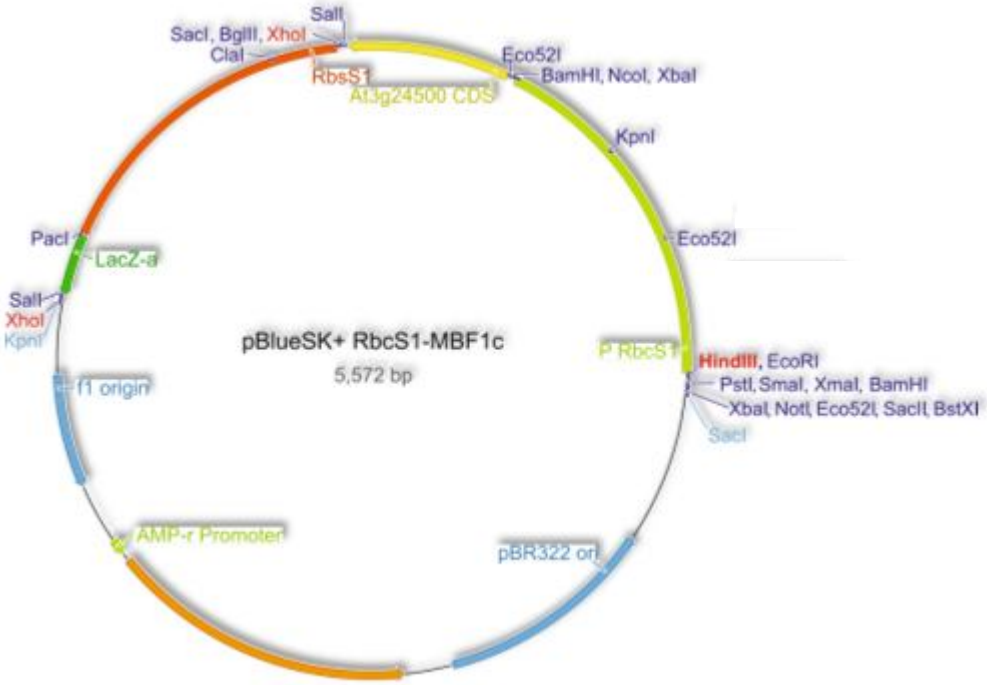

Figure 3.39: Available restriction sites of pBlueSK+RbcS1-MBF1c

3.1.4.3.1.2 Cloning P RbcS1-MBF1c-T RbcS1 from pBlueSK+ to pPZP101.

As it was noted in previous section HindIII and XhoI were the only available restriction sites for recovery of RbcS1-MBF1c cassette for sub cloning into pPZP101 through HindIII and SalI restriction sites. And also it was possible to recover RbcS1-MBF1c casstette in two fragments. Since XhoI and SalI were generating compatible ends that diminish both of the restriction sites upon ligation, the fragmented ligation of the RbcS1-MBF1c cassette into pPZP101 will possibly generate two different clones that are not interchangeable (figure 3.40). Whole cloning practices proceeded till pPZP101 RbcS1-MBF1c unfortunately gave one single colony that resulted to the false clone.

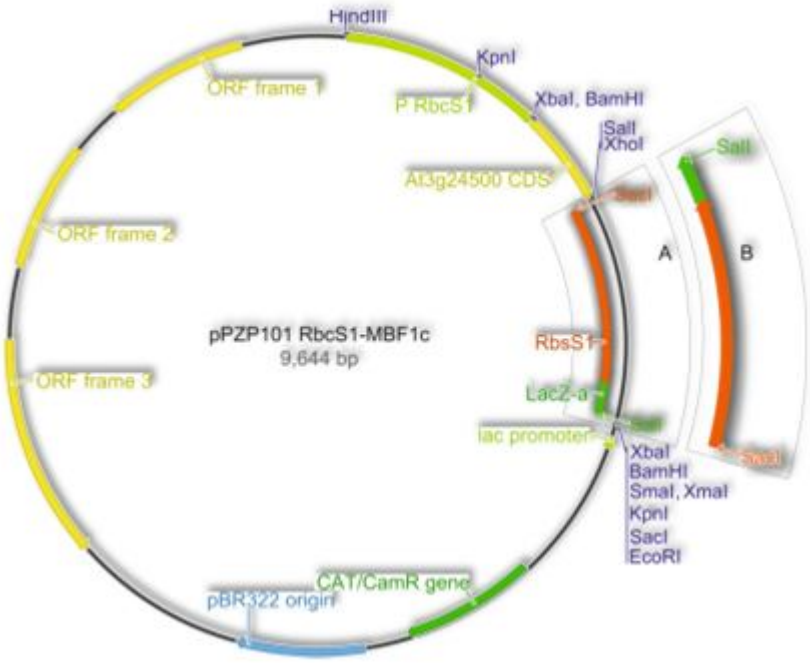

Figure 3.40: The graphical map of the probable pPZP101 RbcS1-MBF1c clones
The HindIII-XhoI digested RbcS1-MBF1c cassette from pBlueSK+ was recovered as two fragments. First fragment was comprised of HindIII-PRbcS1MBF1c-XhoI and the second fragment was comprised of XhoI-TRbcS1-XhoI. Ligation of these two fragments into pPZP101 through HindIII-SalI restriction sites say generate two different non- interchangeable clones. Fist clone it the true clone that has the terminator sequence in in right order (A). The second clone is the false clone that has the terminator sequence in wrong order (B).

3.1.4.3.2 Transformation Vector Based on P35S-MBF1c/ManA-TNOS Cassettes

All of the attempts for generating the ultimate binary transformation vector for lentil transformation were hindered due to some unexpected or unplanned cause. At least some of them were listed in above preceding sections. In order to overcome these hurdles and to find out an exit CaMV35SS promoter and NOS terminator enclosed cassettes for ManA and MBF1c coding sequences were devised in the last steps chronologically. Steps for generation of the two cassettes were given in sections 3.1.3 and 3.1.5.4 respectively. How these two cassettes are bound into pPZP101 is considered in this section.

3.1.4.3.2.1 The pPZP101 ManA-MBF1c Vector

In the section 3.1.3.1 it was mentioned about the restriction sites flanking the CaMV35SS-MBF1c-NOS and CaMV35SS-ManA-NOS cassettes for recovery and sub-cloning. Both of the cassettes were flanked with BamHI-XmaI-EcoRI and HindIII-XmaI restriction sites at 5′ and 3′ ends respectively (Figure 3.41). For cloning the cassettes into pPZP101 the EcoRI and HindIII restriction sites that are bordering the multiple cloning site of pPZP101 were targeted for cassette insertions (figure 3.42).

Figure 3.41: Flanking restriction sites of 35S-ManA/MBF1c-NOS cassettes
Restriction sites designed for sub cloning of CaMV35SS-ManA-TNOS (A) and CaMV35SS-MBF1c-TNOS (B) cassettes. Note the spatial organization of the restriction sites. The BamHI and EcoRI restriction sites at 5′ end of the cassettes are overlapping with an XmaI restriction site in between. That is the three restriction sites each with six bps recognition sequence are stacked into 15 bps length. On the other hand the two restriction sites at 3′ end of the cassettes are not overlapping.

As it is depicted in figure 3.42 ManA and MBF1c cassettes were recovered from their pBlueSK+ clones through XmaI-HindIII and EcoRI-XmaI restriction sites. Cassettes were gel separated and then co-eluted with the EcoRI-HindIII restricted pPZP101.

Ligation is then proceeded and whole ligation product is transformed into competent *E. coli* cells. Emerging colonies were analyzed for both of the cassettes through PCR and restriction digestion.

3.1.4.3.2.2 Preparation of ManA and MBF1c Cassettes and Ligation into pZPZP101

Prior to the restriction digests buffers were selected for maintaining highest enzymatic activities. Enzyme activities in their respective buffers chosen are given in appendix d, table D.29. Restriction-digest mixtures of pPZP101 pBlueSK+CaMV35SS-MBF1c-NOS and pBlueSK+CaMV35SS-ManA-NOS are given in appendix d, table D.30. Following restriction digestion both of the CaMV35SS ManA NOS and CaMV35SS MBF1c NOS cassettes were separated in gel electrophoresis. Both of the cassettes were recovered from gel with gel elution kit. And pPZP101 was cleaned with PCR cleaning kit. At final step all of the fragments were bound to the same column and eluted to 25µl total volume. Finally ligation was done with quick ligation kit at room temperature with 10 µl of elute 10 µl of 2X ligation buffer and 10 units (1 µl) of T4 DNA Ligase. Whole ligation mixture was transformed into competent *E. coli* cells and clone analysis was performed from the emerging colonies in the next day.

3.1.4.3.2.3 Analysis of Putative pZPZP101 MBF1c-ManA Clones

For analysis of the right pPZP101 ManA-MBF1c clones initially 20 colonies were checked with PCR. Amplification of both cassettes was targeted with CamV335S forward primer and NOS reverse primer pair (figure 3.43/ A). Since a very high percent of colonies gave positive signal only six of them were considered here. A double check was performed for these six clones through amplification of the ManA and MBF1c coding sequences only (figure 3.43/ B). Also the same six colonies also gave positive results for both ManA and MBF1c amplifications. Final check was performed through restriction digestion of the clones separately with HindIII and EcoRI enzymes (figure 3.44/ A and B) in order support the colony PCR results for whole cassette amplifications. According to the results we may say that all of the six clones were clean and had full, intact copies of 35S ManA NOS and 35S MBF1c NOS

cassettes. PCR reaction mixtures and PCR conditions were given in appendix d, tables D.31, D.32, D.33 and D.34. Restriction digestion mixes are given in appendix d, table D.35.

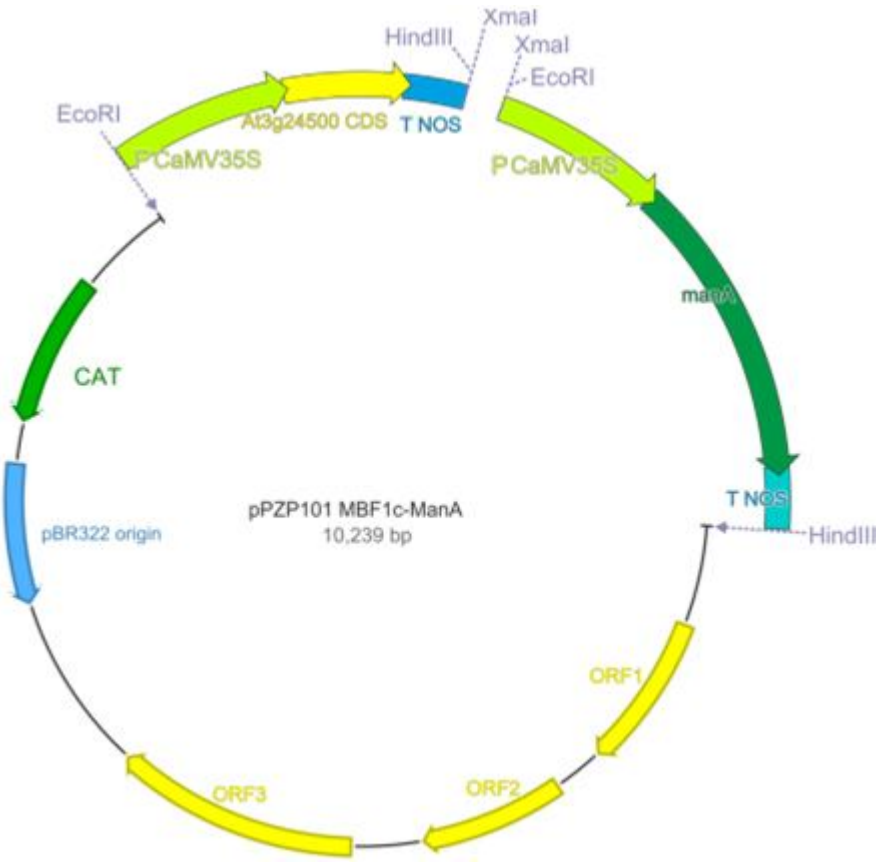

Figure 3.42: Graphical map of constructed pPZP101 MBF1c-ManA vector
Cloning of ManA and MBF1c cassettes into pPZP101 are shown in the figure. The T-DNA bordering restriction sites of pZPZ101 were used for cloning the cassettes where MBF1c cassette (At3g24500) was prepared with EcoRI-XmaI restriction sites and ManA cassette was prepared with XmaI-HindIII restriction sites. Ligation was straightforward.

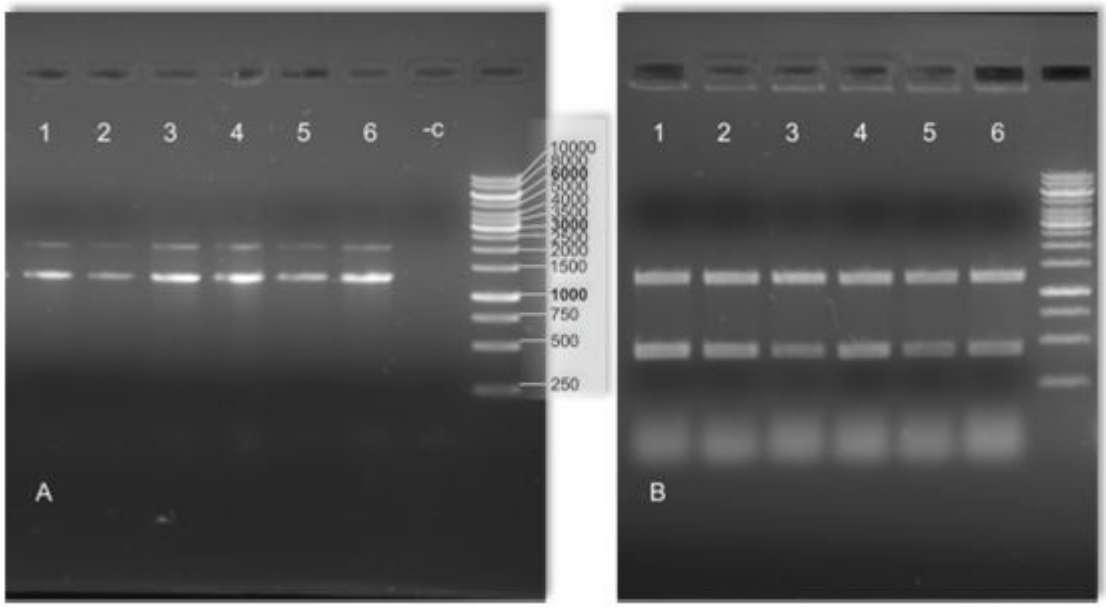

Figure 3.43: Cassette and cds PCR results for pPZP101 ManA-MBF1c clones
Gel images are showing colony PCR analysis of randomly selected 6 colonies for pPZP101 ManA-MBF1c clones: (A) CaMV35SS-ManA-NOS and CaMV35SS-MBF1c-NOS amplifications, 2010 and 1282 bps respectively; (B) ManA and MBF1c amplifications, 1176 and 447 bps respectively. "-c" is for negative control. Clones analyzed in A and B are the same.

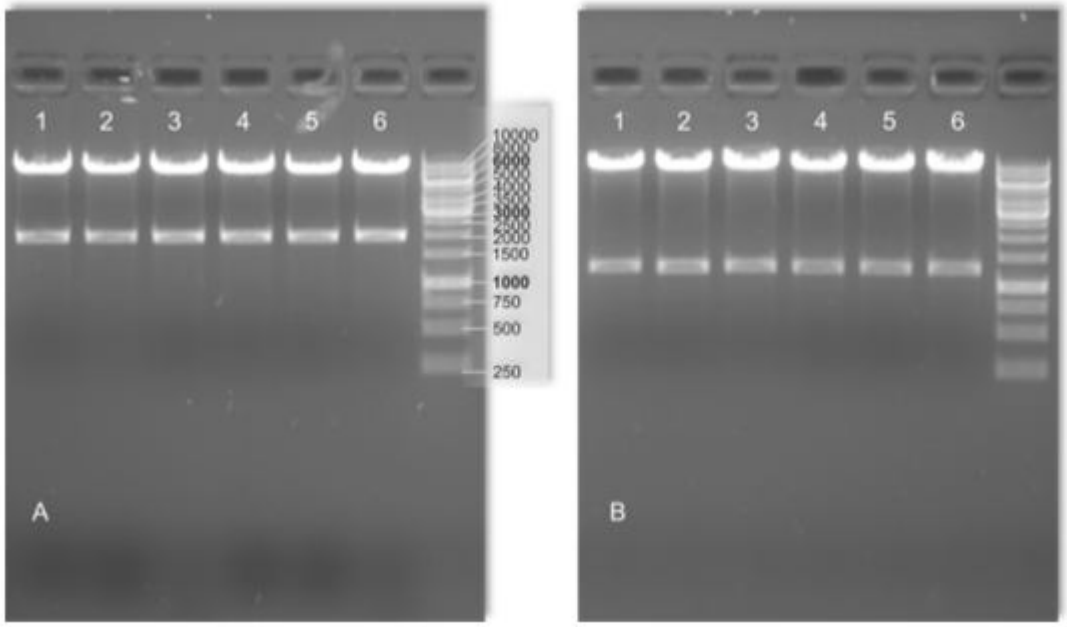

Figure 3.44: HindIII and EcoRI digests of pPZP101 MBF1c-ManA clones
Restriction digests of the colony PCR analyzed six clones for HindIII (A) and EcoRI (B) liberating ManA and MBF1c cassettes respectively. Compare the lover fragment sizes with the colony PCR results for CaMV35SS-ManA-NOS and CaMV35SS-MBF1c-NOS amplifications.

3.1.4.3.3 The pPZP101 ManA-GUSint-MBF1c Vector

Cause for integration of GUSint cassette into pPZP101 ManA-MBF1c vector was noted in section 3.4.6. In this section how the GUSint cassette was integrated into the transformation vector is explained.

The XmaI restriction site linearizes the pZP101 ManA-MBF1c in between the ManA and MBF1c cassettes without fragmenting the vector. Also the same restriction site is available to the 3′ end of the GUSint cassette in pBlueSK+ multiple cloning site (figure 3.45). For transfer of the GUSint cassette to pPZP101 ManA-MBF1c the whole pBlueSK+GUSint vector is integrated to the pPZP101 ManA-MBF1c through this XmaI site (figure 3.46). Then pBlueSK+ vector backbone was excised from the pPZP10 ManA-pBlueSKGUSint-MBF1c hybrid through BcuI(SpeI)-XhoI restriction. Finally the recovered pPZP101 ManA-GUSint-MBF1c vector was circularized through blunting and ligation (figure 3.47).

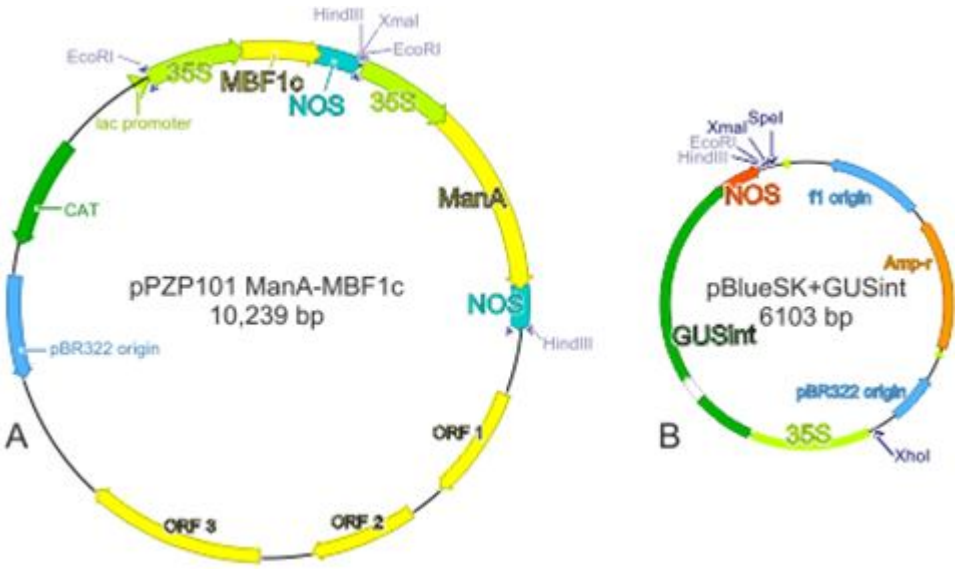

Figure 3.45: Restriction sites for sub-cloning GUSint into pPZP101 ManA-MBF1c
XmaI restriction sites for integration of pPZP101 ManA-MBF1c (A) and pBlueSK+GUSint (B) are shown. Two different clones were generated as a result of using single restriction site. With respect to pBlueSK+GUSint integration the pPZP101 ManA-pBlueSK+GUSint-MBF1c clones were named as forward and reverse clones. See figure 3.46 for details.

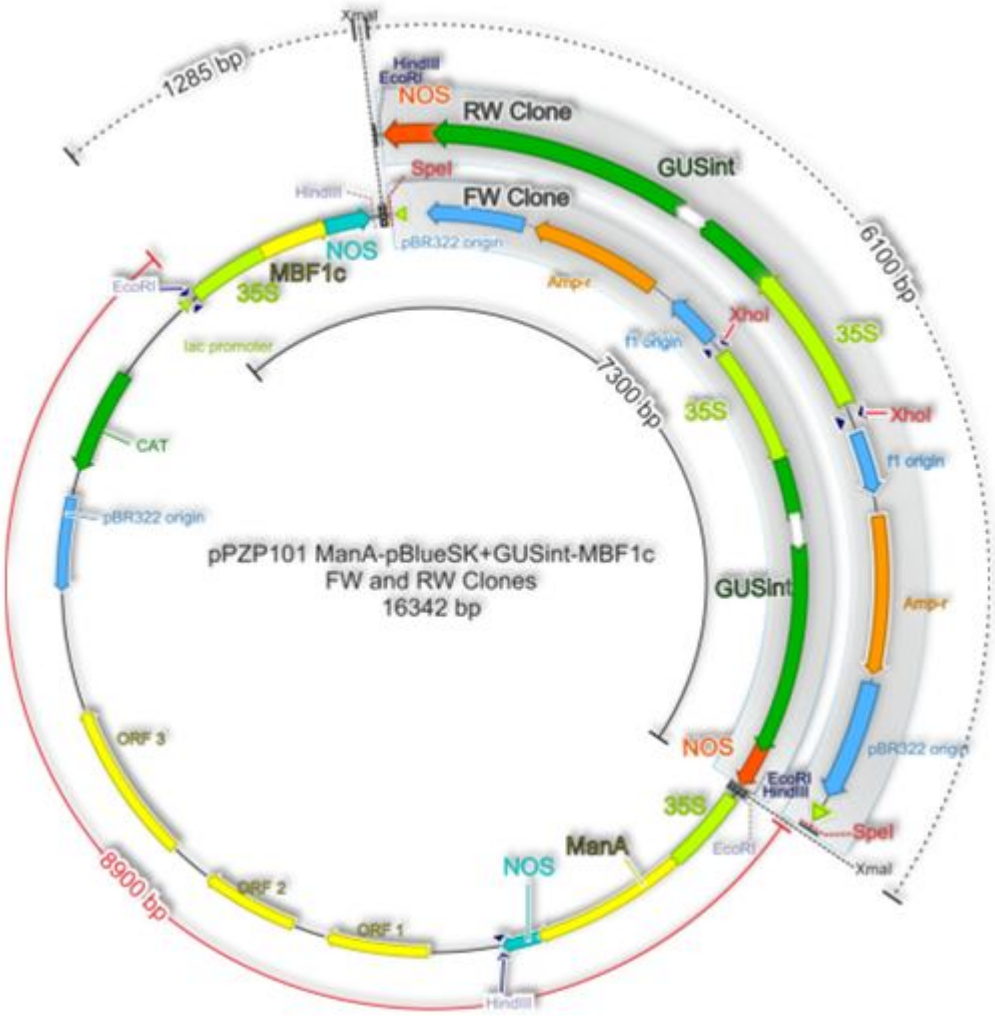

Figure 3.46: Graphical maps of pPZP101 ManA-pBlueSK+GUSint-MBF1c clones
The pPZP101 ManA-pBlueSK+GUSint-ManA hybrid vector is shown. Integration of the two vectors at single restriction site results in two different hybrids with respect to pBlueSK+GUSint vector orientation. The ligation products are named forward and reverse clones. Consider the red highlighted SpeI-XhoI restriction sites through which the pBlueSK+ backbone is excised from the hybrid. Also consider EcoRI fragments that are given with arcs and fragment lengths for analysis of insert direction. *İn-silico* EcoRI digest of the hybrid vector clones gave 7300 bp fragment for forward clone and 6100, 1285 bp fragments for reverse clones. Whereas 8900 bp backbone fragment is common to both of them.

