T.R.
NİĞDE ÖMER HALİSDEMİR UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF AGRICULTURAL GENETIC ENGINEERING

OVEREXPRESSION OF *BABY BOOM (BBM)* GENE IN COTTON (*GOSSYPIUM HIRSUTUM L.*)TO ENHANCE SOMATIC EMBRYOGENESIS

SHAKHNOZAKHON TILLABOEVA

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MASTER THESIS

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Supervisor

Asst. Prof. Dr. Allah BAKHSH

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The study entitled "**Overexpression *of BABY BOOM (BBM)* Gene in Cotton (*Gossypium hirsutum L.*) to Enhance Somatic Embryogenesis**" and presented by Shakhnozakhon TILLABOEVA under the supervision of Asst. Prof. Dr. Allah BAKHSH has been accepted as Master thesis by the jury at the Department of Agricultural Genetic Engineering of Niğde Ömer Halisdemir University, Graduate School of Natural and Applied Sciences.

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CONFIRMATION:

This thesis has been found appropriate on this date of …./…./2021 by the jury member above who have been designated by the Board of Directors of Graduate School of Natural and Applied Sciencesand has been confirmed with the resolution of Board of Directors on …./…./2021and has been accepted by the decision numbered…............................

....../...../2021

Prof. Dr. Murat BARUT

MÜDÜR

DECLARATION

It is certified that I have written this thesis by myself. I further confirm that all information included in this thesis is scientific and is in accordance with the university rules and regulations. Any materials that I have used from external sources as well as help received and all sources used in finalizing this research work and preparing this thesis, all have been acknowledged in the thesis.

Shakhnozakhon TILLABOEVA

ÖZET

PAMUKTA (*GOSSYPIUM HIRSUTUM L.*) SOMATİK EBRİYOGENEZİ ARTIRMAK AMACIYLA *BABY BOOM (BBM)* GENİNİN İFADESİNİN ARTIRILMASI

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Pamuk dünyadaki önemli endüstri bitkilerinden bir tanesidir. Dünya üzerinde seksenden fazla ülkede, yüz milyondan fazla çiftlik sahibi küçük aileler pamuk yetiştiriciliği yapmaktadır. Genetik mühendisliği, transkripsiyon faktörlerinin ifadesi artırılarak rejenerasyon süresinin kısaltılmasıyla gelecek vadeden bir araç olabilir. *BABY BOOM* (*BBM)* geni, bitkilerin temel aşamalarından ve patojen savunma mekanizmalarından sorumlu bitkilere özgü AP2/ERF transkripsiyon faktörü ailesine aittir. Bu çalışmada, pamuk doku kültürü sürecini kısaltmak, geliştirmek ve bitki somatik embriyo oluşum mekanizmasının aydınlatılması amacıyla pamuktaki *BBM* gen ifadesi artırılmıştır. *35S:BnBBM*, *35S:GmBBM*, *UBI:BBM* ve boş vektör içeren tasarım Agrobakteri *LB4404* suşu aracılığıyla pamuk ve tütün bitkilerine aktarılmıştır. Transgenik bitkiler, *LEAFY COTYLEDON, FUSCA3, LEAFY COTYLEDON LIKE, ABSCISIC ACID INSENSITIVE* (*LAFL)* yolağı ve somatik embriyogenezinin negatif regülatörünün ifade profillerinin ortaya çıkarılması için kontrol edilmiştir. Elde edilen sonuçlar, morfojenik genlerin ifadesinin arttığını göstermiştir. Bu sonuçlar, *BBM* geninin bitki rejenerasyon yolağını düzenleyen genlerin üzerinde olduğunu öne sürmektedir.

Anahtar Sözcükler: Somatik embriyogenez, artırılmış gen ifadesi, AP2/ERF transkripsiyon faktörleri, *BBM* geni, pamuk

SUMMARY

OVER EXPRESSION OF *BABY BOOM (BBM)* GENE IN COTTON TO ENHANCE SOMATIC EMBRYOGENESIS

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Cotton is one of the most significant cash crops of the world. More than 100 million small family-owned farms are cultivating cotton crop in more than 80 countries around the globe. Nevertheless, cotton genetic engineering is lengthy and labor intensive due to difficult tissue culture. Genetic engineering can be a promising tool for shortening regeneration period by overexpressing transcription factors. *BABY BOOM (BBM)* gene belongs to the plant specific transcription factor family AP2/ERF, which modulates plant fundamental processes, as well as pathogen defense mechanisms. To shorten and improve regeneration of cotton tissue culture period, *BBM* gene was overexpressed in cotton to understand mechanisms of plant somatic embryogenesis. In this work, 4 constructs were introduced into cotton plants harboring *35S:BnBBM*, *35S:GmBBM*, *UBI:BBM* and empty vector in Agrobacterium strain of *LB4404*. Transgenic plants were checked for expression profiles of *LEAFY COTYLEDON*, *FUSCA3*, *LEAFY COTYLEDON LIKE, ABSCISIC ACID INSENSITIVE* (*LAFL)* network and one negative regulator of somatic embryogenesis (SE). Obtained results showed that expression profiles of morphogenic genes are upregulated. These results proposed that *BBM* gene is upstream of genes that modulate plant regeneration network.

Keywords: Somatic embryogenesis, AP2/ERF Transcription factor family, overexpression, BBM gene, cotton

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SYMBOLS AND ABBREVIATIONS

Symbols	**Descriptions**
%	Percent
°C	Degree centigrade
g/l	Gram per liter
mg/l	Milligram per liter
ml	Milliliter
mM	Milli-molar
W	Watt
µl	Microliter
µM	Micromolar

Abbreviations	**Descriptions**
2,4 D	2,4-dichlorophenoxyacetic acid
AP2	Apetala2
Aux/IAA	Auxin/Indoleacetic acid
BBM	Baby Boom
bp	BasePair
CIM	Callus Induction Medium
ddH2O	Double-distilled Water
DNA	Deoxyribonucleic Acid
EDTA	Ethylene Diamine TriaceticAcid
EEL	Enhancer Element Locator
ERF	Ethylene Response Factors
FAO	Food and Agriculture Organization of the United
IAA	Indole-3-acetic acid
ICAC	International Cotton Advisory Committee ()
ISAAA	International Service for the Acquisition of Agri-biotech Applications
Kb	Kilobase

KT	Kinetin
LAFL	LEC1, LEC1-LIKE (L1L), and AFL (LAFL) network
LB	Luria-bertani Medium
LDCs	Least Developed Countries
MDE	Microscope Derived Embryos
MS medium	Murashige and SkoogMedium
NAA	1-Naphthaleneaceticacid
Ng	Nano Gram
NPTII	Neomycin Phosphotransferase
PCR	Polymerase Chain Reaction
pH	Power of Hydrogen
RNA	Ribonucleic Acid
SE	Somatic embryogenesis
TBE	Tris/Borate/EDTA
TEM	Mean Transformation Efficiency
TFs	Transcription Factors
UNCTAD	United Nations Conference on Trade and Development
UV	Ultraviolet
WTO	World Trade Organization
WUS	Wuschel

CHAPTER I

INTRODUCTION

Genus *Gossypium* L. is a big group of flowering plants that refer to the Malvaceae family, famous for their natural cotton fiber. It includes more than 50 species in the *Gossypium* family, including the most famous *Gossypium hirsutum*. The genus is extremely popular among scientists of different backgrounds, such as geneticists, taxonomists, biologists, biotechnologists, etc. (Wendel et al., 2009).

Most of the cotton species are distributed in arid and semi-arid regions of tropics and subtropics. The origin of *Gossypium* goes back to 5-10 million years ago with quick genome groups diversification. It is believed that allopolyploid cotton arose 1-2 million years ago as a result of A genome trans-oceanic spreading and further hybridization with the D genome. Dispersal of A genome taxon to the New World gave path to more modern and commercially significant species, such as *G. hirsutum* and *G. barbadense* (Wendel et al., 2009).