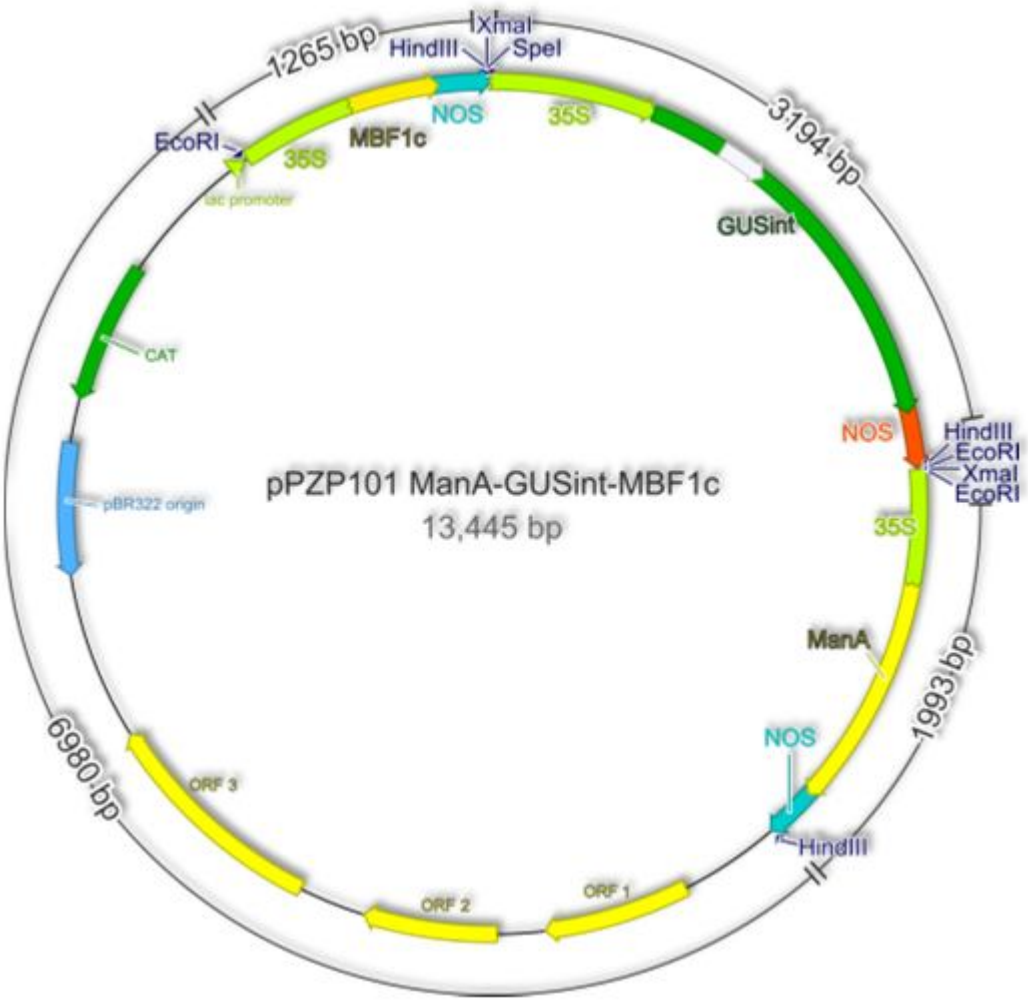

Figure 3.47: Graphical map of pPZP101 ManA-GUSint-MBF1c
The figure is showing the transformation vector generated through emptying the pBlueSK+ backbone from the pPZP101 ManA-pBLueSK+GUSint-MBF1c forward clone. All of the EcoRI, HindIII, XmaI, SpeI restriction sites are shown also. Consider the fragment lengths that are given with the arcs resulting from the EcoRI-HindIII digestion of the vector.

3.1.4.3.3.1 Integration of pBlueSK+GUSint and pPZP101 ManA-MBF1c Vectors

The initial steps undertaken for integration pBlueSK+GUSint and pPZP101 ManA-MBF1c vectors are first PCR confirmation of GUSint, MBF1c and ManA coding sequences (figure 3.48) and then confirmation of the prospected restriction sites (XmaI and SpeI-XhoI) on pPZP101 ManA-MBF1c and pBlueSK+GUSint vectors (figure 3.49).

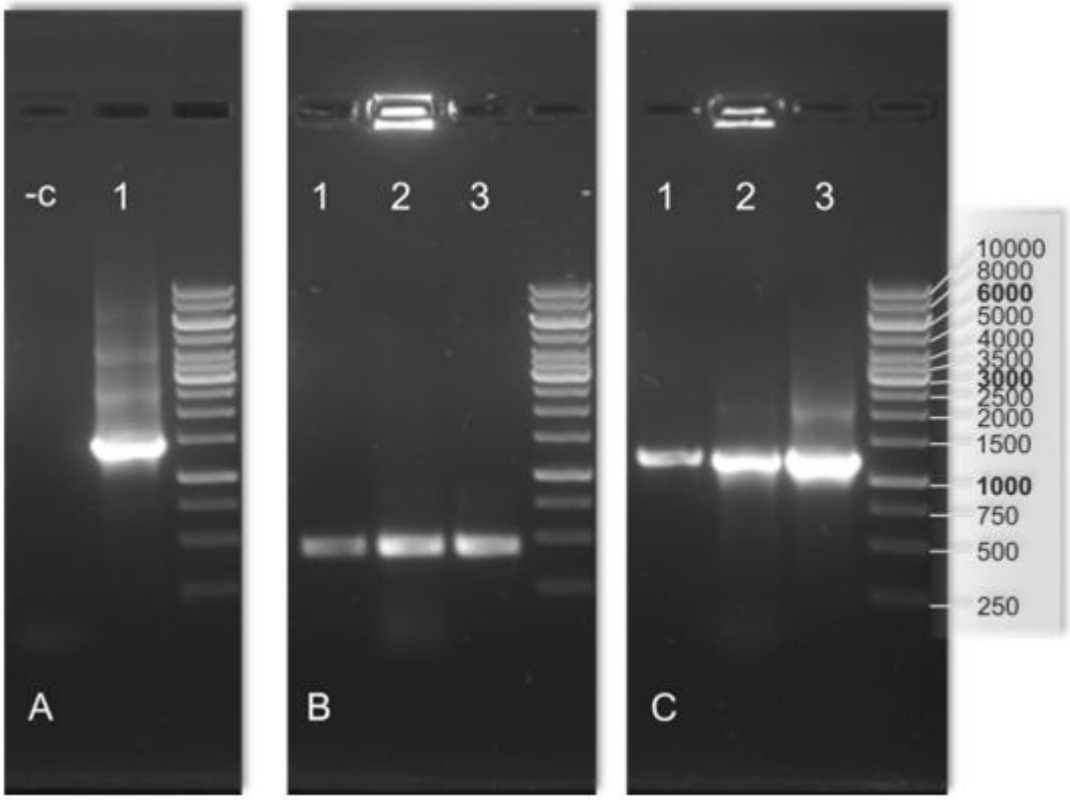

Figure 3.48: PCR confirmation of GUSint and MBF1c, ManA cassettes to be used
PCR analysis for GUSint seqeunce in pBlueSK+GUSint clone (A), MBF1c (B) and ManA (C) coding sequences in pPZP101 ManA-MBF1c. Single pBlueSK+GUSint-SK clone was used for PCR analysis and cloning (A). three pPZP101 ManA-MBF1c clones were used for both MBF1c (B) and ManA (C).

As long as the purity of the plasmid preps and prospected restriction sites were confirmed the integration of the pPZP101 ManA-MBfF1c and pBlueSK+GUSint vectors is undertaken. For this purpose both of the vectors were digested with XmaI and CIP was added to pZPZP101 ManA-MBF1c digest mix in order to minimize self circularization of pPZP. Both of the digest mixes were cleaned with PCR cleaning kit and co-eluted. Then fast ligation was carried out with this elution mix. Finally ligation product was transfromed into competent *E. coli* cells.

The PCR conditions, restriction digestion and ligation reactions are given in appendix d, tables D.36-D.40 respectively. Analysis of the pPZPManA-pBlueSK+GUSint-MBF1c hybrid vector is given in the next section.

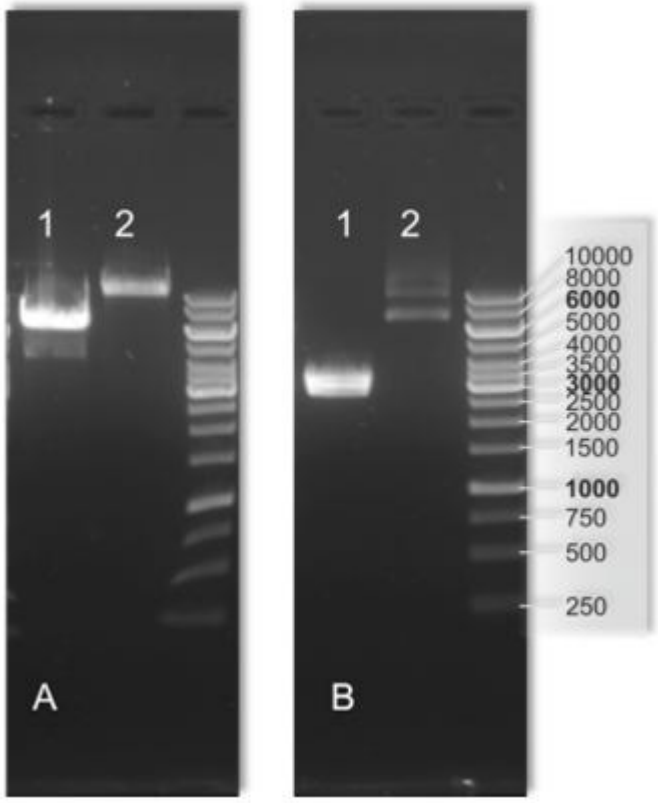

Figure 3.49: Restriciton of pPZP101 ManA-MBF1c and pBlueSK+GUSint vectors
Restriciton analysis of both pPZP101 ManA-MBF1c and pBlueSK+GUSint was done. The XmaI digestion of pBlueSK+GUSint (A-1) and pPZP101 ManA-MBF1c (A-2) was linearised both of the vectors yielding the expected 6 kbps and 10 kbps fragments respectively. Consider the incomplete digestion of pBlueSK+GUSint vector due to its higher concentration. The same vectors were digested with BcuI(SpeI)-XhoI (B). Gel image is showing that pBlueSK+GUSint vector is digested into 3.2 and 2.9 kbps GUSint cassette and pBueSK+ fragments respectively (B-1). It is also showing that pPZP101 ManA-MBF1c does not contain these two restriction sites.

3.1.4.3.3.2 Generating pPZP101 ManA-GUSint-MBF1c from the Hybrid Vector

Randomly selected six colonies for the putative pPZP101 ManA-pBlueSK+GUSint-MBF1c clones were first analysed on size basis. Fast clone detection of the hybrid vector was carried out with respect to pPZP101 ManA-MBF1c (figure 3.50/ A). Five of these colonies that gave arbitrarily larger fragments are further analyzed through EcoRI restriction digestion (see appendix d, table D.41 for restriction digest mix). EcoRI digests revealed three prospective clones with forward and reverse orientations (figure 3.50/ B). For confirmation of one of the reverse clones (clone number 2) and the forward (clone number 6) BcuI(SpeI)-XhoI double digestions were

carried out. This double digestion also removes the pBlueSK+ vector backbone and releases the pPZP101 ManA-GUSint-MBF1c fragment (figure 3.50/ C) which is then re-circularized.

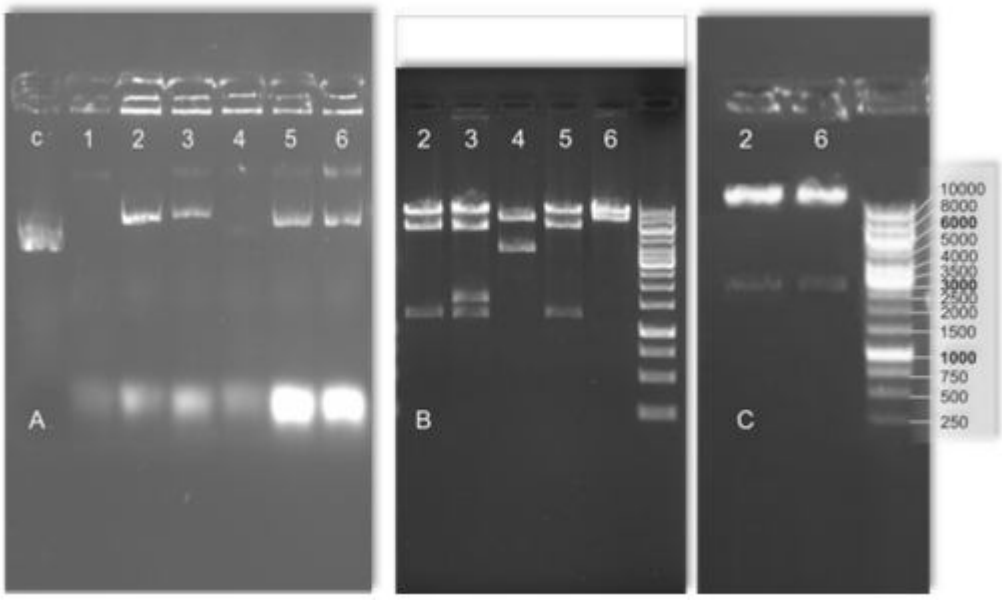

Figure 3.50: Analysis of pPZP10 ManA-pBlueSK+GUSint-MBF1c clones
(A) Size analysis of pPZP10 ManA-pBlueSK+GUSint-MBF1c clones from randomly selected six colonies. "c" stands for pPZP101 ManA-MBF1c plasmid as size control. Colonies numbered 2-6 showed plasmid bands with higher sizes with respect to the control. (B) Five clones selected for their sizes (2-6) are further digested with EcoRI in order to detect the direction of the pBlueSK+GUSint insert mentioned in figure 3.46. According to the *in-silico* analysis of the prospective hybrid vectors lanes numbered 2 and 5 are showing the expected reverse clones whereas lane 6 is showing the forward clone. (C) Clones showed in lanes 2 and 6 are further digested with BcuI-XhoI for release of the pBlueSK+ vector backbone. The upper bands that contain the pPZP101 ManA-GUSint-MBF1c fragments are re-circularized.

For re-circularization of the pPZP101 ManA-GUSint-MBF1c fragment, the BcuI-XhoI digested hybrid vector is first blunted with NEB blunting kit and then the blunted digest mix is separated through gel electrophoresis and gel extracted with Gel-Purification kit. Finally the pPZP101 ManA-GUSint-MBF1c fragment is re-circularized with fast ligation kit and transformed into competent *E. coli* cells. Refer to the appendix d, table D.42 for BcuI-XhoI restriction digest mix.

3.1.4.3.3.3 *Analysis of the pPZP101 ManA-GUSint-MBF1c

Analysis of the pPZP101 ManA-GUSint-MBF1c vector was performed through size analysis and restriction digestion only. Figure 3.51/ A is showing the putative clones that are resolved on size basis through fast clone detection method. The putative clones revealed through fast clone method were then double digested with EcoRI-HindIII for confirmation of intact release of pPZP101 backbone, GUSint, ManA and MBF1c cassettes (figure 3.51/ B).

Finally one of the pPZP101 ManA-GUSint-MBF1c clone is used for electro-transformation into C58C1 and KYRT1::pTJK136 strains.

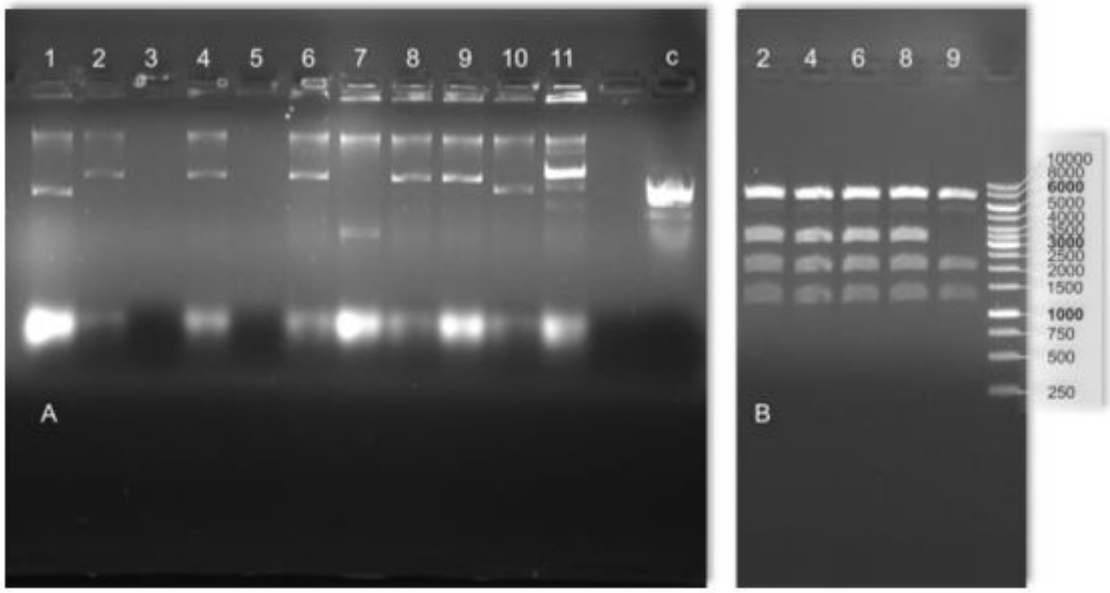

Figure 3.51: Detection and analysis of pPZP101 ManA-GUSint-MBF1c clones
(A) Fast clone detection for the circularized pPZP101 ManA-GUSint-MBF1c clones. Randomly selected 11 colonies were lysed and gel electrophoresed with respect toz pPZP101 ManA-MBF1c vector (c). (B) Five colonies with higher fragment sizes (2, 4, 6, 8 and 9) were further digested with EcoRI-HindIII for releasing the pPZP101 backbone, GUSint, ManA and MBF1c cassettes. The first four of the clones gave the expected cassette fragments. See figure 3.47 for EcoRI-HindIII fragment lengths.

3.1.4.4 pEarleyGATE Series for Gateway Cloning

As an alternative to pPZP101 ManA-MBF1c cassette cloning Gate-Way cloning technology was considered. Evaluation of Arabidopsis-Tair stocks revealed pEarleyGATE vector series as the most plausible vectors that can be used for

Agrobacterium mediated lentil transformation. Namely the pEarleyGate100 was considered for plant expression and pEarleyGate103 was considered for revealing localization with GFP-tag.

For utilizing the Invitrogen-Gateway technology the entry vector pCR8 was used which is also an Invitrogen brand commercial solution for generating entry clones from PCR fragments. pCR8 is served as linear fragment with TOPO clonase enzymes at its free ends. Any blunt ended DNA fragment can be prepared for TOPO cloning through Taq-DNA polymerase addition of single A to its 3' ends. In our case Herculase-II amplified MBF1c fragment prepared with Taq-DNA Polymerase and cloned into pCR8, generating pCR8-MBF1c entry vector. pCR8-MBF1c clones were first PCR analyzed and then sent to sequencing. Among the two clones sent to sequencing first turned out to be empty and the second was revealed as reverse entry of MBF1c. since pBlueSK+35S-ManA/MBF1c-NOS clones were already prepared concomitantly as an alternative plan Gateway cloning was ceased. But GFP tagging is still a plausible option of sub-cellular localization of MBF1c.

3.2 Agrobacterium Culture Works

Lentil transformation starts with *Agrobacterium tumefaciens* KYRT1::pTJK136 strain-binary plasmid couple that shows transient GUS gene expression in intact lentil leaves. Our laboratory reported stable transformation to lentil Sultan-1 cv. with this strain- binary plasmid couple (Akçay et. al. 2009). In this thesis work lentil transformation with functional gene MBF1c was planned also with KYRT1 strain (Torisky et. al. 1997) and pTJK136 binary plasmid (Kapila et. al. 1997) that was to be engineered for the MBF1c. Problems with pTJK136 and causes for elimination of this binary vector was given in section 3.1.6.3. Emptying the KYRT1::pTJK136 was another problem that should be resolved prior to transformation experiments. It was not possible to empty the KYRT1::pTJK136 strain-plasmid couple. From culturing at sub-optimal temperatures to topoisomerase inhibitors many experiments conducted have been proven unsuccessful for curing KYRT1. In depth evaluation of the problem also revealed an interesting report that pKYRT1 engineered from pTiChry5 contains still another border sequence (Palanichelvamet. al. 2000). Presence of another active T-DNA region is obviously problematic during functional gene transfer to plants. Inability in curing and presence of another T-DNA region in *vir* helper plasmid pKYRT1 were the two causes for setting KYRT1 in second queue in this thesis transformation works. C58C1 was shown to be the second potent strain after KYRT1 (Çelikkol 2002) in lentil transformation. So along with C58C1, KYRT1 was also prepared for lentil transformation studies.

The pPZP101 ManA-MBF1c and pPZP101 ManA-GUSint-MBF1c transformation vectors generated were electro-transformed to both C58C1 and KYRT1 strains. Culturing temperatures for C58C1 and KYRT1 strains were re-defined as 22 and 28^{o}C respectively (Baron et. al. 2001).

Growth bacteria in YEB-MES medium for plant transformation and harvesting the bacteria at specific growth phase (OD_{600}=0.8) was critical step for successful transformation experiment. For this purpose growth rate calculations were made during culturing. Sharp up and down shifts at growth rates calculated, raised

suspects about the culture conditions. This poly-cryptic growth was addressed as signs for metabolic shift of bacteria for different nutrients upon depletion of one. The main nutrient source of the YEB-MES media is LB. And LB was noted to be non-standard from batch to batch. Still another problem about the LB media was reported to be in sufficient and nonstandard carbon source, which is the cause of poly-cryptic growth (Nikaido 2009). So we eliminate using YEB media and the most suitable alternative of YEB was found to be MG/L media (Arlene et al. 2006). Analysis of bacterial growth in MG/L media showed that the poly-cryptic growth was eliminated.

3.2.1 Curing KYRT1 for Elimination of pTJK136

Curing can be simply explained as plasmid elimination from the bacterial cell. Approaches for curing can be listed under two headings. First is direct loss of plasmid through physical processes and chemicals applied, second is interference with the replication of genetic material in special the plasmid. Permeabilization of the bacterial membrane and cell wall with SDS or menthol, loss of membrane integrity and cellular components through spheroplasting or electroporating are among the first methods that aims direct loss of the plasmid. Dealing with DNA topologyDNA topoisomerase inhibition or introducing negative supercoiling with DNA interchelating dyes are among the second

KYRT1::pTJK136 curing practices undertaken in this thesis study are listed below.

3.2.2 Culturing at sub-optimal temperatures

Both serial passage and culturing at sub optimal temperatures were studied for curing KYRT1. Only rifampicin (innate resistance of *Agrobacterium* species) and carbenicilin (resistance of pKYRT1; engineered pTi, *vir* helper) resistance of bacteria were utilized during sub culturing period. Streptomycin or spectinomycin resistance was used when pTJK136 screening was performed. For sub culturing 50 µl of overnight batch cultures of KYRT1::pTJK136 in liquid LB with rifampicin and carbenicilin were sub cultured into 10 ml fresh media within 50 ml falcon tubes both

at 28 and 37°C. Starting from the third sub-culture 100 µl of diluted (10^{-5}X) bacterial suspension was spreaded to LB agar with rifampicin and carbenicilin. Two days incubation at 28oC was followed by replica plating selected colonies on separate LB agar plates with rifampicin, carbenicilin or rifampicin, carbenicilin and stpretomycin media. For recovery of any cured KYRT1 colony up to ten sub cultures were successively performed, but it only maintained phenotypic elimination of streptomycin resistance that reverted in subsequent days.

3.2.3 Topoisomerase inhibitors

Idea of using DNA gyrase inhibitors in special topoisomerase II inhibitors is for maintaining interference with topology of the plasmid DNA and hence its' replication, partitioning during growth. "Siprofloksasin" is a synthetic floroquinolon derivative that was utilized as topoisomerase II inhibitor in this study. Its' commercial formulation named CIPRO® 500 was supplied from a local pharmaceutics. The minimal inhibitory concentration for most of the microorgansims was given as 0.005-2.0 µg/ml. A 500mg tablet was dissolved in 20 ml sterile distilled H_2O with the aid of a few drops of glacial hydrochloric acid. The stock of 25mg/ml CIPRO (siprofloksasin) was filter sterilized and kept at room temperature in dark for use in curing experiments.

For curing experiments a 10 mg/ml of LB CIPRO stock with rifampicin and carbenicilin was prepared and stored at 4°C. In order to test the minimal inhibitory dosage for KYRT1 two independent tests were made. In the first test 0.2, 0.1, 0.05, 0.025 mg/ml CIPRO concentrations were analyzed with respect to no CIPRO. In the second test 0.025, 0.015, 0.010, 0.005 mg/ml CIPRO concentrations were analyzed with respect to no CIPRO. At the end of overnight culturing due to inconsistency in bacterial growth CIPRO aided curing was omitted from the study.

3.2.4 SDS and Electrocuring

SDS is noted to be efficient curing agent in many works and still many others find it useless. Idea behind using SDS as curing agent was to interfere with the plasmid-

plasma membrane anchoring determinants. The curing experiments with SDS were performed at three different temperatures namely 17, 28 and 37°C and for an SDS concentration range of 0.1-8.4%.

Only three experiments were conducted with electroporator for electrocuring. Reports concerning the electrocuring of *E. coli* and single step Ti plasmid transfer from *Agrobacterium* to *E. coli* through electroporation were the two studies for commencing electrocuring KYRT1.

Replica plates for both of the SDS and electrocuring studies also failed in recovery of cured KYRT1.

3.2.5 Chelating Divalent Cations with EDTA

EDTA is known to be the divalent cation chelator with different affinities to Ca, Mg and other divalent cations. Its use as curing agent is thought to be indirect through Mg ion which is critical in DNA replication.

The outweighing nutrient source of LB is yeast extract. And the concentration of Mg ions in yeast extract was found to be around 0.001M (Grant 1962). For curing experiments EDTA was tested in the range of 4 to 0.0625 mM. It has inhibited the bacterial growth at 4 mM, showed in consistent growth rates at 2, 1 and 0.5 mM and had approximately no effect at 0.25 mM concentrations.

3.2.6 Combination of Curing Strategies

The problems that lead to failure in curing *Agrobacterium* should be addressed to the curing agents and strategies suitable for other bacteria are applied to *Agrobacterium* with minimal modifications. It is obvious that special techniques should be developed for curing KYRT1 as the gram negative soil bacteria. Differences of *Agrobacterium* with respect to *E. coli* that should be considered when developing curing protocols can be stated as follows. *Agrobacterium* cell densities are higher with respect to that of *E. coli* for a certain optical density measurement.

Also *Agrobacterium* cells have thicker cell wall then *E. coli* cells. Also mobility and stability regions of binary transformation vector from pSV1 should be taken into account along with the transformation vector sizes.

Considering all of these information and experience a probable curing method can be outlined as follows. Continuous sub-culturing *Agrobacterium* in a defined media with Ca and Mg chelator (EDTA) should maintain the cell density at a critical state that inhibits conjugal transfer of plasmids. With this way Mg should be maintained as a limiting factor of DNA replication and also Ca should be delimited for loosening bacterial cell wall material. And also presence of SDS and or menthol should be considered for attacking membrane integrity. Final event of curing should be considered as heat shock applied. Heat shocking for a brief period of time around 60-70^{o}C may result in loss of cell wall and membrane patches along with the plasmids. Heat shock might also be replaced with electroporation. The most critical step might be pre-conditioning the cured bacteria in SOC or other recovery media prior to spreading. All of the steps considered can be handled in a separate optimization work that might be postponed to a proper time period.

3.3 Binary Vector Transformation to C58C1 and KYRT1

Preparation of C58C1 and KYRT1 cells for electroporation was done according to "Cold Spring Harb. Protoc. (2006) pdb.prot4665". Transformation of pPZP101 ManA-MBF1c and pPZP101 ManA-GUSint-MBF1c into C58C1 and KYRT1::pTJK136 were done with BioRad Micro Pulser Electroporator in 0.1 cm electroporation cuvettes. Following bacterial growth and subculture *Agrobacterium* cells electroporation of plasmids were confirmed through colony PCR Analysis.

3.4 Plant Transformation Works

A schematic diagram is given below (figure 3.52) for showing the main steps of lentil transformation in this thesis work. The target tissue for transformation is cotyledonary nodal meristem of 4 days old seedlings. The initial step is surface sterilization of the seeds. Then germination is maintained in dark on water agar.

Next cotyledonary nodal segment is isolated from these 4 days old seedling. Along with the injury, explant isolation is the most labor intensive and hence the least standard step of the transformation works. In a standard cotyledonary nodal segment isolation practice cotyledonary nodal meristems are engraved with cotyledonary petioles. In order to injure meristem zones special glass needles were devised and injury practices were made under dissecting microscope which already increased the labor load. In order to leap over this labor load and standardize the explant isolation, injury steps an optimization work was committed. Aim was isolation of explant during which the process also injures the meristematic zones. Following each explant isolation practice meristematic zones was stained and highlighted red with TCC. Improper and nonstandard sorting of the meristems revealed with TCC staining in either section of the prepared explant leaded us to conventional explant isolation method and then injury in a separate process. However at least in order to simplify the injury process cotyledonary petioles removed completely from the embryo axis and meristems left. In this case the labor load was doubled since the explant isolation and injury were to be made under dissection microscope.

Along with the explant preparation *Agrobacterium* preparation was also modified. Raise of new transgenic plants and accumulation of new procedures with enhanced gene transfer rates lead us to modify our protocols accordingly. For this purpose we have modified our inoculation and co-cultivation media formulation along with the germination process and transformation timeline. Also as the emerging selection agent in plant transformation, mannose selection system was worked out prior to transformation studies.

Plantlet regeneration from the putative transgenic shoots as the last step of the study is still carried out through micro grafting. The problematic step of the grafting also remains to be the stem girth of the root stock. The girth of the root stock ranges approximately between 0.8-1.3 mm. And insertion of the putative transgenic

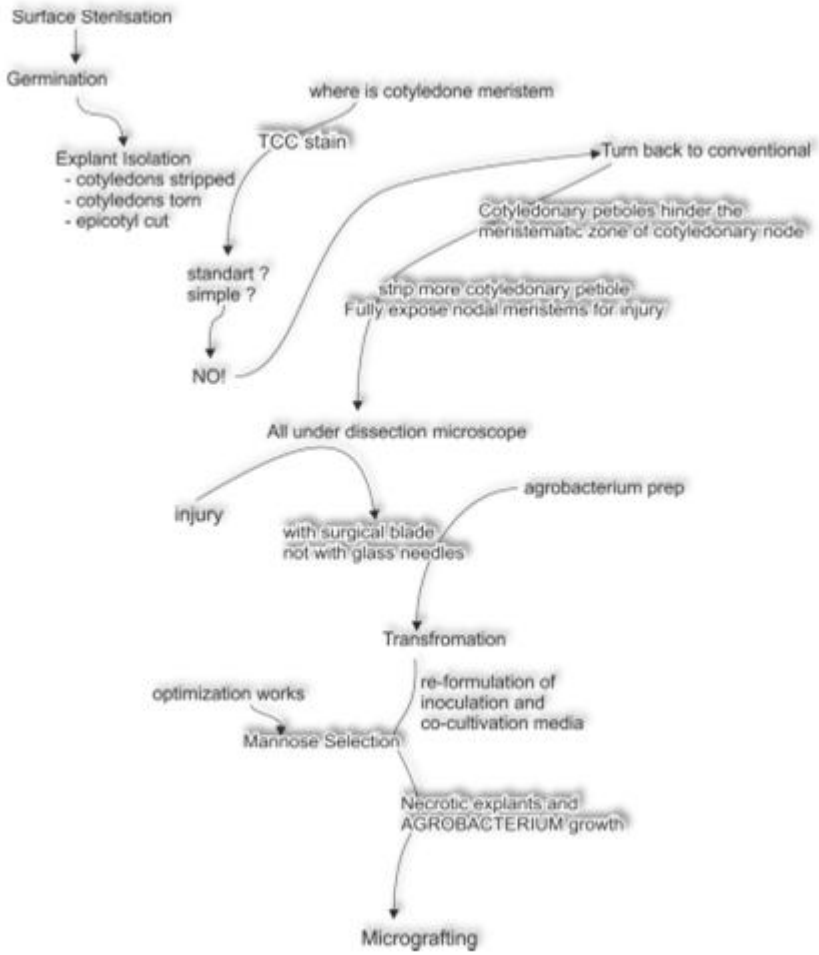

Figure 3.52: Diagram of lentil transformation steps for this thesis works

scion in to the root stock stem requires practice and expertise. Although the grafting is done under dissection microscope, still not every graft could be recovered as a whole plant. In order to overcome the stem girth problem many attempts of stem girth exaggeration were done. But yield expected was at least 50% enhancement and it was not reached in any.

3.4.1 Modification in Lentil Seed Germination Media

Generally seed germinations are maintained in full strength plant growth media like MS salts, Vitamins and sucrose. Utilizing a full media during three to four days of germination is like wasting the sources since germination requires water, oxygen and support material only. In order to prove this statement lentil seeds are

germinated on water agar (M_0) and full strength MS media (M_1; MS nutrients with vitamins, sucrose and agar). At the end of three days dark germinated lentils were scored for rate of germination, seedling weights and hypocotyl lengths. As it is evident from the collected data and visual observations (figure 3.53), full complement media (M_1) for 3 days germination does not result in superior seedlings compared to water-agar (M_0) germinated seedling. Furthermore there is a slight insignificant depression of germination parameters in M_1 group with respect to M_0.

Depending on these preliminary findings, germination steps are going to be carried out in M_0 media (water-agar) for subsequent experiments.

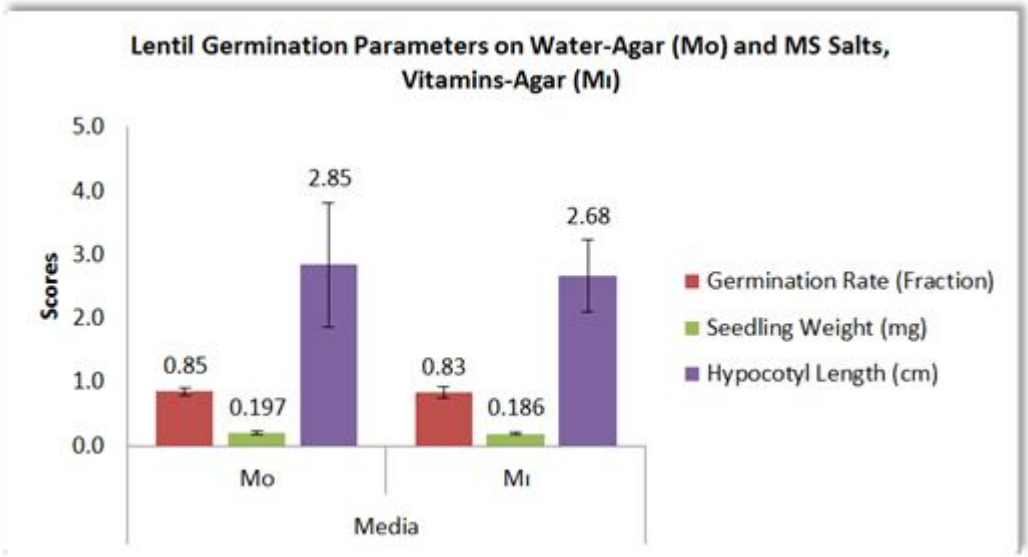

Figure 3.53: Germination scores of Sultan 1 on Water Agar and MS media
Graphic interpretation of germination scores given in table E.1, appendix e.

3.4.2 Explant Preparation and Transformation

General considerations on methods of explant preparation were given in materials and methods section. It can be stated here that explant isolation from the cotyledonary nodal segment is the most labor intensive and hence least standard step of transformation. Conventionally explant isolation and injury steps are separated. Binding them into one step may depress the labor load to tolerable limits

while a standard can be exercised in expense. The requirement for a standard arises mainly from the physiologic effect of injury and bacterial inoculation on explant. Extensive injury applied during explant isolation and wounding made on meristems plus bacterial inoculation results in necrosis of a proper fraction of explants, especially the tissues targeted to transformation. And necrotic explant number is even multiplied as more concentrated *Agrobacterium* suspension is used for explant inoculation. To minimize these necrotic tissues explant isolation and wounding should be limited within the boundaries of a standard.

Conventionally, explant injuries were performed under dissection microscope. Injury practices with un-aided eye may also speed up the process but the quality of the prepared explant may drop. Major obstacle for explant preparation without microscope is the indiscernible millimeter scale tissue and inadequate resolution. Activity staining of the regenerating meristematic tissues sounded plausible gateway to overcome this obstacle. For this purpose explants were stained with 3-phenyl, tetrazolium chloride (TCC). Preliminary staining gave inadequate resolution to un-aided eye but it served quite good resolution under dissecting microscope. Comparison of its toxicity and resolution maintained, staining the explant with TCC was omitted.

In order to couple explant isolation and injury steps two different explant isolation methods were generated and tested along with the conventional cotyledonary nodal segment isolation. For diagnosis of the devised explant isolation methods injured and dead zones and the active meristematic zones of explants were differentially stained with TCC and cresol blue. Evaluation of the results leaded us to conclude using conventional explant isolation method with minor modifications and requirement of dissection microscope during both explant isolation and injury practices.

3.4.2.1 Activity Staining of the Seedlings and Cotyledonary Nodes

For initial analysis, germinated plantlets were incubated with different concentrations of TTC (tri phenyl tetrazolium chloride) solutions at room temperature. Thirty minutes of staining resulted in faint to dark pink colored cotyledons (figure 3.54). Among them explants stained at 0.05 and 0.1 mg/l concentrations were excised to reveal the staining profile of the cotyledonary nodal meristems (figure 3.55).

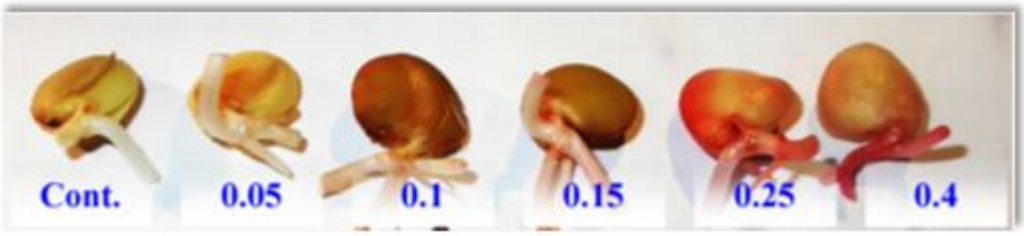

Figure 3.54: TCC staining profiles of lentil cotyledonary segments
TTC stained explants. The TCC concentrations (mg/l) of the explant incubation solutions are given with the explants in the figure. Notice the TCC stained explants coloring from faint to dark-pink.

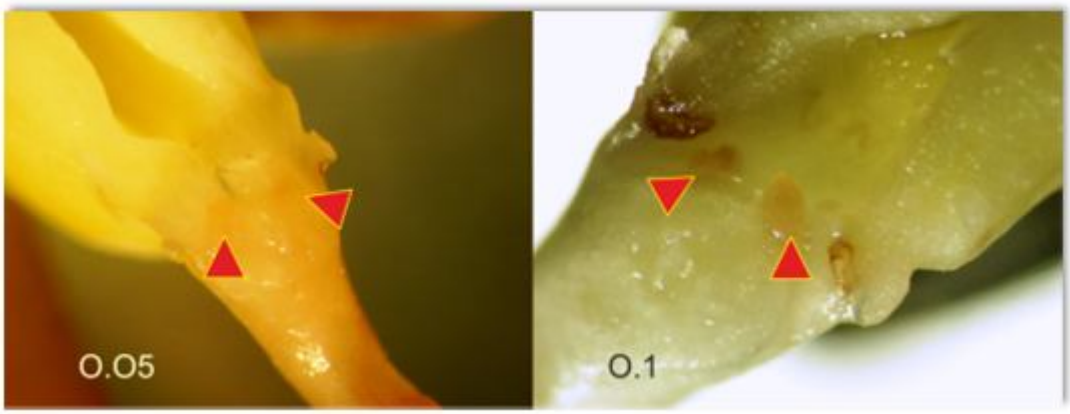

Figure 3.55: TCC stained active axial meristematic sections of Sultan 1
TCC staining at cotyledonary nodal meristems are shown for 0.05 and 0.1 mg/l concentrations of TCC. Notice the red arrow heads that are directed towards the active meristematic zones stained faint pink.

Although the TTC staining may help in detection of the meristematic zones with un-aided eye during injury applications insertion of the glassware needle right into the stained zone requires more resolution, meaning that a dissecting microscope may serve great for our purpose in wounding. That is TTC staining makes our target tissue more discernible but injury practice right into the correct meristematic zones require more resolution.

3.4.2.2 Evaluation of Lentil Explant Preparation Practices

Explant isolation methods are covered in materials and methods section 2.2.3.6. Following explant isolations with the three different methods for revealing the viable and dead tissues explants were TCC stained and incubated in cresol blue solution. Viable and dead sections were discriminated with red and dark blue stains respectively. Staining profiles of for different explant isolation procedures are given in figures 3.56, 3.57, 3.58, for conventional, epicotyl excised, cotyledons stripped methods for explant isolation respectively.

3.4.2.3 Regeneration Potentials of Explants Isolated with Different Methods

Cotyledonary nodal segments isolated with three different methods namely, conventional, epicotyl excised and cotyledons stripped methods were explained above. Explants recovered with three different isolation styles are further wounded at their meristematic zones with fine glass needles. In order to reveal the regenerative potential of these meristems, wounded explants were cultured three days in 1 mg/l BA in M_1 media. Explants that maintain and further raise their shoots were scored after the three day culture period. According to the results, it can be stated that meristematic zones can raise their shoots weather explants are extensively injured during explant preparation or not. Stated in other words once shoot is initiated at the meristem base, dead of surrounding embryonic tissues and

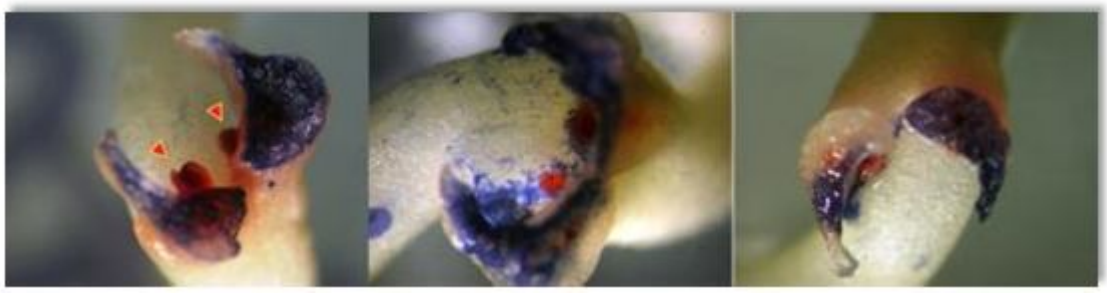

Figure 3.56: Viability stainings of explants isolated with conventional method
Explants prepared through standard isolation method. The dark blue (cresol blue) stained sections are dead tissues mainly composed of cotyledonary petioles. Red stained sections noted with arrow heads are the shoot initial from the meristems. Consider the accessibility of the meristem zones for wounding.

Figure 3.57: Viability stainings of explants isolated with epicotyl excised method
Explants prepared through epicotyl excised method. See the detailed steps of explant isolation in figure 2.1. Consider the dead tissues stained with cresol blue sequestering the active meristematic zones stained with TCC (arrow heads in pictures b and c). During explant preparation through this method the proper place of second incision (figure 2.1/ 2b) could not be located on both of the meristems with reputation. This observation is also confirmed with the TCC stained meristematic zones scattered among the pictures a-c.

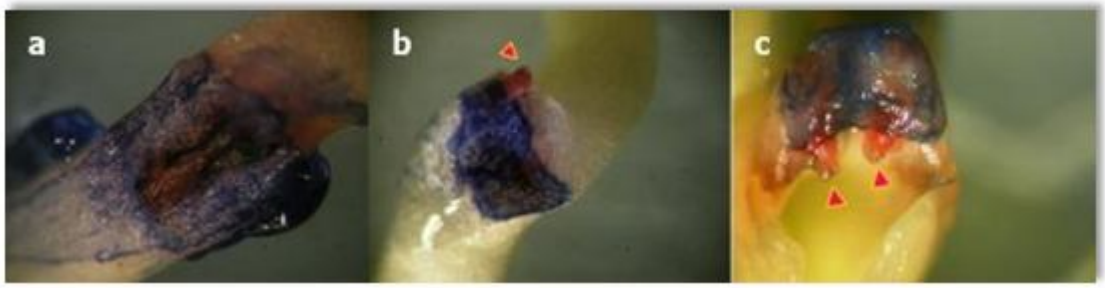

Figure 3.58: Viability stainings of explants isolated with cotyledons stripped method
Explants prepared through cotyledons torn method. Consider the intensity of cresol blue staining on the embryo axis in pictures (a) and (b). Intensity of dead tissues is highest with this explant isolation method. Departing of the shoot meristems is rather random. Both of the meristems may be stripped with the cotyledons (c) or at least one of the meristem may be left on the embryo axis (b).

proper wounding to the shoot initials does not depress shoot raise (appendix e, table E.2 and figure 3.59). But the physiological response of the prepared explant upon *Agrobacterium* inoculation remains to be a question mark.

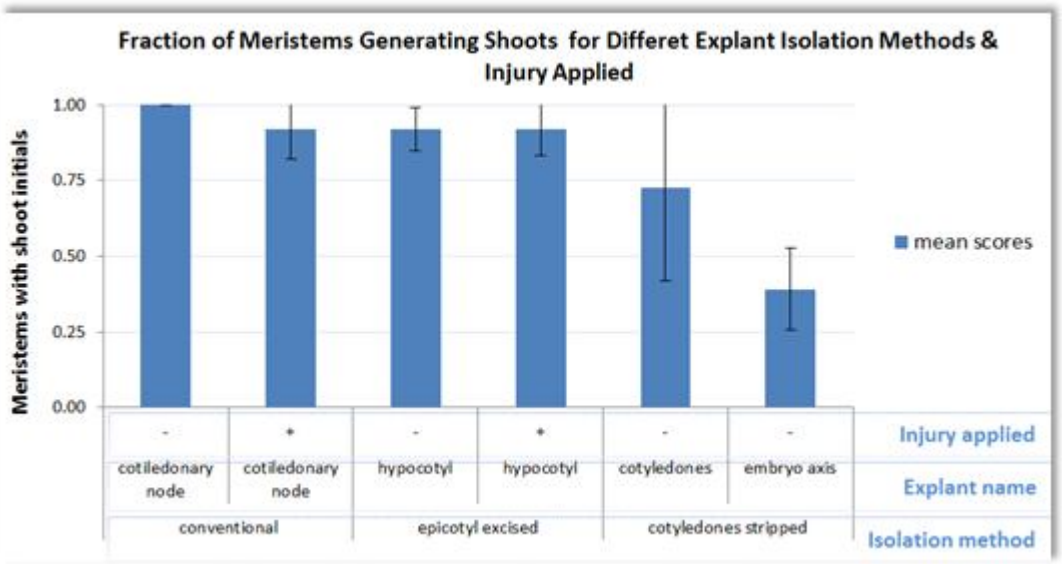

Figure 3.59: Scores for shoot initials of different explant isolation methods
Shoot regeneration potentials are figured out for the cotyledonary nodal meristems from different isolation methods. Shoot regeneration capacities of the meristems are not significantly different for explants generated through conventional and epicotyl excised methods. For cotyledons stripped method on the other hand, due to un-balanced sorting of the meristematic tissues on either cotyledons or embryo axis regenerative capacity drops down.

3.4.2.4 Conventional Explant Isolation Method

Although different methods of explant isolation were experimented, evaluation of the results in detail revealed that these methods did not impose standard on the cotyledonary nodes generated. In the expense of simplicity and less labor epicotyl excised and cotyledons stripped methods yielded inconsistent injury and random departing of the meristems on either section of the excised explant. In the end conventional cotyledonary node isolation was employed again. In order to reveal the meristematic zones explants were isolated under dissection microscope and cotyledonary petioles were excised at the embryo axis junction point leaving the

meristems on the axis to open access. This slight modification served great during explant injury such that special glass needles were not required further and injury can be made with the surgical blade.

3.4.3 *Agrobacterium* Inoculation and Co-cultivation Media Formulation

Modification of culture temperatures for KYRT1 and C58C1 strains and growth media were explained within the concept of *Agrobacterium* culture works in section-3.2. In this section modification in *Agrobacterium* inoculation and co-cultivation media is explained.