With the rapid development of molecular technologies, studies of cotton are giving insight into the evolutionary origin of the crop, relation of crop species and groups. Moreover, the difference of domesticated species from wild progenitors is being intensively studied. The history of cotton crops unites ancient civilization and people from XXI century: several continents and different evolutionary pathway. This domestication process involved two species from America namely *G. hirsutum* and *G. barbadense* and two *G. arboreum* and *G. herbaceum* from Africa-Asia (Wendel et al., 2009).

Each of the species has special domestication history, utilization by aborigines and different application properties. Several aspects of the evolutionary pathway were clarified by Phylogenetic analyses; one of them is a demonstration of the single natural lineage constitution of Gossypium species regardless of worldwide distribution. Another important statement is discovering close relative species of the genus, such as genera of *Gossypioides* and *Kokia* (Wendel et al., 2009).

Commercialization of transgenic cotton started in 1995 when herbicide resistant cultivars were introduced to the world (Blanche et al., 2006). First transgenic cotton cultivars were carrying resistance to bromoxynil herbicide, and later on in 1996 cultivars harboring insecticidal Cry1Ac protein were introduced in the USA (Hardee and Herzog, 1997). The next year in 1997 cotton varieties resistant to glyphosate were introduced in the USA (Kerby and Voth, 1998). The introduction of biotech cotton having resistance to insects and herbicides opened a new era of genetically engineered plants in modern agriculture. Tobacco budworm and bollworm reduced US cotton field yields by 3.97% in 1995, and more than 80% of the acreage was infected (Hardee and Herzog, 1996). In 2000, situation changed and 39% of cotton lands were planted with *Bt* cotton that resulted in 1.43% reduction of damage from the insect population (Hardee and Burris, 2001).

Numerous benefits such as agronomic, economic, social, health and others were acknowledged from adopting biotech crops and the first 21 years of growing GM crops confirmed this trend (ISAAA, 2016). Large and small-scaled farms realized the advantages of biotech crops after rapid adoption in the first years of commercialization. In 2017, global accumulated farmland of biotech crops reached to 2.15 billion hectares, from which cotton counted up for 0.34 billion hectares. Those 2.15 billion hectares provided better food and feed for the growing population of the world (ISAAA, 2017). Therefore, there is a high need in improving yields due to predicted population numbers of 11.2 billion in 2100 (UN, 2017).

In 2017, US biotech cotton counted for 4.58 million hectares, whereas it was 3.9 million hectares in 2016 (ISAAA, 2017). Brazil is counted as the second largest country in term of biotech crops. Biotech cotton counted for 940,000 hectares in 2017. Most of the exported Brazilian cotton goes to the EU and China. India planted biotech crop on 11.4 million hectares in 2017. Paraguay adopted biotech crops in 2004, and in 2017 biotech cotton land reached 10,000 hectares. In the growing season of 2017-18, Pakistan cultivated 3 million hectares of biotech cotton. China has been one of the leaders in cultivating GM crops. In 2017, China planted 2.8 million hectares of cotton, while in 2013 it was 4.2 million hectares. Mexico is considered as one of the pioneer countries in adopting GM crops, and 110,000 hectares were planted in 2017.

One of the first initial steps of genetic crop improvement for commercial plants is tissue culture techniques. Eventually mature plants need to be obtained from tissue culture. Different aspects involved in embryogenesis of *Gossypium* species are being described (Davidonis and Hamilton, 1983). Regeneration of cotton through tissue culture has been problematic since the first advent of genetic transformation. Since the last century, several scientists were successful in obtaining mature plants trough somatic embryogenesis (Trolinder and Goodin, 1988). Another reported somatic embryogenesis was done back in 1983 in G.hirsutum L (Davidonis and Hamilton, 1983).