The inoculation and co-cultivation media were different in our previous *Agrobacterium* mediated transformation protocol for lentil (Kamçı 2004). Inoculation media was so called MMA medium containing half strength MS macro and micro nutrients with 3% sucrose buffered to pH 5.6 with MES. Frequent precipitation and contamination was seen in this media. Co-cultivation media was half strength Hoagland's-E medium. Advent of new procedures and raise of new transgenic plants along with the new agents for enhanced transformation rates leaded us to reformulate the inoculation media which was agar solidified and used also as co-cultivation media.

3.4.3.1 Inoculation and Co-cultivation Media Re-formulation

For *Agrobacterium* inoculation and co-cultivation media reformulation a basis medium was to be selected. And for this purpose Hoagland's-E medium and MS medium were compared. Nutrient concentrations of the media considered are approximately equal. MS medium contains cobalt, iodide and ammonium nitrate which are not present in Hoagland's formulation. Compared to the ionic strength and preparation, Hoagland's-E medium was simpler then MS medium. Easy preparation and control over the media ingredients were the two causes for selection of Hoagland's–E medium as basis. See appendix d table D.43 for comparison of the compositions for the two media.

Ammonium, nitrate, phosphate and calcium were the components to be omitted from inoculation and co-cultivation media according to Azadi et. al. (2010). Hoagland's-E medium lacks ammonium but contains calcium, nitrate and phosphate as in the forms $Ca(NO_3)_2.4H_2O$, KNO_3 and KH_2PO_4 of. As the first modification, these are omitted from the Hoagland's solution

As the initial additions to the media, we have considered thiamin and myo-inositol which are essential vitamin components of plant tissue culture optimized during tobacco studies. Another additive is tryptophan. It was noted in the reviews above that tryptophan is converted into an auxin analog that avoids plant defense against *Agrobacterium* (Escobar et. al., Article in press). And also ascorbic acid and silver nitrate were the two other additives that take effect in clearance of H_2O_2 and H_2O_2 mediated ethylene response clearance respectively. The final additive was l-cysteine. It was used due to its irreversible (covalent) binding to poly phenol oxidases' Fe/Cu containing reaction center (Olhoft et. al. 2001).

Components omitted from and added to the Hoagland's-E medium were listed above. Special care was considered during preparation of the inoculation the media since from amino acids, vitamins, antioxidants to metal nitrate very different components were considered in the media mixture.

Main solution of the media was prepared with the simplified Hoagland's-E medium plus sucrose as the carbon source and MES as the buffering agent. This main solution was prepared and pH was titrated to 5.6 and stored at 4°C prior to use. Rest of the components was filter sterilized or dissolved in DMSO due to stability concerns and heat sensitivities. Thiamin, myo-inositol and tryptophan were filter sterilized and stored as mixture at -20°C till use. Ascorbic acid and silver nitrate was also filter sterilized but stored separately at -20°C. Since DMSO was used for preparation of acetosyringone and l-cysteine these additives were not filter sterilized but freshly prepared before use. Prior use all of the components were brought together to form the inoculation media.

The concerns about the concentration of components in inoculation media were discussed below. The base media constructed through omission of some of the components from Hoaglands' E media is tabulated below (table D.44). Sucrose added was 3%. For addition of MES as buffering agent first ionic strength of the base media was calculated. Micronutrients were omitted from this calculation since their effect to ionic strength calculation was in µM scale. Only the two abundant components, $MgSO_4$ and $FeCl_3$ were considered for ionic strength calculation. For buffering the medium with an ionic strength of 4.12mM to pH 5.6 at 20°C the amount of MES was calculated as 3.9g.

The concentrations of the additives of the modified Hoagland's-E medium are as follows. The amounts of thiamin and myo-inositol used in the modified inoculation media was directly taken from the MS vitamins recipe. The amounts were 10 and 100 mg/l for thiamin and myo-inositol respectively. For tryptophan concentration gram negative bacteria tryptophan auxotroph's culture addition was considered as

reference. Final concentration for tryptophan added to inoculation media was taken as 10 mg/l. Acetosyringone in inoculation media was not modified (200µM). Final concentration of ascorbic acid was taken as 100µM. For silver nitrate final concentration soybean transformation was taken as reference (5 mg/l). The final component, l-cystein was used at a final concentration for 1 gr/l.

Preparation of the inoculation media is depicted in the figure 3.60 below. The base media with MES and sucrose is prepared and stored at 4ºC. For the rest of the components liability and fortification concerns leaded us to filter sterilization or preparation of components just before use. Exact formulation of the inoculation and co-cultivation media are tabulated in materials and methods section

Inoculation media without sucrose and solidified with 0.6% agar was used as co-cultivation media also. The preparation is the same with the inoculation media described above.

3.4.1 Initial Transformation Experiments

In order to make a brief diagnosis of the lentil transformation with new media formulations and new explant isolation methods devised, the initial two transformation experiments were conducted as preliminary experiments. In these

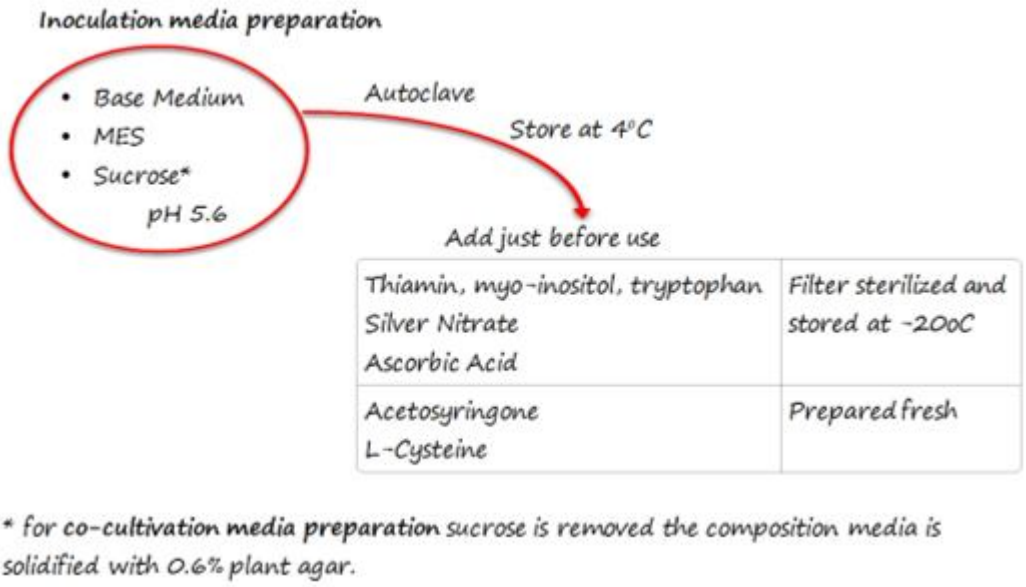

Figure 3.60: Inoculation media preparation precoutions

experiments three days old lentil seedlings germinated on water agar were used as explant sources. Explant isolation was performed according to the section 3.4.2 as conventional and epicotyl excised methods. Immediately after isolation, half of the explants prepared through conventional method were injured through poking into the meristem and the rest were injured through slicing into meristem along the embryo axis. Then explants were *Agrobacterium* inoculated for two hours. Next explants were decontaminated in Augmentin or Timentin solution and transferred to co-cultivation media composed of MS media with vitamins and supplemented with BA, TDZ and Augmentin. Seven days of co-culture at 25±2^{o}C resulted in high rates of necrosis in whole sets. Hence in order to figure out the possible cause(s) of necrosis, experimental setup and results were diagnosed. Data summary of the

results are given in appendix e, table E.3 and graphical interpretation of the data is given in figure 3.61 below.

Necrosis or full bleaching was the most prominent observation of the preliminary transformation experiment. Even the necrosis rate was scored between 30 to 63% for the control groups. Also the control and transformation applications of the same injury group are not significantly different. That is necrosis pattern does not change significantly with *Agrobacterium* inoculation. It is also noted that necrosis was clearly observed at the third day of co-culture period. Visual analysis of the explants under dissection microscope revealed that the necrotic zones were prominent at the hypocotyl and epicotyl sections. During explant injury practices these sections are the zones that we handle the explants with tweezers. That is, during explant isolation un-intended injuries are made on the explant. Also during preparation of cotyledonary nodal meristems for transformation another round of injury is undertaken. In such a condition generation and vascular transport of H_2O_2 cannot be avoided. As a solution a delicate pre-culture period could be considered prior to or after injury for introducing a recovery time to the explants isolated and injured. Pre-culture media is the same as co-cultivation media.

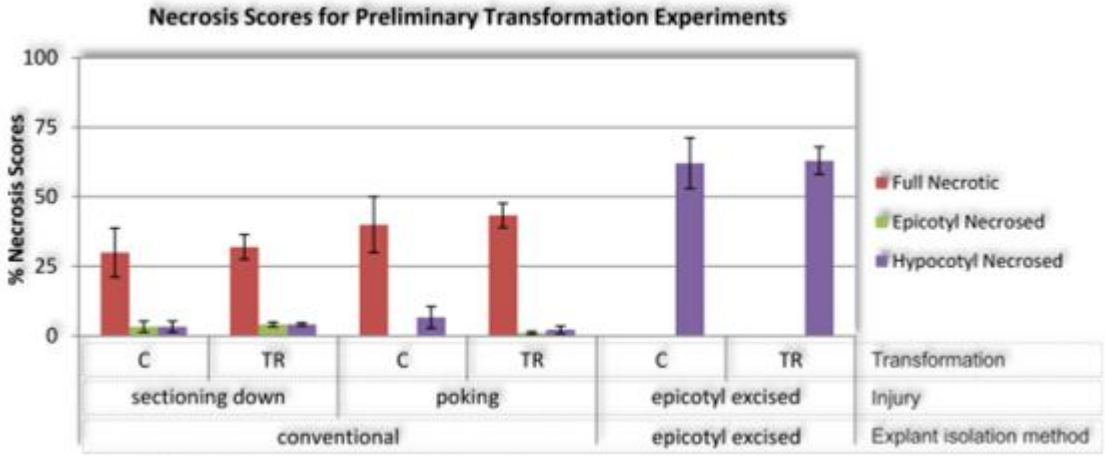

Figure 3.61: Necrosis of different explant isolation types after trasnformation
Graphical interpretation of the data summary in appendix e, table E.3

3.4.2 Transformation Experiments Continued

Interpretation of the results from the first transformation experiments and formulation of new pre-culture, inoculation and co-culture media and explant isolation studies conducted aided for re-planning of the transformation. As it was explained in section 3.4.2.4 cotyledonary nodal segments prepared were without cotyledonary petioles. In the second set of transformation experiment, explants isolated were either left to pre-culture for one or two days and then injured or first injured and then left into pre-culture media prior to *Agrobacterium* inoculation. Also germination media (H2O-agar) contained 2mg/l TDZ or BA or 1mg/l TDZ+BA for inducing the cotyledonary nodal meristems. Following inoculation and co-culture explants are sub cultured in M1 media under mannose (3%) selection, timentin and TDZ or BA for maintaining regeneration and selection.

Since the necrotic behavior was very prominent in the first transformation experiment, in the second transformation set explants were closely examined in the first two weeks of sub-culture. Data and its graphical interpretation are given in appendix e table E.4 and figures 3.62 and 3.63 respectively.

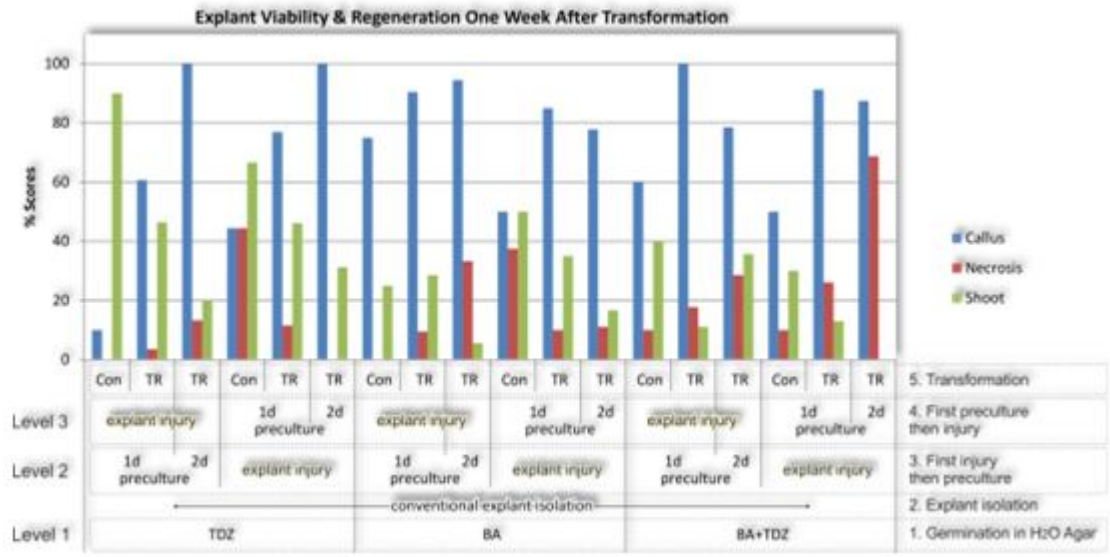

Figure 3.62: Explant viability and regeneration data in the first week
Explant viability and regeneration data in the first week of the second transformation set is figured.

In the first week of sub-culture it is likely to find an inverse correlation between the callus formations and shoot regeneration among the experimental sub groups at level 2 (blue and green bars in figure 3.62). Necrotic phenotype was observed without any trend in the very first week of the sub-culture.

Depending on the second week data it can be stated that both callus formation and shoot regeneration are in an increasing and decreasing trend respectively. That is in the second week as there is increasing callus formation for the explants grouped at level 2 sub-set (blue bars in figure 3.63), the same explant sub-groups show decreasing shoot regeneration (green bars in figure 3.63). According to the figure 3.63 it can be stated that explants processed through subsequent injury and pre-culture (or pre-culture and injury) periods plus cytokinins (2 mg/l) used mediate more callus as the pre-culture period shift from one day to two days. Inverse of the same trend holds for the shoot regeneration pattern.

Besides callusing and shot regenerating explants there is also another sub set of explants emerging in the second week that do not show any regenerative or callus response but remain dormant (red bars in figure 3.63). It is not possible to trace any trend for this explant group. They have scattered behavior for different experimental groups starting from the level 1.

As the data for both weeks compared we may state that in the first week necrotic explants are removed from the culture dish. Amount of callus induction is most prominent in this week while regeneration is also started. In the second week some of the explants that show callus bodies started to give shoot and shoot forming explant number are almost equal to the callus forming explant number.

Next data are recorded on monthly basis. Experiment groups prepared with TZD and TDZ+BA combination were all necrossed at the end of the fourth sub-culture. So these data sets are omitted from evaluation. According to the results it can be evaluated that one of the four experimental group progressed superior over the

others as necrotic behavior, shoot regeneration capacity and callus formation rates are considered (figures 3.63, 3.65 and 3.66).

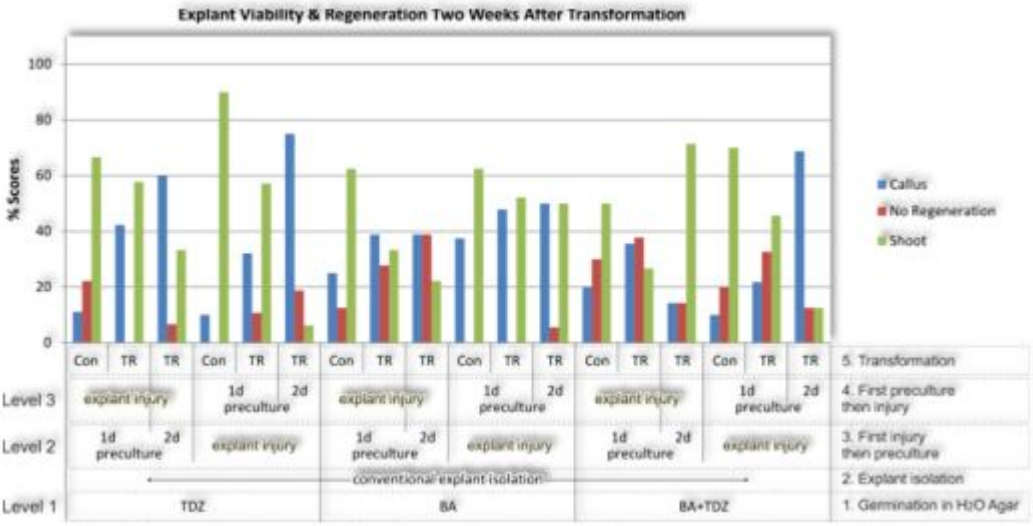

Figure 3.63: Explant viability and regeneration data in the second week
Explant viability and regeneration data in the second week of second transformation set is figured.

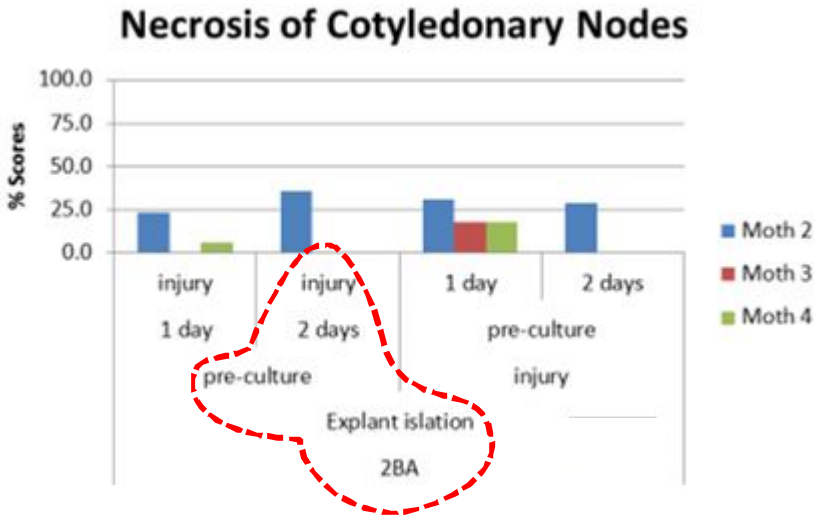

Figure 3.64: Necrosis scores for transformed explants
Highest rate of necrosis was observed at second month for the application group enclosed with red lines. The same group performs superior over the others as scores for third and fourth months are considered. This superior group of explants comes from explants isolated from seedlings germinated in 2 mg/l BA in water agar plates and pre-cultured in co-cultivation media for two days and then injured and transformed.

Evaluation of the results target a specific explant preparation method that performs well with low rate of necrosis, higher rates of shoot regeneration and low rates of callus formation from the meristematic tissues throughout the carried sub-cultures. Shoots regenerated from these applications were grafted and also initial PCR analysis of transgene expression was made from the fresh leaves. Although some primary amplification were observed these amplification signals (figure 3.67) seen in fresh leaves lost as the plants grew further.

Figure 3.65: Shoot regeneration scores for transformed explants
Shoot regeneration potential of the same superior group depicted in figure 3.64 is shown.

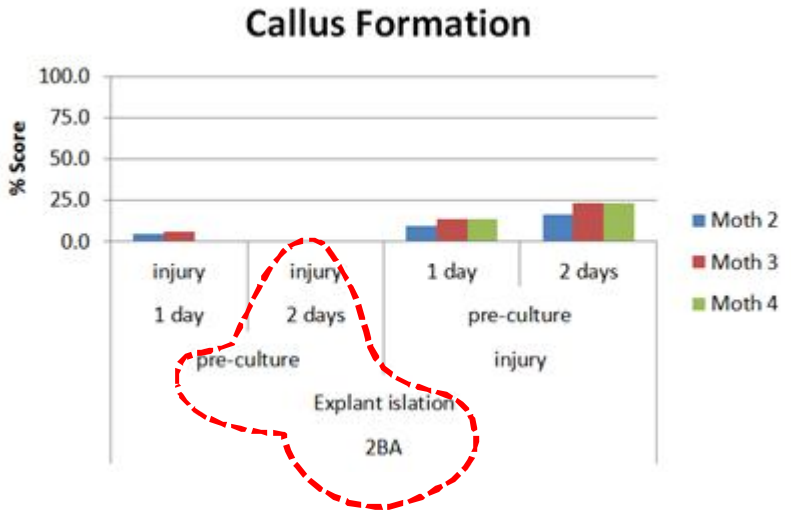

Figure 3.66: Callus formation scores for transformed explants
Callus formation potential of the same superior group depicted in figure 3.64 is shown

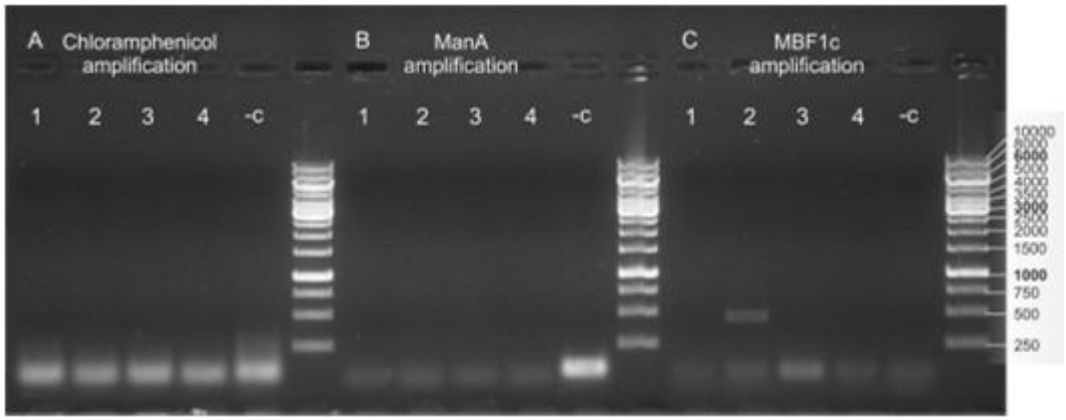

Figure 3.67: Leaf PCR agarose gel image
Weak amplification signal of MBF1c (447bp) (C) was observed from the fresh leaves of second sample (grafted putative transgenic shoots). However a complementing signal for ManA (1176bp) amplification was not seen (B). Chloramphenicol (antibiotic resistance gene of the plant transformation vector, located outside the T-DNA) amplification (800bp) is also not observed for the samples scanned.

3.4.3 Transient GUS Expression Analysis with pPZP101 MAnA GUSint MBF1c

Transformation experiments made with C58C1::pPZP101 ManA MBF1c strain binary vector couple will reside in the range of transformation optimization works as long as no transgenics are recovered. The main handicap can be attributed to use of C58C1 instead of KYRT1. In order to grasp a vision about what may be happening at tissue level upon transformation, GUSin cassette is integrated into the present pPZP101 ManA MBF1c vector. And transient visual marker (GUS) expression analysis was performed on explants transformed with both C58C1 and KYRT1 strains (figure 3.68).

Explants transformed with C58C1 and KYRT1 strains loaded with pPZP101 ManA GUSint MBF1c transformation vector are shown. C58C1 contains only pPZP101 ManA GUSint MBF1c vector but KYRT1 contains both pTJK136 and pPZP101 ManA GUSint MBF1c vectors that contain GUSint cassette. Consequently histochemical GUS analysis of KYRT1 transformed explants showed considerably higher GUS staining. Further transformation experiments should be conducted with pPZP101 ManA GUSint MBF1c vector in order to reveal the dynamics behind the fainting transgene expression in putative transgenic tissues.

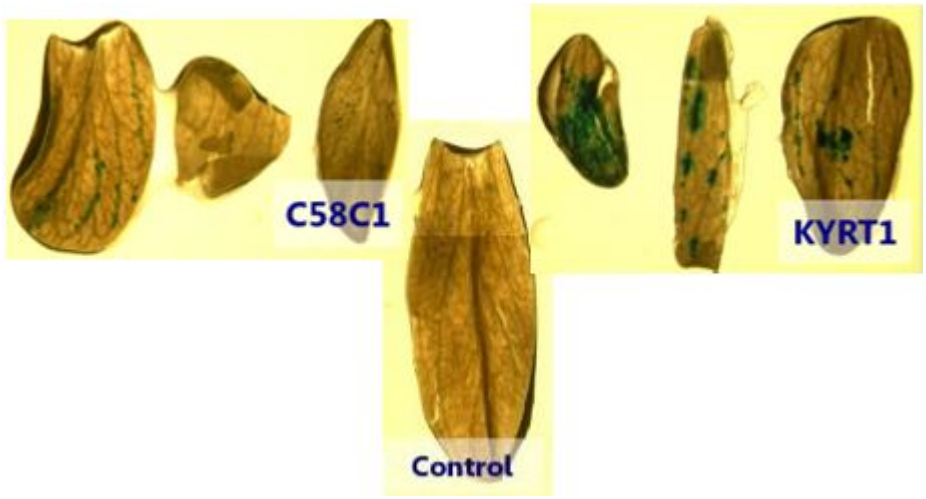

Figure 3.68: Transient GUS expression analysis on lentil leaves

3.4.4 Mannose selection system

Mannose selection should also be regarded as negative selection since accumulating mannose in the form of mannose 6-phosphate inside the cytoplasm is toxic to the cell. Mannose selection is an emerging trend in transgenic studies and its adoptability to lentil transformation is not studied yet. In order to make a brief introduction to mannose selection system applicability in lentil mannose effect on germination and secondary shoot formation was evaluated.