The next successful work was obtained by Price and Smith in *G. klotzschianu*m but without obtaining mature plants (Price and Smith, 1979). Further effort was launched back in 1983 successfully in *G. hirsutum* L. but it took 2 years until spontaneous development of embryos (Trolinder and Goodin, 1988). A year later, Mitten (Mitten, 1985) and a group of scientists with Rangan (Rangan et al., 1984) reported about somatic embryogenesis and further plant development in *G. hirsutum* L. Furthermore, somatic embryogenesis was obtained by a group of scientists and Shoemaker in 1986 (Shoemaker et al., 1986); by Trolinder and Goodin in 1985 (Trolinder and Goodin, 1985).

The progress in transformation techniques and the success of GMO crops led a pathway to the development of other genetic manipulation methods. Unfortunately, most of the delivery systems remain inefficient and slow. Moreover, the chance of getting a transgenic plant is low and labor-intensive. Most academic labs face different problems with plant transformation including too much specialized technologies, the necessity of optimizing tissue culture work, contamination problem and many others. Future demands will need a new way of engineering beyond traditional genome engineering methods (Jones et al., 2019) The idea of stimulating morphogenesis using different genes that can increase growth are in the literature since the 80s of last century (Smigocki and Owens, 1988).

The research work covering maize transformation showed improved transformation by morphogenic TF – BBM using special combination of cassettes that allow successful transformation of explants. Moreover, rapidly formed embryos allow direct germination in mature plants avoiding a callus stage (Jones et al., 2019).

BABY BOOM (BBM) gene is one of the morphogenic genes involved in somatic embryogenesis (SE). This gene is a member of AP2/ERF TFs super family, main functions are growth, developmental processes and response to environmental signals. AP2 subfamily consists of several genes participating exclusively in developmental processes and initiation of SE. *BBM*-like genes share similar DNA binding domain sequences that have common bbm-1 conserved motif. Research regarding their motifs propose similar functions based on their conserved motifs (Boutilier et al., 2002).

The idea of this current research is original since there is no report on overexpression of *BBM* gene in cotton in order to get better regeneration and higher transformation efficiency. This is the first study of cotton harboring *UBI:BBM*, *35S:BnBBM* and *35S:GmBBM* constructs. Obtained results may explain the real function and role of *BBM* gene in initiation of SE in cotton. Moreover, provided work illustrates interaction of negative regulator with the initiation of SE and levels of morphogenic genes in obtained transgenic callus.

This thesis work is focused on overexpression of *BBM* gene in cotton to improve somatic embryo gene-genesis and regeneration capacity. For achieving this task following stages of research were conducted:

1) Isolation of *BBM* gene from soybean Nova cultivar.
2) Cloning of *BBM* gene in plant expression vector (pEarleyGate 100).
3) Agrobacterium-mediated transformation of cotton cultivar (Coker 312) with plant expression vector carrying target gene.
4) Confirmation of the target gene integration and expression in transformed calli by PCR and qRT-PCR, respectively.

CHAPTER II

REVIEW OF LITERATURE

2.1 Importance of Cotton

Cotton became a huge part of day to day lives of millions of people all over the globe. Cotton is involved in almost every aspect of life, starting from food that we eat and finishing clothes that we wear. However, these features are not only remarkable parts of this crop, but cotton is also significant part of every national economy in more than 75 cotton producing countries. Small family-owned farmlands and huge corporations in developed, developing and least developed countries equally see importance of this crop. Moreover, cotton crop cultivation and manufacturing are giving a job for millions of people worldwide. Cotton is accepted as global commodity. Cotton unites international trade by involving different nations and cotton producers. Trading is allowing cotton producers to earn, invest, and create workplace that allow growing national economy. However, distortions in cotton prices of trade markets create inequality for farmers especially in least developed countries (LDCs). Therefore, WTO created special policies for LDCs allowing creating better trading field (World Cotton Day, 2019).

Cotton is considered as significant sector for FAO for numerous reasons. FAO shares that cotton is a major component of national economic and driver of growth and development for different countries all over the world. This huge and significant industry involves more than 75 countries and more than 100 million family-owned farmlands that generate as a raw product more than 50 billion USD each year. FAO states that cotton production providing income and employment for millions of farmers. LDCs see cotton as a perfect income from exporting cotton goods that allow surviving. Production and trade of cotton goods allow to face problems of poverty and achieve some of the goals of FAO project named the Zero Hunger Goal. Moreover, this project is supporting and empowering women who are actively involved in cotton sector. FAO admits that cotton industry faces a lot of uncertainties regarding production, manufacture and trading (World Cotton Day, 2019).

According to the International Trade Centre (ITC), cotton industry provides income to millions of people living in Africa, particularly to people that live in villages, including women. The ITC is helping to strengthen economic basis of cotton industry by increasing export potential and adding greater value to the industry. Different international organizations work together to provide better understanding of supply chains and trading specificities in different countries. These organizations include ITC, WTO, FAO, UNCTAD, ICAC and others. International organizations highlight importance of cotton production, celebrate cotton crop and improve livelihoods of millions of farmers. ITC tries to improve resilience of farmers to external difficulties by technical support. Farmer trainings, seed quality improvements, source value and enhancement of technical background are the main highlights of international organizations. New projects of international scale are being signed each year, for instance, the recent project of UNCTAD and WTO will consider processing cotton by-products. Such projects were performed in Zambia that reduced income dependence of local farmers from harvesting time and made possible to have year-round income by introducing spinning and weaving technologies. This project improved livelihood of 400 Zambian cotton growers. Moreover, hand-weaving projects were performed in Burkina Faso and Mali by the Ethical Fashion Initiative of ITC (World Cotton Day, 2019).

2.1.1 Cotton production worldwide

Cotton crop is a major mainstay of worldwide economies, involving different participation of wide board services and market demands. Besides essential fiber, cotton vegetable oil is rich in amino acids and seed hulls are extremely popular and important for the industry. Stock markets are showing necessity in the cotton crop and increasing consumption and production scales year by year. Moreover, each stage of cotton production requires a wide range of workforce (Wilkins, 2000). Production rates of world’s leading ten countries reached up to 23.485 metric tons totally in 2019 (Figure 2.1) (Statista, 2021).

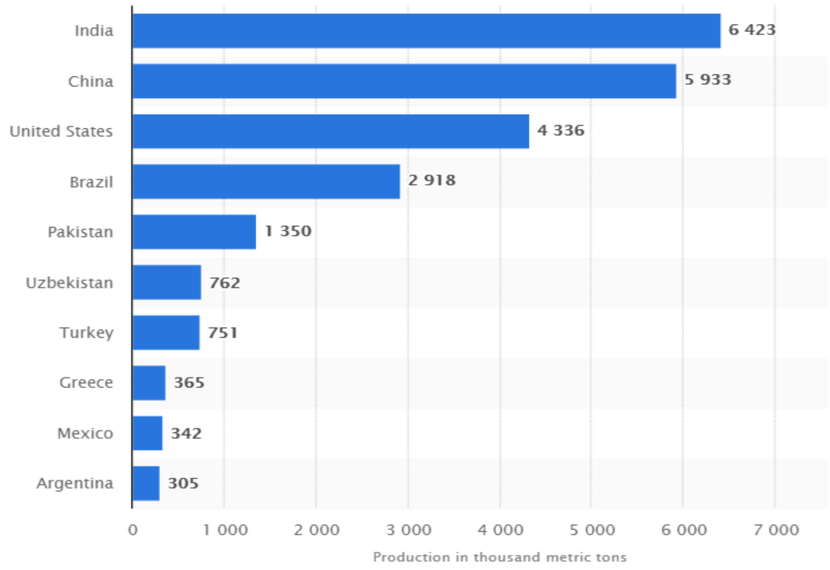

Figure 2.1. Leading cotton producing countries worldwide in metric tons in 2019/2020. (Statista, 2021)

Nowadays, cotton is a major and first leading natural fiber source and the world second oilseed plant. All production stages are expensive including costs of crop protection. Therefore, biotech crops influence and importance are growing year by year, with several leading countries growing biotech crops in a large scale (Wilkins, 2000). According to the Statista, total area of GM crops in the USA and Brazil reached up to 71.5% and 52.8% respectively in 2019 (Figure 2.2), (Statista, 2021).