3.4.4.1 Mannose Effect on Germination

A simple germination experiment was conducted for diagnosis of effect of mannose on lentil seedling development. For this purpose lentil seeds are germinated on water agar supplemented with wearying concentrations of either mannose or sucrose. At the end of three days epicotyl and hypocotyl raise for seedling are measured. Depending on the data it can be stated that higher concentrations of both carbon sources are inhibitory to epicotyl formation while hypocotyl can be raised in any concentration of both carbon source. On the other hand it can be

clearly stated that 4% mannose is completely inhibitory to epicotyl formation with respect to 4% sucrose application and control (figure 3.69).

It can be concluded that 4% mannose in water agar plates can be used to select the transgenic lentil seedlings according to their epicotyl raise.

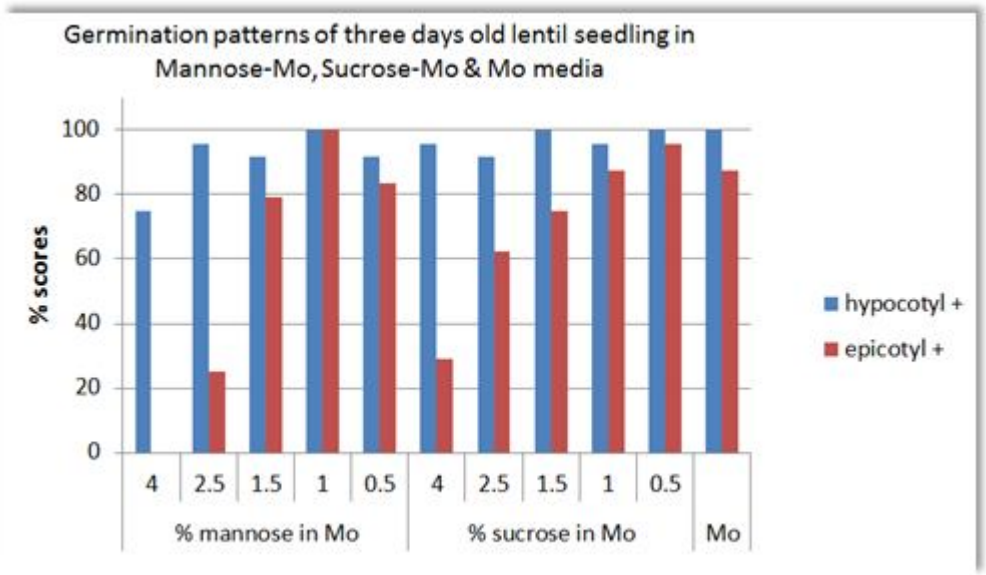

Figure 3.69: Epicotyl and hypocotyl elongation of seedlings on sucrose and mannose

3.4.4.2 Mannose Effect on Regeneration from Cotyledonary Nodes

In order to make an assessment of mannose effect on axillary shoot regeneration, the cotyledonary node explants are cultured in MS salts and vitamins media supplemented with varied amounts of mannose and sucrose and 1mg/l BAP. At the end of four weeks culture mean dry weight of the shoots and mean number of shoots per explants was scored. For convenience mean dry weight scores was multiplied by 100 in order to show the data in the same graph with mean shoot number (figure 3.70).

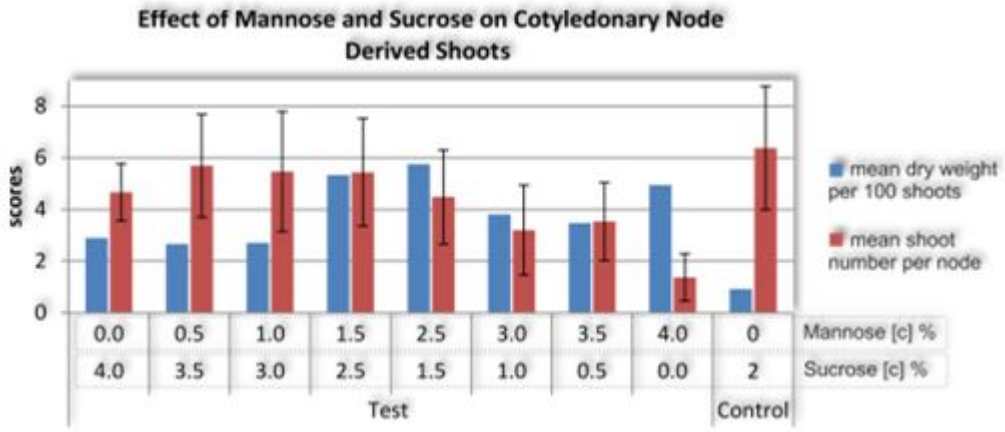

Figure 3.70: Effect of mannose and sucrose on axillary shoot regeneration

Compared to the control and other test groups mean shoot number is depressed significantly when 4% mannose is used as the only carbon source. Also as it is evident from the mean dry weight data non transgenic shoots generated in the presence of 4% mannose would show swelled morphology as mean shoot number to mean dry weight ratios for other test groups are considered.

3.4.5 Micro-grafting

Micro-grafting was first optimized as cleft and whip type grafts in our laboratory (Kamçı, 2004). Up to date two minor modifications were done for enhancing the efficiency. First modification was elimination of the graft suture. In the initial optimized method spirally prepared lab tubing was used to squeeze the graft union at the neck. Insertion of the scion into stock and squeezing the spiral lab tubing to the union region was quiet difficult to practice. Another method was devised during thesis studies and also used in our first genetically modified lentil (Akçay et. al. 2009). In this method the stem of the root stock was not excised along the stem axis but rather an incision was introduced so that as the scion is inserted the stem itself holds it in place. But another problem was raised with this method. This time it was problem to open the incision sites apart. The two halves of the stem were immediately collapsing onto each other prior to scion insertion so scions were forced into the stem and they were broken usually. In order to solve this problem

lentil relative species with larger stem girths were searched. And a preliminary experiment was conducted with a Turkish lentil cultivar which returned unsuccessful. So exaggeration of stem girth was hypothesized as a solution.

3.4.5.1 Stock Stem Girth Exaggeration

3.4.5.1.1 Auxin Application during Micrografting

Although the applied amount of auxin (1 mg/l) did not directly affect the root stock secondary shoot formation it resulted in improvement of the health status of the scions. Either application of auxin did not result in any enhancement on lowering the number of secondary shoots generated from the nodal segment with respect to the control (see bars Normal, Pre/Post-Auxin in figure 3.71). However application of auxin after micrografting to the graft union region (directly from the NaOH stock due to volumetric concerns) resulted in approximately 9 fold depression in health status of the scion (compare wilting bars for Post-Auxin versus Normal in figure 3.71). However if we compare the Pre-Auxin application with Normal we can note a double fold improvement on wilting status of the scions.

3.4.5.1.2 Effect of Auxin on Secondary Shoot Regeneration of Root Stocks

Following the grafting practices at the end of the two weeks the micrografts begin to recover after a period close nursery for removing their secondary shoots. It is the first week of the grafting practice that secondary shoot formation boosts and accounts for the 12-14 days of the root stock from germination. So root stocks with 12-14 days age old were decapitated re-cultured with different concentrations of auxin. At the end of 1 week scores for secondary shoot formation were scored (figure 3.72). According to the observations at least one half of the grafts of control group were showing secondary shoot regeneration from their nodal segments. While being the most active the 12 days old root stocks` secondary shoot regeneration depressed almost tree fold under the effect of 2.6 mg/l auxin concentration with respect to the control group. The secondary shoot regeneration

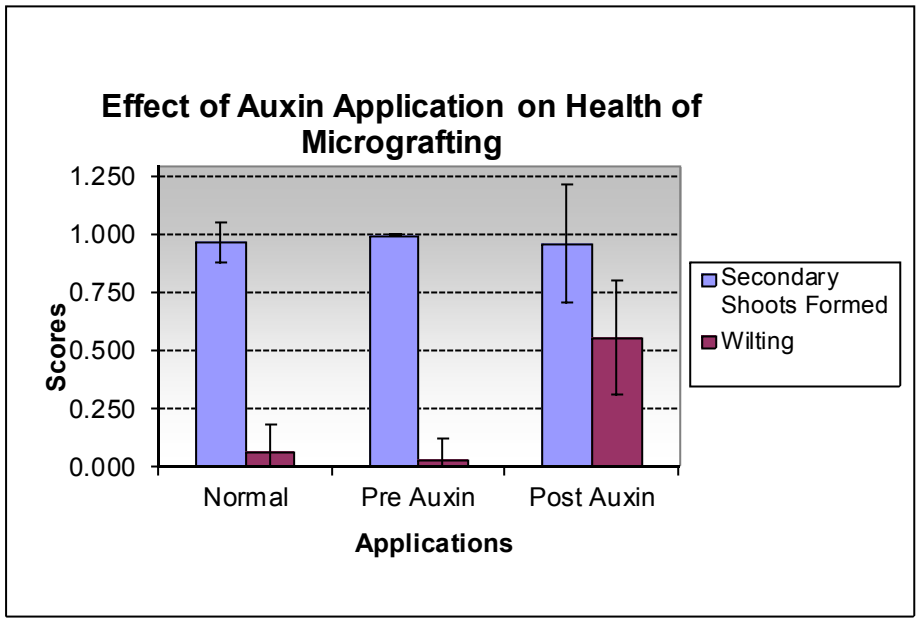

Figure 3.71: Effect of auxin on health of scions and secondary shoot of root stock
Effect of auxin on health status of scions and secondary shoot formation of the root stock. Scores were taken at the end of 6 days after micrografting. Pre-Auxin application: scions wetted with 1mg/l Auxin. Post-Auxin application: graft unions wetted with 1mg auxin from 4mg/ml auxin stock.

activity of the other two 13 and 14 days old groups were met approximately at thesame plateau with that of 12 days old group for 2.6 mg/l auxin application. That is with 2.6 mg/l auxin application the secondary shoot regeneration capacity of the root stocks are depressed with a rate of almost 3 fold with respect to control while age of the root stock does not matter.

3.4.5.1.3 Auxin Effect on Scions

While the effect of auxin applied to the grafts were noted as favorable for scion health for a full micrograft it was not shown to be so when scions were incubated solely on auxin supplied with Hoglands` solution. Varying auxin concentrations applied from 0.4 to 4.0 mg/l resulted in different wilting patterns in incubated scions under light and approximately 22 ° C culture temperature environment. When compared to the success of grafted scion health status noted in previous sections the wilting effect might be due to continuous induction with auxin in liquid culture

and triggering hypersensitive response or due to the lack of adequate carbon source; first proposal being the most plausible. Experiment should be revisited with appropriate precautions in order to see the sole auxin effect on health status of the scions (figure 3.73).

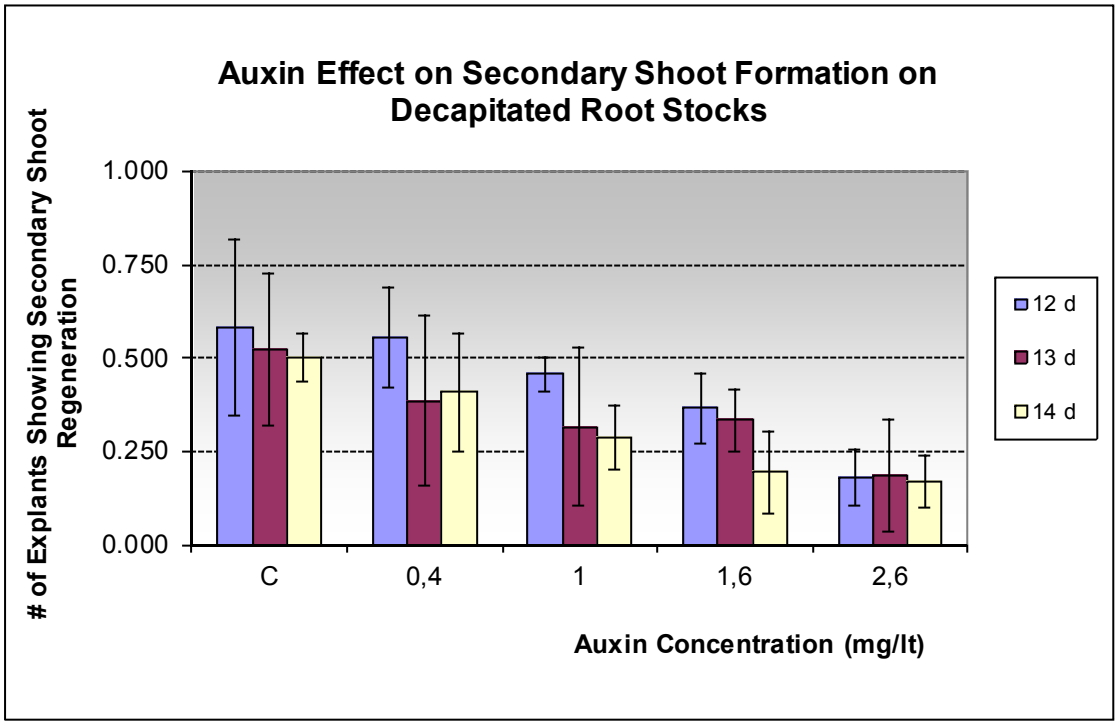

Figure 3.72: Auxin effect on secondary shoot formation of decapitated root stocks Bars denoting for 12, 13 and 14 days old root stocks respectively

Figure 3.73: Auxin effect on scion wilting
Numbers indicating mg/l of auxin applied.

3.4.5.1.4 Decapitation and Hormone Effect on Stem Girth of Root Stocks

For this part of the experimentation since the root stocks for grafting practices were germinated at dark conditions aim was to depress etiolation with Ag and to enhance the vascularization with auxin in order to increase the stem girth of the decapitated root stocks. For this purpose cytokinin, auxin and AgNO3 along with auxin were applied separately to the cultures of germinated 3 days old root stocks. Among the groups only the control group showed slender weak stem segments while the test groups showed highly fleshy and erect stem segments.

All of the different applications resulted with a relative enhancement on stem weight and length with respect to control group, but any of the application did not yield a respectable growth in stem girt compared to the control. On the whole the maximum enhancement in stem girth was at most 0.1 mm with 4mg/l auxin and 1 µg/l AgNO3 application (figure 3.74).

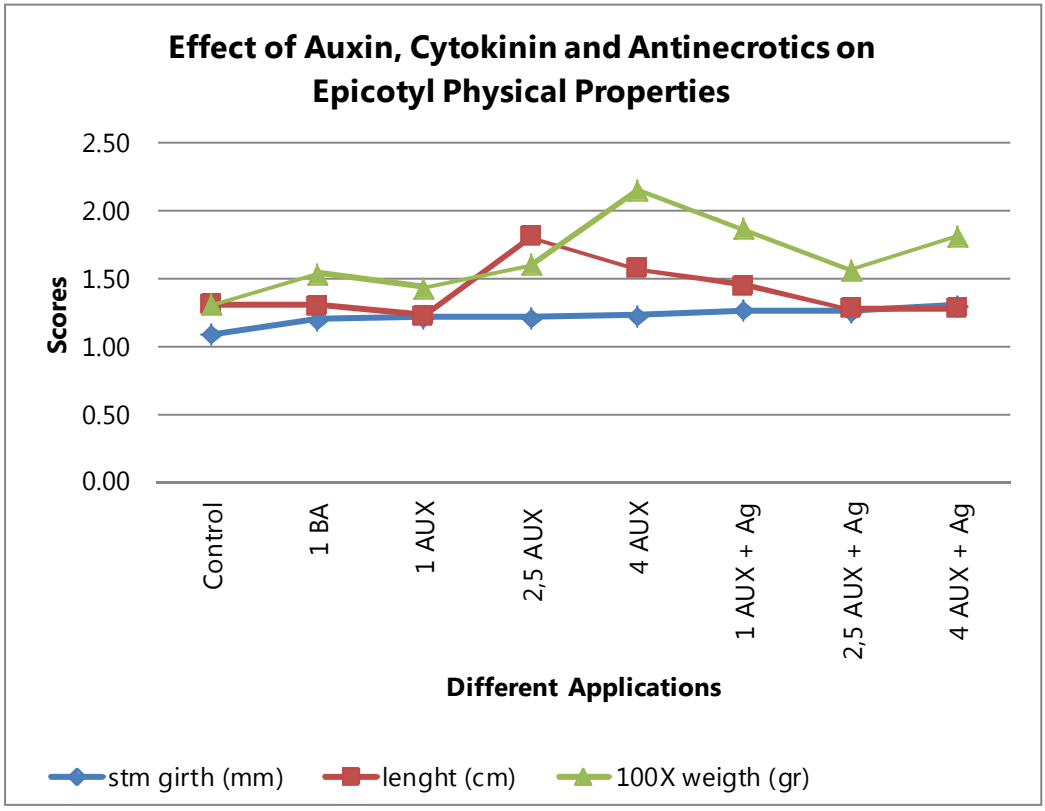

Figure 3.74: Effect of phytormones and $AgNO_3$ on epicotyl growths of seedlings

3.4.5.1.5 A Slight Modification in Grafting Solved the Problems

Since stem girth exaggeration practices did not produce adequate enlargement in stem radius and also due to unpredictable post effects of hormone applications, we

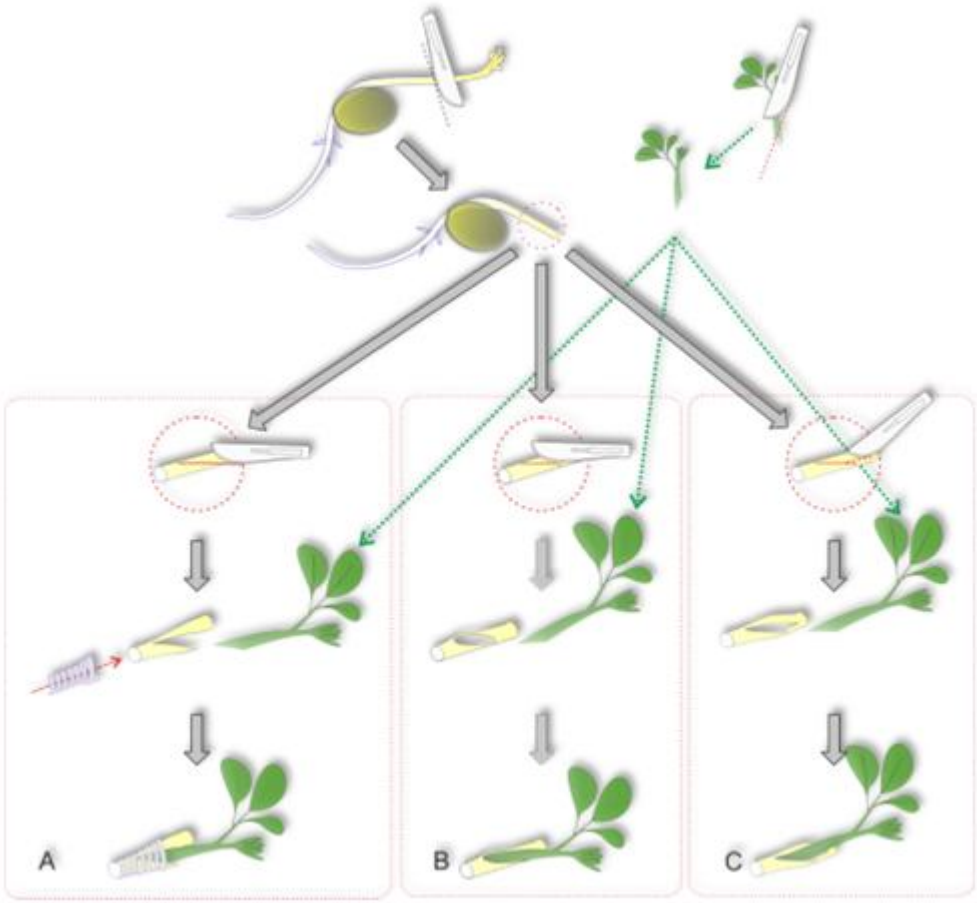

Figure 3.75: Micro-grafting method optimization studies schematized
(A) Cleft type micro-grafting was adapted to lentil in master thesis study by Kamçı (2004). Most critical issue for the cleft type of grafting in lentil was holding the scion and stock united. (A) For this purpose spirally prepared lab tubing was devised first (Kamçı 2004). Both preparation and use of this spiral was not handy so a small modification in stock preparation let us grafting without any device. (B) The stock was prepared through a lateral incision (not excision) into the stem axis. In such a preparation scion inserted into the stem was squeezing the right in place inside the axis of the stem. Avoidance of any device both decreased the labor load and contamination that may rise from it. However a new problem emerged with this new method was the break of scion while forcing it into the incision on the stem axis. (C) For solution of the problem another incision was made on the top of the first one along the stem axis. The problems about non matching stem-scion girth, insertion of the scion in to the incised stem and holding the scion and stem united were avoided with this new modification.

have again returned our focus on grafting. And a simple but effective modification was introduced to stock stem preparation. It was just another incision on the top of the first one for elongating the cut site so that during scion insertion to the stem the two halves of the stem may easily accept the scion without any damage. With this slight modification the grafting practice and regeneration of lentil can be maintained in a very short time. The comparisons and the grafting progress are schematized in figure 3.75.

CHAPTER IV

CONCLUSION AND FUTURE PROSPECTS

Transformation of lentil with MBF1c coding sequence was initially planned on the optimized transformation method with KYRT1::pTJK136 *Agrobacterium* strain and binary plasmid (Akçay, 2008). And transgenic lentil lines that would be recovered were to be analyzed for abiotic stress resistance thereafter. However engineering the pTJK136 for MBF1c expression was hampered due to unknown plasmid and T-DNA sequences. Also the available restriction maps for the T-DNA region of both pTJK136 and its ancestor pTHW136 returned false results of restriction digests. The other problem with the KYRT1::pTJK136 pair was the strain itself. It was not possible to empty the *Agrobacterium* strain so that in place of pTJK136 an alternative plant transformation vector can be used. As a result both the *Agrobacterium* strain and the plant transformation vector were changed to C58C1 and pPZP101 respectively, meaning that a new transformation vector construction should be worked out and lentil cotyledonary node transformation with C58C1 optimized first. Then analysis of stress resistance for transgenic lentils can be undertaken.

Search for an alternative plant transformation vector leaded to the pPZP101 binary plasmid. First point in vector construction was expression cassette construction for the MBF1c. coding sequence. Immediate solution was recovered among the lab sources as Impact Vector 1.1 (IV 1.1). A RbcS1 promoter deriving the MBF1c expression would provide light inducible expression of the protein. So MBF1c coding sequence retrieved from Arabidopsis Tair center was first cloned into IV 1.1. The recovery of RbcS1-MBF1c cassette and sub-cloning into pPZP101 was not possible

due to MBF1c flanking restriction sites retained from successive sub-cloning practices from the Arabidopsis Tair stock to IV 1.1. In order to eliminate these unwanted restriction sites, MBF1c coding sequence was amplified with PCR while specific restriction sites were integrated to the 5′ and 3′ ends. With the new restriction sites integrated it was possible to generate fully defined RbcS1 cassette in IV 1.1 and new MBF1c cassette based on CaMV35SS-NOS sequences. The same procedure for CaMV35SS-MBF1c-NOS construction was used for a ManA selection cassette construction. Concomitantly with PCR based cloning of MBF1c, Gateway technology was applied for plant transformation vector construction. For this purpose pEarleyGate vectors (100-104 series) were requested from Arabidopsis-Tair. Initial cloning practices for MBF1c cloning through Gateway technology was undertaken and pCR8-MBF1c entry clones were generated. Sequencing of the pCR8-MBF1c and CaMV35SS-MBF1c/ManA-NOS cassette clones revealed that only CaMV35SS-NOS cassettes were proper. As a result transformation vector construction was carried out with pPZP101 and CaMV35SS-NOS clones and pPZP101 ManA-MBF1c vector was constructed.

The initial transformation experiments were conducted with C58C1 strain and pPZP101ManA-MBF1c transformation vector on differentially prepared lentil cotyledonary node explants. Initial transformation experiments resulted in full necrosis of the plant material regardless of the explant preparation type. Diagnosis of this necrotic behavior revealed that unintended injuries with tweezers and successive wounding for transformation were triggering plant hyper sensitive response during co-cultivation with *Agrobacterium*. In the continuing transformation experiments explant isolation, injury and transformation practices were separated from each other with a pre-culture period. Also a new formulation of inoculation and co-cultivation media was proposed. And also in order to minimize injury special emphasis was ascribed to explant preparation. The second and third transformation experiments yielded putative transgenic tissues that substantially generated healthy shoots under mannose selection. At the end of 5 months of selection some of the putative transgenic shoots were grafted and simple PCR analysis were made for

MBF1c coding sequence presence. Fading signals of MBF1c as the plantlet grew leaded us elimination of these first grafts. Then at the end of 8 moths of selection healthy shoots regenerated were grafted again.

Molecular analyses for these putative transgenic plantlets are being carried out at the moment.

Following confirmation of the transgenic plantlets and generation of transgenic F1 lines analysis of abiotic stress tolerance will be performed.

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APPENDIX A

PLANT AND BACTERIAL CULTURE MEDIA

A.1 Bacterial Culture Media

A.1.1 LB media

10 g/L tryptone
10 g/L NaCl
5 g/L yeast extract

pH 7

Autoclave

* for solidification use 1.5% bacteriological agar

A.1.2 SOC medium

20 g/L tryptone
5 g/L yeast extract
0.5 g/L NaCl

Autclave

Add 20 mM final glucose before use.

A.1.3 YEB media

5 g/L tryptone,
1 g/L yeast extract
5 g/L nutrient broth
5 g/L sucrose
0.49 g/L MgSO 4·7H2O

pH 5.6

Autoclave

A.1.4 MG/L media

5 g/L	tryptone
2.5 g/L	yeast extract
5 g/L	NaCl
5 g/L	mannitol
0.1 g/L	MgSO47H2O
0.25 g/L	K2HPO4
1.2 g/L	L-glutamate
1.2 g/L	thiamine* (10% solution, filter-sterilized).

pH 5.6.

Autoclave

* Add thiamine before use

A.2 Basal Plant Tissue Culture Media

Basal plant rissue culture media used throughout this study is MS salts and MS vitamins plus 3% sucrose and 0.6% agar. pH is adjusted to 5.6 prior to agar addition. Required additions to this basal media are made while considering the heat and pressure liabilities of the addtives.

APPENDIX B

PRIMERS, CODING SEQUENCES AND SEQUENCE OF NEW CONSTRUCTS

B.1 Multi Protein Bridging Factor 1c CDS

```
LOCUS       MBF1c_CDS               447 bp    DNA     linear   PLN 22-FEB-2003
DEFINITION  Arabidopsis thaliana At3g24500 gene, complete cds.
ACCESSION   BT004761
VERSION     BT004761.1  GI:28466836
KEYWORDS    FLI_CDNA.
SOURCE      Arabidopsis thaliana (thale cress)
  ORGANISM  Arabidopsis thaliana
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            Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
            rosids; eurosids II; Brassicales; Brassicaceae; Arabidopsis.
REFERENCE   1
  AUTHORS   Kim, C.J., Chen, H., Cheuk, R., Shinn, P., Bowser, L., Carninci,
            P., Chan, M.M., Chang, C.H., Dale, J.M., Hayashizaki, Y., Hsuan,
            V.W., Ishida, J., Jones, T., Kamiya, A., Karlin-Neumann, G., Kawai,
            J., Lam, B., Lee, J.M., Lin, J., Miranda, M., Narusaka, M., Nguyen,
            M., Onodera, C.S., Palm, C.J., Quach, H.L., Sakurai, T., Satou, M.,
            Seki, M., Southwick, A., Tang, C.C., Toriumi, M., Wong, C., Wu,
            H.C., Yamada, K., Yu, G., Yuan, S., Shinozaki, K., Davis, R.W.,
            Theologis, A., andEcker, J.R.
  TITLE     Arabidopsis ORF clones
  JOURNAL   Unpublished
REFERENCE   2
  AUTHORS   Kim, C.J., Chen, H., Cheuk, R., Shinn, P., Bowser, L., Carninci,
            P., Chan, M.M., Chang, C.H., Dale, J.M., Hayashizaki, Y., Hsuan,
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            J., Lam, B., Lee, J.M., Lin, J., Miranda, M., Narusaka, M., Nguyen,
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            H.C., Yamada, K., Yu, G., Yuan, S., Shinozaki, K., Davis, R.W.,
            Theologis, A., andEcker, J.R.
  TITLE     Direct Submission
  JOURNAL   Submitted (22-FEB-2003) Salk Institute Genomic Analysis Laboratory
            (SIGnAL), Plant Biology Laboratory, The Salk Institute for
            Biological Studies, 10010 N. Torrey Pines Road, La Jolla, CA 92037,
            USA
COMMENT     RIKEN Genomic Sciences Center (GSC) members carried out the
            collection and clustering of RAFL cDNAs (RAFL cDNA : 'RIKEN
            Arabidopsis Full-Length cDNA') : Seki, M., Narusaka, M., Ishida,
            J., Satou, M., Kamiya, A., Sakurai, T., Carninci, P., Kawai, J.,
            Hayashizaki, Y. and Shinozaki, K.  The Salk, Stanford, PGEC (SSP)
            Consortium members constructed and sequenced the pUNI (ORF) clones
            using the RAFL cDNAs: Kim, C.J., Chen, H., Cheuk, R., Shinn, P.,
            Bowser, L., Chan, M.M., Chang, C.M., Dale, J.M., Hsuan, V.W.,
            Jones, T., Karlin-Neumann, G., Lam, B., Lee, J.M., Lin, J.,
            Miranda, M., Nguyen, M., Onodera, C.S., Palm, C.J., Quach, H.L.,
            Southwick, A., Tang, C.C., Toriumi, M., Wong, C., Wu, H.C., Yamada,
            K., Yu, G., Yuan, S., Davis, R.W., Theologis, A., and Ecker, J.R.
            Kim, C.J. (SSP/Salk) and Seki, M. (RIKEN GSC) contributed equally
            to this work. Shinozaki, K. (RIKEN GSC) and Ecker, J.R. (SSP/Salk)
            contributed equally to this work as PIs.
```

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      361 aatggtaaag ctgttcctaa tcaggctgtg cttgcgaaga tggagaaggt tctaggtgtt
      421 aaacttaggg gtaaaattgg gaaatga
//
```

B.2 Hypothetical pUNI51-MBF1c sequence data

```
LOCUS       pUNI51-MBF1c            2986 bp    DNA     circular UNA 26-JUL-2011
DEFINITION  Concatenation of 2 sequences.
ACCESSION
VERSION
KEYWORDS    .
SOURCE      .
  ORGANISM
            .
FEATURES             Location/Qualifiers
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                     /Original_Bases
                     /note="Geneious name: "
                     /note="Geneious type: Editing History Insertion"
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     CDS             239..685
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ligation        231..233
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ligation        691..693
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rep_origin      1645..2130
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rep_origin      1240..1645
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                /note="modified from pUNI50 originally constructed by
                Stephen Elledge (Liu Q, et al. Current Biology. 8 (24):
                1300-1309, (1998))"
                /note="Geneious name: source Cloning vector pUNI51"
source          239..685
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source          1..>233
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                Stephen Elledge (Liu Q, et al. Current Biology. 8 (24):
                1300-1309, (1998))"
                /note="Geneious name: source Cloning vector pUNI51"
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misc_feature    1..37
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misc_feature    705..730
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misc_feature    219..>233
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                /note="Geneious name: SfiI"
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      121 cagacactgc ttgtccgata tttgatttag gatacaggta ccaattaacc ctcactaaag
      181 ggataacttc gtatagcata cattatacga agttatctgg aattcggccg tcaaggccat
      241 gccgagcaga tacccaggag cagtaacaca agactgggaa ccagtagttc tccacaaatc
      301 aaaacaaaag agccaagacc tacgcgatcc gaaagcggtt aacgcagctc tgagaaacgg
      361 tgtcgcggtt caaacggtta agaaattcga tgccggttcg aacaaaaagg ggaaatctac
      421 ggcggttccg gtgattaaca cgaagaagct ggaagaagaa acagagcctg cggcgatgga
      481 tcgtgtgaaa gcagaggtga ggttgatgat acagaaagcg agattggaga agaagatgtc
      541 acaagcggat ttggcgaaac agatcaatga gaggactcag gtagttcagg aatatgagaa
      601 tggtaaagct gttcctaatc aggctgtgct tgcgaagatg gagaaggttc taggtgttaa
      661 acttaggggt aaaattggga aatgaggcct catgggccgt cgactagaat tgtgagcgct
      721 cacaattcta gcggccgccg agatcatatc actgtggacg ttgatgaaag aatacgttat
      781 tctttcatca aatcgtggtc gatcgacgag ctcgctatca gcctcgactg tgccttctag
      841 ttgccagcca tctgttgttt gcccctcccc cgtgccttcc ttgaccctgg aaggtgccac
      901 tcccactgtc ctttcctaat aaaatgagga aattgcatcg cattgtctga gtaggtgtca
      961 ttctattctg gggggtgggg tggggcagga cagcaagggg gaggattggg aagacaatag
     1021 caggcatgct ggggattcta gaagatccgg ctgctaacaa agcccgaaag gaagctgagt
     1081 tggctgctgc caccgctgag caataactag cataacccct tggggcctct aaacgggtct
     1141 tgaggggttt tttgctgaaa ggaggaacta tatccggata tcatgcatcg cgagagagag
     1201 agagagagag agagagagag agagagagag agagacgtgg gcccaattct gtcagccgtt
     1261 aagtgttcct gtgtcactga aaattgcttt gagaggctct aagggcttct cagtgcgtta
     1321 catccctggc ttgttgtcca caaccgttaa accttaaaag ctttaaaagc cttatatatt
     1381 ctttttttc ttataaaact taaaacctta gaggctattt aagttgctga tttatattaa
     1441 ttttattgtt caaacatgag agcttagtac gtgaaacatg agagcttagt acgttagcca
     1501 tgagagctta gtacgttagc catgagggtt tagttcgtta aacatgagag cttagtacgt
     1561 taaacatgag agcttagtac gtgaaacatg agagcttagt acgtactatc aacaggttga
     1621 actgctgatc aacagatcct ctacactagt ctaaattgta agcgttaata ttttgttaaa
     1681 attcgcgtta aatttttgtt aaatcagctc atttttaac caataggccg aaatcggcaa
     1741 aatccttat aaatcaaaag aatagaccga gatagggttg agtgttgttc cagtttggaa
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     1981 ggcgaacgtg gcgagaaagg aagggaagaa agcgaaagga gcgggcgcta gggcgctggc
     2041 aagtgtagcg gtcacgctgc gcgtaaccac cacacccgcc gcgcttaatg cgccgctaca
     2101 gggcgcgtcc cattcgccat tcaggctgca cgcgtttcga accccagagt cccgctcaga
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     2221 aaagcacgag gaagcggtca gcccattcgc cgccaagctc ttcagcaata tcacgggtag
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     2401 gatcctcgcc gtcgggcatg cgcgccttga gcctggcgaa cagttcggct ggcgcgagcc
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     2521 ctcgctcgat gcgatgtttc gcttggtggt cgaatgggca ggtagccgga tcaagcgtat
     2581 gcagccgccg cattgcatca gccatgatgg atactttctc ggcaggagca aggtgagatg
     2641 acaggagatc ctgccccggc acttcgccca atagcagcca gtcccttccc gcttcagtga
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     2761 cctcgtcctg cagttcattc agggcaccgg acaggtcggt cttgacaaaa agaaccgggc
     2821 gcccctgcgc tgacagccgg aacacggcgg catcagagca gccgattgtc tgttgtgccc
     2881 agtcatagcc gaatagcctc tccacccaag cggccggaga acctgcgtgc aatccatctt
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B.3 Hypothetical pBlueSK-MBF1c sequence data

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VERSION
KEYWORDS    .
SOURCE      .
  ORGANISM
            .
FEATURES             Location/Qualifiers
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     propeptide      536..696
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                     /note="Geneious name: lacZ a"
     rep_origin      2298..2917
                     /created_by="User"
                     /note="Geneious name: pBR322 ori"
     rep_origin      714..1020
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                     /note="Geneious name: f1 origin"
     source          19..465
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                     Stephen Elledge (Liu Q, et al. Current Biology. 8 (24):
                     1300-1309, (1998))"
                     /note="Geneious name: source Cloning vector pUNI51"
     source          <474..>479
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                     Stephen Elledge (Liu Q, et al. Current Biology. 8 (24):
                     1300-1309, (1998))"
                     /note="Geneious name: source Cloning vector pUNI51"
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                /note="Geneious name: AMP-r Promoter"
promoter        3325..3344
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                /note="Geneious name: T3 Promoter"
promoter        complement(514..532)
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                /note="Geneious name: T7 Promoter"
primer_bind     complement(548..570)
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primer_bind     3269..3291
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                /note="Geneious name: M13 Rw-primer"
primer_bind     complement(539..555)
                /created_by="User"
                /note="Geneious name: M13 fw20-primer"
primer_bind     3380..3396
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misc_feature    3364..3369
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                     /note="Geneious name: BamHI"
                     /note="Geneious type: restriction site"
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                     /annotation_group="ACTAGT"
                     /note="Geneious name: SpeI"
                     /note="Geneious type: restriction site"
     misc_feature    <480..484
                     /Recognition_pattern="G^TCGAC"
                     /annotation_group="GTCGAC"
                     /note="Geneious name: SalI"
                     /note="Geneious type: restriction site"
     misc_feature    <480..484
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      121 aacgcagctc tgagaaacgg tgtcgcggtt caaacggtta agaaattcga tgccggttcg
      181 aacaaaaagg ggaaatctac ggcggttccg gtgattaaca cgaagaagct ggaagaagaa
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      361 gtagttcagg aatatgagaa tggtaaagct gttcctaatc aggctgtgct tgcgaagatg
      421 gagaaggttc taggtgttaa acttaggggt aaaattggga aatgaggcct catgggccgt
      481 cgacctcgag gggggcccg gtacccaatt cgccctatag tgagtcgtat tacaattcac
      541 tggccgtcgt tttacaacgt cgtgactggg aaaaccctgg cgttacccaa cttaatcgcc
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      721 gcgcggcggg tgtggtggtt acgcgcagcg tgaccgctac acttgccagc gccctagcgc
      781 ccgctccttt cgctttcttc ccttcctttc tcgccacgtt cgccggcttt ccccgtcaag
      841 ctctaaatcg ggggctccct ttagggttcc gatttagtgc tttacggcac ctcgacccca
      901 aaaaacttga ttagggtgat ggttcacgta gtgggccatc gccctgatag acggtttttc
      961 gccctttgac gttggagtcc acgttcttta atagtggact cttgttccaa actggaacaa
     1021 cactcaaccc tatctcggtc tattcttttg atttataagg gattttgccg atttcggcct
     1081 attggttaaa aaatgagctg atttaacaaa aatttaacgc gaattttaac aaaatattaa
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     1261 ataatattga aaaaggaaga gtatgagtat tcaacatttc cgtgtcgccc ttattccctt
     1321 ttttgcggca ttttgccttc ctgtttttgc tcacccagaa acgctggtga aagtaaaaga
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     1681 caacttactt ctgacaacga tcggaggacc gaaggagcta accgcttttt tgcacaacat
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     2041 ctcccgtatc gtagttatct acacgacggg gagtcaggca actatggatg aacgaaatag
     2101 acagatcgct gagataggtg cctcactgat taagcattgg taactgtcag accaagttta
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     3061 gtcagtgagc gaggaagcgg aagagcgccc aatacgcaaa ccgcctctcc ccgcgcgttg
     3121 gccgattcat taatgcagct ggcacgacag gtttcccgac tggaaagcgg gcagtgagcg
     3181 caacgcaatt aatgtgagtt agctcactca ttaggcaccc caggctttac actttatgct
     3241 tccggctcgt atgttgtgtg gaattgtgag cggataacaa tttcacacag gaaacagcta
     3301 tgaccatgat tacgccaagc tcgaaattaa ccctcactaa agggaacaaa agctggagct
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B.4 IV 1.1-MBF1c XSS sequence data

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LOCUS       3_IV_1.1-MBF1c_X        5047 bp    DNA     circular UNA 26-JUL-2011
DEFINITION  Concatenation of 2 sequences.
ACCESSION
VERSION
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SOURCE      .
  ORGANISM
            .
FEATURES             Location/Qualifiers
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terminator      471..1388
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                /note="Geneious name: amp R (b-lactamase)"
gene            1404..1563
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ligation        461..464
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      181 aaatctacgg cggttccggt gattaacacg aagaagctgg aagaagaaac agagcctgcg
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      361 tatgagaatg gtaaagctgt tcctaatcag gctgtgcttg cgaagatgga gaaggttcta
      421 ggtgttaaac ttaggggtaa aattgggaaa tgagtcgacg agctctgatc tcataagccc
      481 gatggctact aagttttact atttaccaag acttttgaat attaaccttc ttgtaacgag
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      601 ctttaattcc ctaattgtct gttcatttcg ttgtttgttt ccggatcgat aatgaaatgt
      661 aagagatatc atatataaat aataaattgt cgtttcatat ttgcaatctt ttttttacaa
      721 acctttaatc gttgtatgta tgacattttc ttcttgttat attaggggga aataatgtta
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      841 acacctttcc atatacttcc tacatgaagg cattttcaac attttcaaat aaggaatgct
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      961 aagattttat tatagtttcg ttatcttctt ttctcattaa gcgaatcact acctaacacg
     1021 tcattttgtg aaatattttt tgaatgtttt tatatagttg tagcattcct cttttcaaat
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     1381 atagtggacg cattaattaa caattcactg gccgtcgttt tacaacgtcg tgactgggaa
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     1981 taatattgaa aaaggaagag tatgagtatt caacatttcc gtgtcgccct tattcccttt
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     4021 gaccatgatt acgccaagct ggcgcgccaa gcttagacaa acacccccttg ttatacaaag
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B.5 Hypothetical pBlueSK PMUbi1-PMI-TCaMV35SSPolyA sequence data

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LOCUS       6_pBlueSK_PMUbi1        6350 bp    DNA     circular UNA 06-JUL-2011
DEFINITION  Concatenation of 2 sequences.
ACCESSION
VERSION
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SOURCE      .
  ORGANISM
            .
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                     VAGAHPAIAHFLQQPDAERLSELFASLLNMQGEEKSHALAILKSALDSQQGEPWQTIR
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                     LTPKYIDIPELVANVKFEAKPANQLLTQPVKQGAELDFPIPVDDFAFSLHDLSDKETT
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promoter        626..644
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B.6 pBlueSK CaMV35S-ManA-TNOS sequence data

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DEFINITION  Concatenation of 4 sequences.
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VERSION
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SOURCE      .
  ORGANISM
            .
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     2041 taggccacca cttcaagaac tctgtagcac cgcctacata cctcgctctg ctaatcctgt
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     2161 agttaccgga taaggcgcag cggtcgggct gaacgggggg ttcgtgcaca cagcccagct
     2221 tggagcgaac gacctacacc gaactgagat acctacagcg tgagctatga gaaagcgcca
     2281 cgcttcccga agggagaaag gcggacaggt atccggtaag cggcagggtc ggaacaggag
     2341 agcgcacgag ggagcttcca gggggaaacg cctggtatct ttatagtcct gtcgggtttc
     2401 gccacctctg acttgagcgt cgatttttgt gatgctcgtc aggggggcgg agcctatgga
     2461 aaaacgccag caacgcggcc tttttacggt tcctggcctt ttgctggcct tttgctcaca
     2521 tgttctttcc tgcgttatcc cctgattctg tggataaccg tattaccgcc tttgagtgag
     2581 ctgataccgc tcgccgcagc cgaacgaccg agcgcagcga gtcagtgagc gaggaagcgg
     2641 aagagcgccc aatacgcaaa ccgcctctcc ccgcgcgttg gccgattcat taatgcagct
     2701 ggcacgacag gtttcccgac tggaaagcgg gcagtgagcg caacgcaatt aatgtgagtt
     2761 agctcactca ttaggcaccc caggctttac actttatgct tccggctcgt atgttgtgtg
     2821 gaattgtgag cggataacaa tttcacacag gaaacagcta tgaccatgat tacgccaagc
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     3121 atacagtctc agaagaccaa agggcaattg agacttttca acaaagggta atatccggaa
     3181 acctcctcgg attccattgc ccagctatct gtcactttat tgtgaagata gtggaaaagg
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     4321 tgctgcgtgc gggtctgacg cctaaataca ttgatattcc ggaactggtt gccaatgtga
     4381 aattcgaagc caaaccggct aaccagttgt tgacccagcc ggtgaaacaa ggtgcagaac
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     4561 tgtggaaagg ttctcagcag ttacagctta aaccgggtga atcagcgttt attgccgcca
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     4681 gagtcgagga tcgttcaaac atttggcaat aaagtttctt aagattgaat cctgttgccg
     4741 gtcttgcgat gattatcata taatttctgt tgaattacgt taagcatgta ataattaaca
     4801 tgtaatgcat gacgttattt atgagatggg tttttatgat tagagtcccg caattataca
     4861 tttaatacgc gatagaaaac aaaatatagc gcgcaaacta ggataaatta tcgcgcgcgg
     4921 tgtcatctat gttactagat cgggaattaa agcttcac
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B.7 pBlueSK CaMV35S-MBF1c-TNOS sequence data

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VERSION
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SOURCE      .
  ORGANISM
            .
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ligation        3956..3959
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primer_bind     2940..>2952
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misc_feature    2924..2929
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B.8 pPZP101 35s ManA-MBF1c-TNos CaMV35S-MBF1c-TNOS sequence data

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DEFINITION  Concatenation of 3 sequences.
ACCESSION
VERSION
KEYWORDS    .
SOURCE      .
  ORGANISM
            .
FEATURES             Location/Qualifiers
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B.9 pBluSK p35SGUSintTNos sequence data

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LOCUS       C_pBluSK_p35SGUS        6103 bp    DNA     circular UNA 03-SEP-2011
DEFINITION  Concatenation of 2 sequences.
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VERSION
KEYWORDS    .
SOURCE      .
  ORGANISM
            .
FEATURES             Location/Qualifiers
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     5821 gcaagtgtag cggtcacgct gcgcgtaacc accacacccg ccgcgcttaa tgcgccgcta
     5881 cagggcgcgt cccattcgcc attcaggctg cgcaactgtt gggaagggcg atcggtgcgg
     5941 gcctcttcgc tattacgcca gctggcgaaa gggggatgtg ctgcaaggcg attaagttgg
     6001 gtaacgccag ggttttccca gtcacgacgt tgtaaaacga cggccagtga attgtaatac
     6061 gactcactat agggcgaatt gggtaccggg ccccccctcg agg
//
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B.10 pPZP101 35sManATNos-35SGUSintTNos-35SMBF1cTNos sequence data

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LOCUS       E_pPZP101_ManA_G       13445 bp    DNA     circular UNA 30-JUL-2011
DEFINITION  Concatenation of 2 sequences.
ACCESSION
VERSION
KEYWORDS    .
SOURCE      .
  ORGANISM
            .
FEATURES             Location/Qualifiers
     misc_feature    2860..2865
                     /Original_Bases
                     /note="Geneious name: "
                     /note="Geneious type: Editing History Insertion"
     origin          3191..3190
     rep_origin      complement(9774..10393)
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                     /note="Geneious name: pBR322 origin"
     terminator      2882..3134
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     motif           2833..2859
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                     /Motif="AAACCGCAGCAGGGAGGCAAACAATGA"
                     /annotation_group="AAACCGCAGCAGGGAGGCAAACAATGA: 3, 067 ->
                     3, 093"
                     /note="Geneious name: AAACCGCAGCAGGGAGGCAAACAATGA"
     motif           2867..2891
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                     /%_Identity=100
                     /Motif="AATTGATCCGAAGCAGATCGTTCAA"
                     /annotation_group="AATTGATCCGAAGCAGATCGTTCAA: 3, 095 -> 3, 119"
                     /note="Geneious name: AATTGATCCGAAGCAGATCGTTCAA"
     polyA_signal    13163..13415
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                     /note="Geneious name: polyA signal"
     polyA_signal    4926..5178
                     /note="poly(A) signal from nopaline synthase"
                     /note="Geneious name: polyA signal"
     rRNA            1433
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                     /note="Geneious name: RE Site Mod"
     rRNA            1249
                     /created_by="User"
                     /note="Geneious name: RE Site Mod"
     gene            order(862..>1246, <1436..2859)
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                     /locus_tag="b1617"
                     /gene_synonym="ECK1612; gurA; gusA; JW1609"
                     /db_xref="ECOCYC:EG11055"
                     /db_xref="EcoGene:EG11055"
                     /db_xref="GeneID:946149"
                     /modified_by="User"
                     /note="Geneious name: uidA gene"
     gene            complement(10648..11307)
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                     /note="Geneious name: CAT/CamR gene"
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     primer_bind     complement(13436..>13441)
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                     /note="Geneious name: pBluScrSK primer"
     promoter        <7..813
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                     /note="Geneious name: CaMV 35S promoter"
     promoter        12165..>12700
                     /note="CaMV35SS eukaryotic promoter"
                     /note="Geneious name: CaMV35SS eukaryotic promoter"
     promoter        3200..>3735
                     /note="CaMV35SS eukaryotic promoter"
                     /note="Geneious name: CaMV35SS eukaryotic promoter"
     promoter        12072..12101
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                     /note="Geneious name: lac promoter"
     intron          1247..1435
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     intron          order(1419..1420, 1424..1425, 1427..1428, 1430)