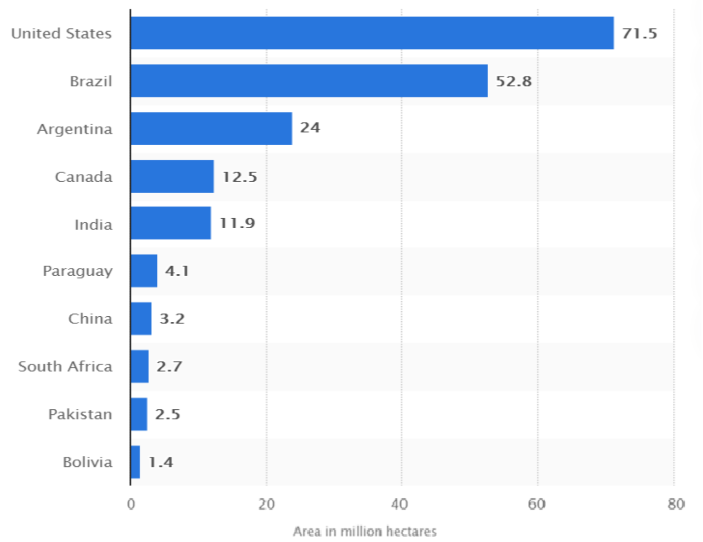

Figure 2.2. Area of genetically modified (GM) crops worldwide in million hectares in 2019 (Statista, 2021)

According to the ISAAA, 2018 (James, 2006), biotech crops total planting area has increased to 2.5 billion hectares. Cotton is the third largest cultivated biotech crop with 7% of total area in the USA.

Moreover, average worldwide cotton price was around 87 US cents/pound in 2018. It was also stated that annual cotton yield losses reach up to 50% due to abiotic stress (Bita et al., 2013). Therefore, there was and still there is a strong need in effective tools against yield loses. One of the possible tools of increasing yields and protecting fields from abiotic and biotic stresses is implementation of genetic engineering (Bakhsh et al., 2009).

Biotech crops adopted by different countries show increased income of farmland due to decrease of pesticide usage and increased yields. In 2019, farmlands of biotech crops in top five biotech adopted countries increased compared to past years (Table 2.1 and Figure 2.3). Expansion of biotech farmlands is due to increasing commercialization of new varieties of major crops that possess better quality of nutrients, resistance to pathogens, diseases, and stresses (ISAAA, 2019).

Table 2.1. Adoption rates of biotech crops in top five biotech countries (ISAAA, 2019)

Country	Adoption rate, % Average for cotton, soybean, maize, and canola
USA	95
Brazil	94
Argentina	100
Canada	90
India	94

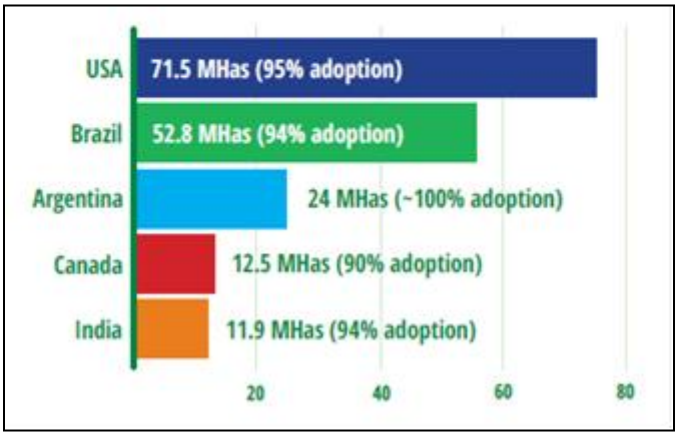

Figure 2.3. Top 5 countries that planted biotech crops in 2019 (area and adoption rate), (ISAAA, 2019)