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nce__for__plant__introns_(Shapiro___and___Senepathy_1987)
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ligation        12161..12164
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ligation        5185..5188
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ligation        3191..3194
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ligation        7..10
                /note="Geneious name: Ligation"
ligation        3152..3155
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ligation        13430..13433
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                /db_xref="GeneID:8113180"
                /translation="MQKLINSVQNYAWGSKTALTELYGMENPSSQPMAELWMGAHPKS
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                VAGAHPAIAHFLQQPDAERLSELFASLLNMQGEEKSHALAILKSALDSQQGEPWQTIR
                LISEFYPEDSGLFSPLLLNVVKLNPGEAMFLFAETPHAYLQGVALEVMANSDNVLRAG
                LTPKYIDIPELVANVKFEAKPANQLLTQPVKQGAELDFPIPVDDFAFSLHDLSDKETT
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CDS             7753..8859
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                RIEAEARPGESWQAAADRIRKESRQPPAAGAPSIRKPPKGDEQPDFFVPMLYDVGTRD
                SRSIMDVAVFRLSKRDRRAGEVIRYELPDGHVEVSAGPAGMASVWDYDLVLMAVSHLT
                ESMNRYREGKGDKPGRVFRPHVADVLKFCRRADGGKQKDDLVETCIRLNTTHVAMQRT
                KKAKNGRLVTVSEGEALISRYKIVKSETGRPEYIEIELADWMYREITEGKNPDVLTVH
                PDYFLIDPGIGRFLYRLARRAAGKAEARWLFKTIYERSGSAGEFKKFCFTVRKLIGSN
                DLPEYDLKEEAGQAGPILVMRYRNLIEGEASAGS*"
                /note="Geneious name: ORF frame 3"
CDS             complement(10648..11448)
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                /translation="MTCFFGVQSMPRASKQQARYAVGRCLMLWSSNDVTQQGSRPKTK
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                RQFLHIYSQDVACYGENLAYFPKGFIENMFFVSANPWVSFTSFDLNVANMDNFFAPVF
                TMGKYYTQGDKVLMPLAIQVHHAVCDGFHVGRMLNELQQYCDEWQGGA*"
                /note="Geneious name: ORF frame 3"
CDS             5744..6430
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                DTTTPAGRMVLTVFAGIAEFERSLIIDRTRSGREAAKARGVKFGPRPTLTPAQIAHAR
                ELIDQEGRTVKEAAALLGVHRSTLYRALERSEEVTPTEARRRGAFREDALTEADALAA
                AENERQEEQA*"
                /note="Geneious name: ORF frame 1"
CDS             6729..7358
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                     IPVQPSPYDIWATADLVELVKQRIEVTDGRLQAAFVVSRAIKGTRIGGEVAEALAGYE
                     LPILESRITQRVSYPGTAAAGTTVLESEPEGDAAREVQALAAEIKSKLI*"
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     CDS             12707..13153
                     /note="'ethylene-responsive transcriptional coactivator,
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                     /db_xref="GI:28466837"
                     /translation="MPSRYPGAVTQDWEPVVLHKSKQKSQDLRDPKAVNAALRNGVAV
                     QTVKKFDAGSNKKGKSTAVPVINTKKLEEETEPAAMDRVKAEVRLMIQKARLEKKMSQ
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                     /note="Geneious name: lacZ a"
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                     /label="M13_pUC_fwd_primer"
                     /note="Geneious name: M13 pUC fwd primer"
     misc_feature    12115..12137
                     /label="M13_pUC_rev_primer"
                     /note="Geneious name: M13 pUC rev primer"
     misc_feature    complement(9355..9377)
                     /label="pGEX_3_primer"
                     /note="Geneious name: pGEX 3 primer"
     misc_feature    12136..12154
                     /label="M13_reverse_primer"
                     /note="Geneious name: M13 reverse primer"
     misc_feature    complement(5193..5209)
                     /label="M13_forward20_primer"
                     /note="Geneious name: M13 forward20 primer"
     misc_feature    2860..2865

/X._Barandiaran_·_A._Di_Pietro_·_J._Martín_(Plant_Cell_Reports_1998)_EcoRI_Restr
iction_Site_is_inserted_instead_of_NNNNNN
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                     sequence 2)"
                     /note="Geneious type: Concatenated sequence"
     misc_feature    3195..13429
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                     sequence 1)"
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     misc_feature    order(1673..>3151, <7..1246)
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                     sequence 2)"
                     /note="Geneious type: Concatenated sequence"
     misc_feature    11..3151
                     /note="Geneious name: A7F SalI NNNNNN Modified extraction
                     (concatenated sequence 1)"
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     misc_feature    1247..1672
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                     1)"
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     misc_feature    <1247..>1672
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                     (concatenated sequence 2)"
                     /note="Geneious type: Concatenated sequence"
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misc_feature    1436..>1672
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                /annotation_group="CCCGGG"
                /note="Geneious name: XmaI"
                /note="Geneious type: restriction site"
misc_feature    13429..13434
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                /annotation_group="CCCGGG"
                /note="Geneious name: XmaI"
                /note="Geneious type: restriction site"
misc_feature    3190..3195
                /Recognition_pattern="CCC^GGG"
                /annotation_group="CCCGGG"
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                /note="Geneious type: restriction site"
misc_feature    13429..13434
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                /annotation_group="CCCGGG"
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                /note="Geneious type: restriction site"
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                /annotation_group="ACTAGT"
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misc_feature    1556..1561
                /Recognition_pattern="G^GATCC"
                /annotation_group="GGATCC"
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                /note="Geneious type: restriction site"
misc_feature    13435..13440
                /Recognition_pattern="G^GATCC"
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                /annotation_group="CTGCAG"
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misc_feature    3184..3189
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                /annotation_group="CTGCAG"
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misc_feature    6..11
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                /annotation_group="GTCGAC"
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                /note="Geneious type: restriction site"
misc_feature    3151..3156
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                /annotation_group="GTCGAC"
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                /note="Geneious type: restriction site"
misc_feature    3145..3150
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                /annotation_group="TCTAGA"
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                /note="Geneious type: restriction site"
misc_feature    2860..2865
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misc_feature    3178..3183
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     1141 tacggcaaag tgtgggtcaa taatcaggaa gtgatggagc atcagggcgg ctatacgcca
     1201 tttgaagccg atgtcacgcc gtatgttatt gccgggaaaa gtgtacgtat gtttctgctt
     1261 ctacctttga tatatatata ataattatca ttaattagta gtaatataat atttcaaata
     1321 ttttttttcaa aataaaagaa tgtagtatat agcaattgct tttctgtagt ttataagtgt
     1381 gtatatttta atttataact tttctaatat atgaccaatt catttttttt ttcaggtatc
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     1741 agcgggactt tgcaagtggt gaatccgcac ctctggcaac cgggtgaagg ttatctctat
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     1861 atccggtcag tggcagtgaa gggcgaacag ttcctgatta accacaaacc gttctacttt
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5041 gcatgacgtt atttatgaga tgggtttta tgattagagt cccgcaatta tacatttaat
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    13441 actag
//
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B.11 Primer Sequences Used in this Study

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>GUSint_FW      GGATCTAGAA CCATGGTAGA TCTGAGGGTA AA
>GUSint_RW      GTCGTCGACT CACACGTGAT GGTGATGGTG ATGG

>ManA_FW        CACTCTAGAA TGCAAAAACT CATTAACTCA GTGCAA
>ManA_RW        ATAGTCGACT CAGAGTTTGT TGTAAACACG CGCTAAAC

>MBF1c_FW       ACTCTAGAAT GCCGAGCAGA TACCCA
>MBF1c_RW       AGTGTCGACT CATTTCCCAA TTTTACCC

>p35s_DE_FW     GGGATCCCGG GAATTCTGCG TATTGGCTAG AGCA
>p35s_DE_RW     TGCGAACTAG TGAGAGAGAT AGATTTGTAG AGAG

>p35s_FW        GGGATCCCGG GAATTCATGG AGTCAAAGAT TCAAA
>p35s_RW        GTCACTAGTC CCCCGTGTTC TCTCC

>tPolyA_FW      ATCTCGAGGA TCGTTCAAAC ATTTGGC
>tPolyA_RW      TCCCCGGGTG AAGCTTTAAT TCCCGATCTA GT

>MBF1c_LR_FW    CACCATGCCG AGCAGATACC CAGGA
>MBF1c_LR_RW    TCATTTCCCA ATTTTACCCC TAAGTTTAAC ACC

>MBF1c_PCR8_FW ATGCCGAGCA GATACCCAGG AGCA
>MBF1c_PCR8_RW TCATTTCCCA ATTTTACCCC TAAGTTTAAC ACC
```

APPENDIX C

PERMISSION LETTERS AND DECLERATIONS FOR INTELLECTUAL AND COMMERCIAL PROPERTIES

C.1 Permission Letter for pTJK136

Laboratorium Genetica
Vakgroep Moleculaire Genetica
K. L. Ledeganckstraat 35
B-9000 Gent, BELGIE
Tel. 32(0)9-2645170/71
Fax. 32(0)9-264 53 49

Gent, 24.08.00

Dear Prof.Dr.Hüsevin Avni ÖKTEM

Please find enclosed the requested material. I would appreciate if you fill in the declaration and send it by return post to me.

Map included

If you need more information, don't hesitate to contact me.

Yours sincerely

Christiane Geneteilo
e-mail: chgengengenp.rug.ac.be

C.2 Letter for pUNI51 Stock of MBF1c Coding Sequence

Arabidopsis Biological Resource Center
DNA Stock Center
Dr. Emma Knee, Research Scientist
Zhen Zhang Research Associate
Natalie Case, Research Associate

1060 Carmack Road
Columbus, OH 43210-1002

Phone: (614) 292-2988
FAX: (614) 292-5379
E-mail: abrc@arabidopsis.org
URL: http://www.arabidpsis.org/

Hamdi Kamci
Orta Dogu Teknik Universitesi
Biyolojik Bilimler Binasi Lab.No: 209
Balgat Ankara TURKEY
Ankara 06531
Turkey

July 23, 2009

DNA Order 70264: U23216

Dear Kamci,

Thank you for your order. If your order included seed stocks, they will be shipped separately.

DISCLAIMER: In order to reduce the need for Material Transfer Agreements and other paperwork, stocks are sent to you with the understanding that:
1. The service is treated as an academic collaboration without issues of indemnity.
2. Although ABRC makes every reasonable attempt to ensure the correctness of stocks, ABRC and the donors of the stock do not imply any warranty whatsoever regarding the material and its use, nor do they guarantee (i) that the material or its use does not infringe on any rights or claims from third parties; or (ii) the material's suitability, novelty or safety for any purpose whatsoever.
3. Any fees that we assess are substantially less than the real cost of storing, producing and distributing the material and therefore the service is not-for-profit and not commercial and all costs passed to you represen partial recovery of our production costs.
4. You have read the available information published on this stock, including our stock descriptions, and are aware of the nature of the material that you have ordered including any special conditions, regulations and requirements for your local handling of the material.
If you are unhappy about any of the above conditions, return the unopened material to us in the original packaging for a full refund (if applicable). We strongly encourage you to characterize all stocks shortly after receipt. Your confirmation/validation of diagnostic results may be useful to other users, so please send them to us if possible.

Please consider donating seed lines and constructed in your laboratory to ABRC. See www.arabidopsis.ora/submit/abrc_submission.jsp for details

Sinerely,
Emma Knee

C.3 Letter for pEarleyGate100-104 Clones

Arabidopsis Biological Resource Center
DNA Stock Center

Dr. Emma Knee, Research Scientist
Zhen Zhang Research Associate
Natalie Case, Research Associate

1060 Carmack Road
Columbus, OH 43210-1002

Phone: (614) 292-2988
FAX: (614) 292-5379
E-mail: abrcarabidopsis.org
URL: http://www.abrc.osu.edu/

Hamdi Kamci
Orta Dogu Teknik Universitesi
Biyolojik Bilimler Binasi Lab.No: 209
Balgat Ankara TURKEY
Ankara 06531
Turkey

October 7, 2010

Order 79597: CD3-683 CD3-684 CD3-685 CD3-686 CD3-724 (Gateway™ clones)

Dear Kamci,

Thank you for your order.

DISCLAIMER: In order to reduce the need for Material Transfer Agreements and other paperwork, stocks are sent to you with the understanding that:
1. The service is treated as an academic collaboration without issues of indemnity.
2. Although ABRC makes every reasonable attempt to ensure the correctness of stocks, ABRC and the donors of the stock do not imply any warranty whatsoever regarding the material and its use, nor do they guarantee (i) that the material or its use does not infringe on any rights or claims from third parties; or (ii) the material's suitability, novelty or safety for any purpose whatsoever.
3. Any fees that we assess are substantially less than the real cost of storing, producing and distributing the material and therefore the service is not-for-profit and not commercial and all costs passed to you represent partial recovery of our production costs.
4. You have read the available information published on this stock, including our stock descriptions, and are aware of the nature of the material that you have ordered including any special conditions, regulations and requirements for your local handling of the material.
If you are unhappy about any of the above conditions, return the unopened material to us in the original packaging for a full refund (if applicable). We strongly encourage you to characterize all stocks shortly after receipt. Your confirmation/validation of diagnostic results may be useful to other users, so please send them to us if possible. Please consider donating seed lines and constructed in your laboratory to ABRC. See www.arabidopsis.org/submit/abrc_submission.jsp for details

Sinerely,
Emma Knee

C.4 Limited Use Label License Letter for pEarleyGate100-104 Clones

Limited Use Label License

This product and its use is the subject of one or more of U.S. Patent Nos. 5,888,732, 6,143,557, 6,171,861, 6,270,969, and 6,277,608 and/or other pending U.S. and foreign patent applications owned by Invitrogen Corporation. The purchase of this product conveys to the buyer the non-transferrable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for profit entity). The purchase of this product does not convey a license under any method claims in the foregoing patents or patent applications, or to use this product with any recombination sites other than those purchased from Invitrogen Corporation or its authorized distributor. The right to use methods claimed in the foregoing patents or patent applications with this product for research purposes only can only be acquired by the use of Clonase™ purchased from Invitrogen Corporation or its authorized distributors. The buyer cannot modify the recombination sequence(s) contained in this product for any purpose. The buyer cannot sell or otherwise transfer (a) this product, (b) its components, or (c) materials made by the employment of this product or its components to a third party or otherwise use this product or its components or materials made with this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) not to transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Transfer of such materials and/or information to collaborators does not convey rights to practice any methods claimed in the foregoing patents or patent applications. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. Invitrogen Corporation will not assert a claim against the buyer of infringement of the above patents based upon the manufacture, use or sale of a therapeutic, clinical diagnostic, vaccine or prophylactic product developed in research by the buyer in which this product or its components was employed, provided that none of (j)) this product, (ii) any of its components, or (iii) a method claim of the foregoing patents, was used in the manufacture of such product. Invitrogen Corporation will not assert a claim against the buyer of infringement of the above patents based upon the use of this product to manufacture a protein for sale, provided that no method claim in the above patents was used in the manufacture of such protein. For information on purchasing a license to use this product for purposes other than those permitted above, contact Licensing Department, Invitrogen Corporation, 1600 Faraday Avenue, Carlsbad, California 92008. Phone (760) 603-7200.

APPENDIX D

RESTRICTION DIGESTS AND PCR REACTION TABLES

Table D.1: Restriction digest of GUSint and nptII cassette for pBlueSK+cloning

	GUSint cassette cloning				nptII cassette cloning			
Vector	pTJK136	3 µl	pBlueSK+	3 µl	pTJK136	3 µl	pBlueSK+	3 µl
Fragment	GUSint		SalI		nptII		BamHI	
Buffer (10X)		2.5 µl		2.5 µl		2.5 µl		2.5 µl
enzyme	SalI	1 µl	SalI	1 µl	BamHI	1 µl	BamHI	1 µl
FAP		-		0.5 µl		-		0.5 µl
supWater		18.5 µl		18.5 µl		18.5 µl		18.5 µl

Table D.2: Fast ligation of GUSint and nptII cassettes sub-coning into pBlueSK+

Ligation of	GUSint and pBlueSK+	nptII and pBlueSK+
Cassette & Vector Mix	6 µl	6 µl
5X Rapid Lig. Buff.	5 µl	5 µl
Roche T4 DNA Lig.	1 µl	1 µl
supH2O	13 µl	13 µl

Table D.3: pBlueSK+ and pPMI-GFP HindIII digest mix compositions
FAP (fast alkaline phosphatase) was also added to pBlueSK+ digest mix, to prevent circularization during ligation reaction.

HindIII Digestion of pBlueSK+			HindIII recovery of PMI		
Vector	pBlueSK+	6 µl	Vector	pPMI-GFP	6 µl
Fragment	-		Fragment	PMI cassette	
10X Buffer Red		2.5 µl	10X Buffer Red		2.5 µl
HindIII		1 µl	HindIII		1 µl
FAP		1 µl	FAP		-
supH_2O		14.5 µl	supH_2O		15.5 µl

Table D.4: Rapid ligation of PMI cassete into pBlueSK+
Since the rapid ligation buffer was old extra ribo-ATP added.

pBlueSK+ and PMI Ligation	
PMI Cassette HindIII Fragment	4 µl
pBluscriptSK+ HindIII Fragment	3 µl
50 mM ATP	2 µl
5X Rapid Lig. Buff.	5 µl
Roche T4 DNA Lig.	1 µl
supH_2O	10 µl

Table D.5: PCR reaction mixtures for CaMV35SS, ManA and TNOS amplifications

P35S; ManA; TNOS PCR Rxn. Mix						
	Final [c]	P 35S/ TNOS			ManA	
Herculase Buffer (5X)	1x		5 µl	5 µl		5 µl
Template	-	pCambia1305.1	1 µl	1 µl	pPMI-GFP	1 µl
Primer Mix (25µM)	0.5µM		(P35S) 1 µl	(TNOS) 1 µl	ManA	1 µl
dNTP Mix (1 mM)	0.25mM		4 µl	4 µl		4 µl
Herculase Enzyme (5u/µl)	2.5u		0.5 µl	0.5 µl		0.5 µl
supH_2O			13.5 µl	13.5 µl		13.5 µl

Table D.6: TD-PCR reaction program for CaMV35SS, ManA and TNOS amplifications

MBF1c (XSS) TD-PCR Program				
Initial Denaturation		96ºC	4 min	
TD Step				
2X for each annealing T	Denaturation	94ºC	45 sec	TD annealing by -1ºC
	Annealing	65-51ºC	90 sec	
	Extension	74ºC	45 sec	
Log Step				
10X	Denaturation	94ºC	45 sec	
	Annealing	50ºC	45 sec	
	Extension	74ºC	45 sec	
Final Extension		74ºC	5 min	

Table D.7: Double digest buffer selections for P35S, ManA and TNOS digests

		Double Digest enzymes	Buffers	% Activity
Vector and Fragments	pBlueSK	BamHI & XmaI	Neb 4	100 & 100
	P CamV35S	BamHI & BcuI(SpeI)	Buffer BamHI	100 & 50-100
	ManA cds	XbaI & SalI	Neb 3	75 & 100
	T NOS	XhoI & XmaI	Neb 4	100 & 100

Table D.8: Restriction reaction mixtures for PCR amplified fragments and vector

pBlueSK+		P CamV35S		ManA		T NOS	
Vector	6 µl	Amplicon	33 µl	Amplicon	30 µl	Amplicon	30 µl
Buffer NEB -4	3 µl	Buffer BamHI	4 µl	Buffer NEB-3	4 µl	Buffer NEB-4	4 µl
BSA (10x)	3 µl	BSA (10x)	-	BSA (10x)	4 µl	BSA (10x)	4 µl
BamHI	1 µl	BamHI	1 µl	XbaI	1 µl	XhoI	1 µl
XmaI	1 µl	BcuI	2 µl	SalI	1 µl	XmaI	1 µl
H_2O	16 µl	H_2O	-	H_2O	-	H_2O	-
∑ V	30 µl	∑ V	40 µl	∑ V	40 µl	∑ V	40 µl

Table D.9: Ligation reaction mixture for PCaMV35SS-ManA-TNOS cassette in pBlueSK+

Rapid Ligation Mix. For 35S-ManA-NOS Cassette		
5X Rapid Ligation Buffer		3 µl
pBlueSK+	digest mixes colected and co-eluted	11 µl
CamV35S Amplicon		
ManA Amplicon		
T NOS Amplicon		
T4-DNA Ligase		1 µl

Table D.10: PCR reaction mixture for CaMV35SS-ManA-NOS cassette detection

PCR Mix for CamV35S-ManA-NOS Cassette Detection		
	Final [c]	1X
dNTP mix	1 mM	5 µl
10X Taq Buffer (NH4)	1X	2.5 µl
$MgCl_2$	25 mM	2 µl
Primer 1 (CamV35S FW)	0.25 µM	0.25 µl
Primer 2 (NOS RW)	0.25 µM	0.25 µl
Taq Polymerase	1.25 unit	0.25 µl
H_2O	-	14.75 µl
∑ V	25 µl	

Table D.11: PCR program for CaMV35SS-ManA-NOS cassette detection

PCR Program for CamV35S-ManA-NOS Amlificaiton			
Initial Denaturation		94ºC	4 min
Log Step			
35 Cycles	Denaturation	94ºC	35 sec
	Annealing	60ºC	35 sec
	Extension	74ºC	2' 30 sec
Final Extension		74ºC	5 min

Table D.12: Restriction reaction mixes for pUNI51-MBF1c cds clone analysis

	MBF1c insert size analysis			HincII direction analysis	
Vector	pUNI51-MBF1c	4 µl	Vector	pUNI51-MBF1c	4 µl
Fragment	MBF1c		Fragment	MBF1c	
Buffer (10X)	O	2.5 µl	Buffer (10X)	Buffer Tango 1X	2 µl
enzyme 1	EcoRI	1 µl	enzyme	HincII	1 µl
enzyme 2	NotI	1 µl	supWater		13 µl
supWater		16.5 µl			

Table D.13: EcoRI-SalI restriction digestion mixes MBF1c sub-cloning into pBlueSK+

pBlueSK+ EcoRI-SalI Double Digestion			MBF1c EcoRI-SalI Double Digestion		
Vector	pBlueSK+	6 µl	Vector	pUNI51-MBF1c	6 µl
Fragment	-		Fragment	MBF1c	
Buffer O (10X)		2.5 µl	Buffer O (10X)		2.5 µl
EcoRI		1 µl	EcoRI		1 µl
SalI		1 µl	SalI		1 µl
supH_2O		14.5 µl	supH_2O		14.5 µl

Table D.14: pBlueScriptSK+ and MBF1c cds ligation reaction mix
Note that EcoRI-SalI restriction sites were utilized for directional cloning

MBF1c, pBlueSK+ Ligation Rxn. Mix.		
EcoRI-SalI Fragments	MBF1c cds	4 µl
	pBlueSK+	3 µl
50 mM ATP		2 µl
5X Rapid Lig. Buff		5 µl
Roche T4 DNA Lig.		1 µl
supH_2O		10 µl

Table D.15: Restriction mixes for the first digests of pBlueSK-MBF1c and IV 1.1

	MBF1c 1st Digest			IV 1.1 1st Digest	
Vector	pBlueSK-MBF1c	5 µl	Vector	IV 1.1	5 µl
Fragment	MBF1c		Fragment	-	
Buffer R (10X)		2 µl	Buffer Tango (10X)		2 µl
XhoI		1 µl	BglII		1 µl
supH_2O		12 µl	supH_2O		12 µl

Table D.16: Blunting of XhoI and BglII digests of pBlueSK-MBF1c and IV 1.1
Final concentration of dNTP mix was 150 µM. Blunting reaction was carried out at 11°C for 20 min.

	XhoI Blunting		BglII Blunting	
Digest Mix	pBlueSK-MBF1c XhoI digest	20 µl	IV 1.1 BglII digest	20 µl
dNTP mix		0.5 µl		0.5 µl
T4 DNA Polymerase		1 µl		1 µl

Table D.17: Compositions of second restriction digest mixes of pBlueSK-MBF1c and IV 1.1

	MBF1c 2nd Digest			IV 1.1 2nd Digest	
Blunted Fragment	MBF1c 1st Digest Elution	27 µl	Blunted Fragment	IV 1.1 1st Digest Elution	27 µl
Buffer Eco52I (10X)		3 µl	Buffer Tango (10X)		3 µl
Eco52I		1 µl	NotI		1 µl

Table D.18: Ligation reaction mix for IV 1.1-MBF1c cassette construction
Ligation was performed with T4 DNA Ligase in total volume of 20 µl at 16°C overnight.

MBF1c, IV 1.1 Ligation Rxn. Mix.	
Eco52I-MBF1c-Blunt (XhoI) Fragment	6 µl
NotI-IV 1.1-Blunt (BglII) Fragment	6 µl
10X Ligation Buffer	2 µl
T4 DNA Ligase	1 µl
supH_2O	5 µl

Table D.19: TD-PCR reaction mix for MBF1c amplification with MBF1c XSS primers
Note that there is no MgCl2 component in the table. It is supplied in Herculase buffer mix at 2mM final concentration

MBF1c (XSS) PCR Rxn. Mix		
	Final [c]	V
Herculase Buffer (5X)	1x	5 µl
Template(pBlueSK-MBF1c)	-	3 µl
Primer Mix (25µM)	0.25µM	2.5 µl
dNTP Mix (2mM)	0.2mM	2.5 µl
Herculase Enzyme (5u/µl)	2.5u	0.5 µl
supH_2O		11.5 µl

Table D.20: The TD-PCR program for MBF1c amplification with MBF1c XSS primers Consider Primer-3 generated Tm values of 55.85oC for Fw and 59.94oC for Rw primers. According to these Tm values Touch Down (TD) step annealing temperature was started from 60°C and ended at 55°C with 1°C decrements and each temperature step was cycled for 5 times. At the logarithmic step annealing temperature was set to 54°C.

MBF1c (XSS) TD-PCR Program				
Initial Denaturation		94ºC	4 min	
TD Step				
5X for each annealing T	Denaturation	94ºC	35 sec	TD annealing by -1ºC
	Annealing	60-55ºC	35 sec	
	Extension	74ºC	35 sec	
Log Step				
20X	Denaturation	94ºC	35 sec	
	Annealing	54ºC	35 sec	
	Extension	74ºC	35 sec	
Final Extension		74ºC	5 min	

Table D.21: XbaI-SacI double digest reaction of MBF1c and IV 1.1

MBF1c XSS XbaI-SacI Double Digestion			IV 1.1 XbaI-SacI Double Digestion		
Amplicon	MBF1c XSS	10 µl	Vector	IV 1.1	6 µl
Buffer NEB4 (10X)		3 µl	Buffer NEB4 (10X)		3 µl
BSA (10X)		3 µl	BSA (10X)		3 µl
XbaI		1 µl	XbaI		1 µl
SacI		1 µl	SacI		1 µl
supH_2O		12 µl	supH_2O		16 µl

Table D.22: Ligation reaction mix for XbaI-SacI digested MBF1c XSS amplicon and IV 1.1

MBF1c XSS and IV 1.1 Ligation Rxn. Mix.	
MBF1c XSS XbaI-SacI Double Digest	4 µl
IV 1.1 XbaI-SacI Double Digest	7 µl
5X Rapid Ligation Buffer	3 µl
T4 DNA Ligase	1 µl

Table D.23: Colony PCR reaction mix compositions for IV 1.1-MBF1c XSS clone detection.

MBF1c (XSS) Colonyn PCR Rxn. Mix		
	Final [c]	V
Colony	-	-
10X Taq Buffer	1X	2.5 µl
1 mM dNTP Mix	0.2mM	5 µl
25 mM $MgCl_2$	2mM	2 µl
25µM Primer mix XSS	0.5µM	0.5 µl
Taq DNA Polymerase	1.25u	0.25 µl
supH_2O		13.75 µl

Table D.24: Colony PCR program of IV 1.1-MBF1c XSS clone detection

MBF1c (XSS) Colony PCR Program			
Initial Denaturation		94ºC	4 min
Log Step			
35 Cycles	Denaturation	94ºC	35 sec
	Annealing	58ºC	35 sec
	Extension	74ºC	45 sec
Final Extension		74ºC	5 min

Table D.25: XbaI-SalI digestion mixture for MBF1c XSS amplicon
The PCR amplified MBF1c fragment is cleaned with PCR cleaning kit and eluted into 40 µ of total volume then digestion was proceeded at 37°C overnight.

MBF1c XSS Digest	
MBF1c XSS	30 µl
Buffer NEB-3	4 µl
BSA (10x)	4 µl
XbaI	1 µl
SalI	1 µl
H_2O	-
∑ V	40 µl

Table D.26: The ligation mix for CaMV35SS-MBF1c-NOS cassette construction

Rapid Ligation Mix. For 35S-ManA-NOS Cassette		
5X Rapid Ligation Buffer		3 µl
pBlueSK+	digest mixes colected and co-eluted	11 µl
CamV35S Amplicon		
MBF1c XSS Amplicon		
T NOS Amplicon		
T4-DNA Ligase		1 µl

Table D.27: PCR reaction mixture for MBF1c coding sequence amplification

Colony-PCR Mix for CamV35S-MBF1c-NOS Clone Detection		
	Final [c]	1X
Template	-	5 µl
dNTP mix	1 mM	5 µl
10X Taq Buffer (NH4)	1X	2.5 µl
$MgCl_2$	25 mM	2 µl
MBF1c XSS Primer mix (25µM)	0.25 µM	0.5 µl
Taq Polymerase	2.5 unit	0.5 µl
H_2O	-	9.5 µl
∑ V	25 µl	

Table D.28: PCR program for MBF1c detection of pBlueSK 35S-MBF1c-NOS clones

Colony-PCR Program for MBF1c Detection			
Initial Denaturation		94ºC	4 min
Log Step			
35 Cycles	Denaturation	94ºC	35 sec
	Annealing	65ºC	35 sec
	Extension	74ºC	45 sec
Final Extension		74ºC	5 min

Table D.29: Double digest buffers for P35S ManA/MBF1c NOS cassette recoveries Buffers were selected on the basis that maintains highest activity for both of the restriction enzymes

		Double Digest	Buffers	% Activity
Vectors	pPZP101	EcoRI & HindIII	Neb 2	100 & 100
	pBlueSK 35S MAnA NOS	HindIII & XmaI	Neb 4	50 & 100
	pBlueSK 35S MBF1c NOS	EcoRI & XmaI	Neb 4	100 & 100

Table D.30: Restriction digest mixes of pPZP101, pBlueSK 35S ManA/MBF1c NOS Digestion was carried out at 37°C overnight. Critical event of Digestion was pBlueSK 35S ManA NOS double digest the vector was first digested with EcoRI overnight and then with XmaI since EcoRI and XmaI restriction sites at 5′ end of the cassette are overlapping (mentioned in figure 3.41 in section 3.1.6.3.2.1).

pPZP101		pBlueSK35S ManA NOS		pBlueSK35S ManA NOS	
Vector	6 µl	Vector	6 µl	Vector	6 µl
Buffer NEB -2	4 µl	Buffer NEB -4	3 µl	Buffer NEB -4	4 µl
BSA (10x)	4 µl	BSA (10x)	3 µl	BSA (10x)	4 µl
EcoRI	1 µl	EcoRI	1 µl	HindIII	2 µl
HindIII	1 µl	XmaI	1 µl	XmaI	1 µl
H_2O	24 µl	H_2O	16 µl	H_2O	23 µl
∑ V	40 µl	∑ V	30 µl	∑ V	40 µl

Table D.31: PCR mixes for P35S-ManA-NOS and P35S-MBF1c-NOS amplifications
Amplifications to be made for analysis of the putative pPZP101 ManA-MBF1c clones.

Colony-PCR Mix for CamV35S-ManA/MBF1c-NOS Cassette Amplificaitons		
	Final [c]	1X
Template	-	5 µl
dNTP mix	1 mM	5 µl
10X Taq Buffer (NH4)	1X	2.5 µl
$MgCl_2$	25 mM	2 µl
35S Fw Primer (50 µM)	0.25 µM	0.125 µl
NOS Rw Primer (50 µM)	0.25 µM	0.125 µl
Taq Polymerase	2.5 unit	0.5 µl
H_2O	-	9.75 µl
∑ V	25 µl	

Table D.32: PCR program for P35S-ManA-NOS and P35S-MBF1c-NOS amplifications
Amplifications to be made for analysis of the putative pPZP101 ManA-MBF1c clones.

Colony-PCR Program for CamV35S-ManA/MBF1c-NOS Cassette Detection			
Initial Denaturation		94ºC	4 min
Log Step			
35 Cycles	Denaturation	94ºC	35 sec
	Annealing	70ºC	35 sec
	Extension	74ºC	1' 30 sec
Final Extension		74ºC	5 min

Table D.33: PCR reaction mixes for ManA and MBF1c cds amplifications
Amplifications to be made for analysis of the putative pPZP101 ManA-MBF1c clones.

Colony-PCR Mix for ManA and MBF1c Amplificaitons		
	Final [c]	1X
Template	-	5 µl
dNTP mix	1 mM	5 µl
10X Taq Buffer (NH4)	1X	2.5 µl
$MgCl_2$	25 mM	2 µl
ManA Primer Mix (25 µM)	0.25 µM	0.25 µl
MBF1c Primer Mix (25 µM)	0.25 µM	0.25 µl
Taq Polymerase	2.5 unit	0.5 µl
H_2O	-	9.5 µl
∑ V	25 µl	

Table D.34: PCR program for ManA and MBF1c cds amplifications
Amplifications to be made for analysis of the putative pPZP101 ManA-MBF1c clones.

Colony-PCR Program for ManA and MBF1c Detection			
Initial Denaturation		94ºC	4 min
Log Step			
35 Cycles	Denaturation	94ºC	35 sec
	Annealing	66ºC	35 sec
	Extension	74ºC	1' 30 sec
Final Extension		74ºC	5 min

Table D.35: Restriction digest mixtures for EcorI and HindIII digests of PCR positive clones

EcoRI digest		HindIII digest	
	1X		1X
plasmids for clones 1-6	5 µl	plasmids for clones 1-6	5 µl
Buffer EcoRI	2.5 µl	Buffer Red	2.5 µl
EcoRI	0.5 µl	HindIII	0.5 µl
H_2O	17 µl	H_2O	17 µl
∑ V	25 µl	∑ V	25 µl

Table D.36: The PCR reaction mixtures for MBF1c, ManA and GUSint amplificaitons Amplifications are for three puative clones of pPZP101 ManA-MBF1c and a single clone of pBLueSK+GUSint. "No template" control was only carried with the pBlueSK+GUSint clone only.

Reaction Mixtures for PCR Check of GUSint, ManA & MBF1c Seqeunces						
Components	**Final [c]**	**1X V**	**M.Mix (9X V)**	**MBF1c (3X)**	**ManA (3X)**	**GUSint (2X)**
Template	-	0.5 µl	-	0.5 µl	0.5 µl	0.5 µl
1mM dNTP mix	1 mM	5 µl	45 µl			
10X Taq Buffer (NH4)	1X	2.5 µl	22.5 µl			
25mM MgCl2	25 mM	2 µl	18 µl			
25µM Primer mix	0.25 µM	0.25 µl	-	0.75 µl	0.75 µl	0.50 µl
Taq	0.125 u	0.25 µl	2.25 µl			
H2O	-	14.5 µl	135 µl			
Total Volumes(µl)	-	25 µl	222.75 µl	75 µl from M.Mix	75 µl from M.Mix	50 µl from M.Mix

Table D.37: The PCR program for MBF1c, ManA and GUSint amplificaitons
Note that amplifications were carried in single run and with single annealing temperature (58°C). Compared to the Tm values of primer sets PCR reaction annealing temperature was approximately 5-10°C below.

PCR Program for GUSint, ManA and MBF1c Analysis			
		Temperature	**Time**
Initial Denaturation		94ºC	4 min
Amplification Step			
Denaturation	35 Cycles	94ºC	35 sec
Annealing		58ºC	35 sec
Extension		74ºC	120 sec
Final Extension		74ºC	5 min

Table D.38: XmaI digest mix for pPZP101 ManA-MBF1c and pBlueSK+GUSint vectors
Note that calf intestinal alkaline phosphatase (CIP) was added to restriction mix of pPZP101 MM digest in order to prevent self circularisation during liantion reaction.

Cfr9I(XmaI) Digest Mixes for pPZP101 ManA-MBF1c & pBlueSKGUSint				
Components	**1X**	**M.Mix (2X V)**	**pPZP101 MM**	**pBlueSKGUSint**
DNA	7 µl	-	7 µl	7 µl
Buffer Cfr9I	4 µl	8 µl	-	-
Cfr9I	2 µl	4 µl	-	-
CIP	-	-	1 µl	-
H2O	27 µl	54 µl	-	-
Total Volume	40 µl	66 µl	33 µl from M.Mix	33 µl from M.Mix

Table D.39: BcuI-XhoI digest mixes for pPZP101 ManA-MBF1c and pBlueSK+GUSint
Note that vectors are first digested with BcuI in 1X Tango Buffer and then XhoI digest was performed within the same reaction mix through completion of Tango buffer strenght to 2X. Also note that the activities of BcuI and XhoI enzymes are 100% in 1X and 2X Tango buffers respectively

BcuI(SpeI)-XhoI Digest Mixes for pPZP101 ManA-MBF1c & pBlueSKGUSint					
	First, BcuI Digest				Second, XhoI Digest
Components	**1X**	**M.Mix (2X V)**	**pPZP101 MM**	**pBlueSKGUSint**	**M.Mix (2X V)**
DNA	4 µl	-	4 µl	4 µl	-
BcuI	2 µl	4 µl	-	-	-
XhoI	-	-	-	-	2 µl
Buffer Tango	4 µl	8 µl	-	-	8.8 µl
H2O	30 µl	60 µl	-	-	Total 10.8 µl
Total Volume	40 µl	72 µl	36 µl from M.Mix	36 µl from M.Mix	Dispense 5.4 µl to the first digests

Table D.40: The ligation mixture for pPZP101ManA-MBF1c and pBlueSK+GUSint Fast liagtion was carried out for integration of the vectors.

pPZP101 ManA-MBF1c & pBlueSK+GUSint Ligation Mix	
Elution Mix	11 µl
5X Rapid Ligation Buffer	3 µl
T4 DNA Ligase	1 µl
Total Volume	15 µl

Table D.41: EcoRI digest mix of the pPZP101ManA-pBlueGUSint-MBF1c hybrids

pPZP101 ManA-pBlueSKGUSint-MBF1c Clones EcoRI Digest Mixes for Direction Analysis			
Components	**1X**	**M.Mix (5X V)**	**Clones 2-6**
DNA	4 µl	-	4 µl
Buffer EcoRI	2.5 µl	12.5 µl	-
EcoRI	1 µl	5 µl	-
H2O	17.5 µl	87.5 µl	-
Total Volume	25 µl	105 µl	21 µl from M.Mix

Table D.42: BcuI-XhoI digest mix for pPZP101 ManA-pBlueSK+GUSint-MBF1c hybrids
The forward and the reverse pPZP101 ManA-pBlueSK+GUSint-MBF1c hybrid clones (#6 and #2 respectively) were double digested for both analysis and ligation.

BcuI(SpeI)-XhoI Digest Mixes for pPZP101 ManA-pBlueSKGUSint-MBF1c Hybrid Vector					
	First, BcuI Digest				Second, XhoI Digest
Components	**1X**	**M.Mix (2X V)**	**RW Clone (#2)**	**FW Clone (#6)**	**M.Mix (2X V)**
DNA	6 µl	-	6 µl	6 µl	-
BcuI	2 µl	4 µl	-	-	-
XhoI	-	-	-	-	2 µl
Buffer Tango	4 µl	8 µl	-	-	8.8 µl
H2O	28 µl	56 µl	-	-	Total 10.8 µl
Total Volume	40 µl	68 µl	34 µl from M.Mix	34 µl from M.Mix	Dispense 5.4 µl to the first digests

Table D.43: Comparison table MS salts and Hoagland`s E solution

MS Micro Nutrients	Hoagland's E Micro Nutrients
$CoCl_2.6H_2O$	-
$CuSO_4.5H_2O$	$CuSO_4.5H_2O$
FeNa.EDTA	$FeCl_3.6H_2O$.EDTA
H_3BO_3	H_3BO_3
KI	-
$MnSO_4.H2O$	$MnCl_2.4H_2O$
$Na_2MoO_4.2H_2O$	$Na_2MoO_4.2H_2O$
$ZnSo_4.7H_2O$	$ZnSO_4.7H_2O$
MS Macro Nutrients	**Hoagland's E Macro Nutrients**
$CaCl_2$	$Ca(NO_3)_2.4H_2O$
KH_2PO_4	KH_2PO_4
KNO_3	KNO_3
$MgSO_4$	$MgSO_4.7H_2O$
NH_4NO_3	-

Table D.44: Base media for inoculation and co-cultivation media preparations Modified Hoagland's-E medium as the basis of the inoculation and co-cultivation media.

Base Media Micro Nutrients	
$CuSO_4.5H_2O$	0.09g/l
$FeCl_3.6H_2O$: EDTA	0.484g/l
H_3BO_3	2.86g/l
$MnCl_2.4H_2O$	1.82g/l
$Na_2MoO_4.2H_2O$	0.09g/l
$ZnSO_4.7H_2O$	0.22g/l
Base Media Macro Nutrients	
$MgSO_4.7H_2O$	24.6g/100 ml

APPENDIX E

DATA TABLES

Table E.1: Germination scores of three days old lentil seedlings
Germination scores of three days old lentil seedlings tabulated. Germination rate is given as fraction. Germination rate X100 is percent germination. Sample size for germination rate calculation is 120. Seedling weights and hypocotyl lengths were pooled from approximately 25 samples.

		Germination Rate (Fraction)		Seedling Weight (mg)		Hypocotyl Length (cm)	
		Mean	Std Dev.	Mean	Std Dev.	Mean	Std Dev.
Media	Mo	0.85	0.07	0.197	0.027	2.85	0.98
	Mı	0.83	0.09	0.186	0.022	2.68	0.56

Table E.2: Regenerative capacities of explants isolated with different methods
Data table for regenerative capacities of explants isolated with different methods. Mean of regenerated shoots are scores as fractions (X100 for% interpretation). (n=20).

Fraction of Meristems Generating Shoots for Differet Explant Isolation Methods & Injury Applied				
	explant	injury	mean scores	std. error.
conventional	cotiledonary node	-	1.00	0.00
	cotiledonary node	+	0.92	0.10
epicotyl excised	hypocotyl	-	0.92	0.07
	hypocotyl	+	0.92	0.09
cotyledones stripped	cotyledones	-	0.73	0.31
	embryo axis	-	0.39	0.14

Table E.3: Data summary for the necrotic phenotype of transformed explants

Preliminary Transformation Experiments							
explant isolation method		conventional				epicotyl excised	
injury		sectioning down		poking		epicotyl excised	
tarnsformation		C	TR	C	TR	C	TR
phenotype % scores	Full Necrotic	30	32	40	43	no epicotyl	
	Epicotyl Necrosed	3	4	0	1		
	Hypocotyl Necrosed	3	4	7	2	62	63
	Sample Number	30	97	30	90	29	100
Std. Dev.	Full Necrotic	8.82	4.44	10.00	4.37	no epicotyl	
	Epicotyl Necrosed	1.92	0.84	0.00	0.45		
	Hypocotyl Necrosed	1.92	0.52	3.85	1.36	9.12	4.93

Table E.4: Second transformation set regeneration and viability scores

Second transformation set regeneration and viability scores are tabulated. Explant sub-groups separated according to hormone application during germination (blue highlighted), pre-culture or injury (blue highlighted) else injury or pre-culture (orange highlighted) during explant preparation were noted as level 1-3 in the figures 5.1-1 and -2.

% Scores for the Viability & Regeneration of the Transformation Set 2																	
germination	TDZ						BA						BA+TDZ					
	conventional explant isolation																	
explant preparation	preculture			injury			preculture			injury			preculture			injury		
	1d		2d	preculture			1d		2d	preculture			1d		2d	preculture		
	injury			1d		2d	injury			1d		2d	injury			1d		2d
transformation	Con	TR	TR	Con	TR	TR	Con	TR	TR	Con	TR	TR	Con	TR	TR	Con	TR	TR
Week 1																		
Callus	10	61	100	44	77	100	75	90	94	50	85	78	60	100	79	50	91	88
Necrosis	0	4	13	44	12	0	0	10	33	38	10	11	10	18	29	10	26	69
Shoot	90	46	20	67	46	31	25	29	6	50	35	17	40	11	36	30	13	0
Week 2																		
Callus	11	42	60	10	32	75	25	39	39	38	48	50	20	36	14	10	22	69
No Regeneration	22	0	7	0	11	19	13	28	39	0	0	6	30	38	14	20	33	13
Shoot	67	58	33	90	57	6	63	33	22	63	52	50	50	27	71	70	46	13

CURRICULUM VITAE

Personal Information

- Nationality: TC
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Education

- Doctorate: 2004–2011/ METU Institue of Natural and Applied Sciences-Biotechnology
- M.Sc.: 2002–2004/ METU Institue of Natural and Applied Sciences-Biotechnology
- B.S: 1995-2001 Middle East Technical University Faculty of Education / Biology Education

Language

- Turkish (moher tongue)
- English

Work Experience

- 2004-2007 General Manager / OBiTEK Ltd. Şti.
  Project Completed in OBiTEK Ltd. Şti.
  - ✓ Horizontal mini gel electrophorsis and power supply
  - ✓ Biolab Ltd. Şti ile ortak yürütülen "Bio-Balistik: Design Production and Optimisation of Microcontroller Based Gene Transfer System (7. Great Technology Award at Technology Price Kongressat at Sabancı Center, İstanbul -11.12.2007)

Military Service

- 2008-2009 Ardahan 25. Mechanized Infantry Brigade

Academic Works and Publications

- 2004-2011/ Doctorate Thesis (This study)
- 2008/ Publication

Agrobacterium tumefaciens-mediated genetic transformation of a recalcitrant grain legume, lentil (Lens culinaris Medik) Ufuk Celikkol Akcay, M. Mahmoudian, H. Kamci, M. Yucel, H. A. Oktem Plant Cell Rep (2009) 28:407–417 GENETIC TRANSFORMATION AND HYBRIDIZATION

- 2008/Book Chapter

Compendium of Transgenic Crop Plants/ Transgenic Legume Grains and Forages-Lentil (Volume3/Chapter 5-2008) Co-Author (Huseyin A. Oktem, Ufuk Celikkol Akcay, Abdullah Tahir Bayrac, Hamdi Kamçı and Meral Yücel Department of Biological Sciences, Middle East Technical University, Ankara, Turkey)

- 2005/Kongress Attended

Türkiye Biyoteknoloji Derneği 14. Bitoteknoloji Kurultayı

- ✓ Presentation Bio-Balistics: Desing, Production and Optimisation of Micro Controller Assisted Mictoprojectile Gene Based Transfer Systems. Hüseyin Avni Öktem, Sinan Vasfi, Didem Demirbaş, Özge Şenyur, İrem Karamollaoğlu, Hamdi Kamçı, Meral Yücel
- ✓ Presentation Optimisation of Regeneration and Gene Transfer Systems in Lentil Hamdi Kamçı-A. Tahir Bayraç, Meral Yücel, Hüseyin Avni Öktem

- 2004: M.Sc Thesis

"Optimisation of Agrobacterium Mediated Gene Transfer and Micrografting Systems in Lentil (*Lens clunaris* Medik.)" ODTÜ Fen Bilimleri Enstitüsü Biyoteknoloji ABD.

- 2000: Training

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Proffessions

- MolecularBiology
  - ✓ PCR
  - ✓ RT-PCR
  - ✓ Q-PCR
  - ✓ Southern Blotting
  - ✓ Gene Cloning and Characterisation
  - ✓ RFLP
- Plant Gene Transfer
  - ✓ Agrobacterium mediated
  - ✓ Microprojectile mediated
- Plant TissueCulture Techniques