ISAAA (2019), states that biotech crops are increased in cultivated lands by 112-fold since 1996. Moreover, it has been shown that biotechnology is the most rapid adopted technology of crop improvement. In 1996, biotech crop farmland was 1.7 million hectares, while this number reached to 190.4 million hectares in 2019. This achievement allowed putting crop biotechnology on the first place among all technologies. Nowadays, GM crops are adopted in 29 countries, from which 24 are developing and 5 countries are industrial. Developing countries contributed 56% from total, whereas industrial 44%. Moreover, statistics show that 42 countries, from which 26 are EU countries, imported GM crops in terms of food and feed. Therefore, it is counted that 71 countries adopted GM crops in 2019 (ISAAA, 2019).

According to the ISAAA, 2019 report, GM soybeans were constituting 48% from total global cropland. Most of the adopted and cultivated GM crops in 29 countries were cotton, maize, soybean, and canola (Table 2.2).

Table 2.2. Global biotech crop area for individual crops

Crop	Area	Global cropland
Soybean	91.9 million hectares	74%
Maize	60.9 million hectares	31%
Cotton	25.7 million hectares	79%
Canola	10.1 million hectares	27%

However, the big four group are not the only crops that expanded their crop are, other crops such as alfalfa, sugarcane, rice, banana, potato, wheat, eggplant, sugar beet increased in cropland as well (Figure 2.4).

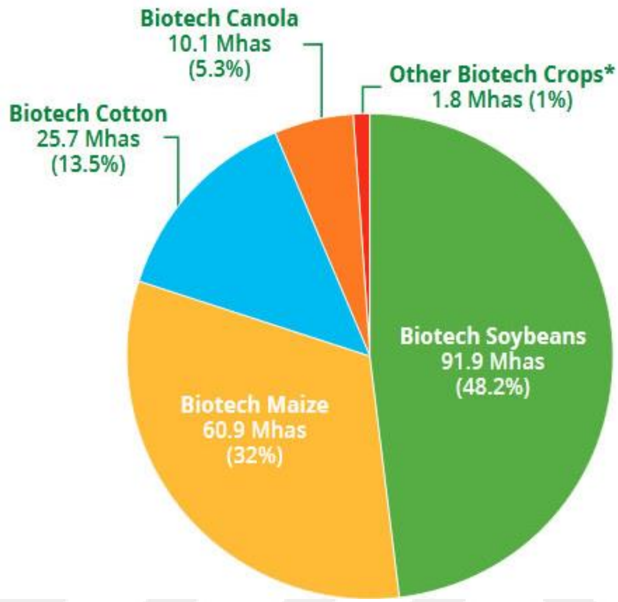

Figure 2.4. Biotech crops in 2019. Area and adoption rate (ISAAA, 2019)

Most of the cultivated biotech crops possess insect and herbicide resistance (IR/HT). These resistant crops reached to 85.1 million hectares which counts to 45% of total cropland. Most of the cotton growers preferred herbicide tolerant cotton cultivars until 2018, whereas this trend was changed in 2019 with reduced cropland of HT cotton. In 2019, 12% of global biotech cropland was planted with IR crops. Moreover, in 2019 several crops with resistance to glyphosate, 2,4-D, glufosinate, isofluxatole and dicamba were approved. This trend is observed all over the world preferring resistant genetically modified cotton cultivars that allow to have increased yields due to protection from insects and having resistance to common herbicides. Table 3 illustrates global are of crops situated in different countries. In 2019, 19 countries cultivated biotech cotton (ISAAA, 2019).

Table 2.3. The global area of Biotech crops in 2019; by country (million hectares) (ISAAA, 2018)

	Country	2017	%	2018	%	+/-	%
1	*USA**	75.0	39	75.0	39	0	0
2	Brazil*	50.2	26	51.3	27	1.1	2
3	Argentina*	23.6	12	23.9	12	0.3	1
4	*Canada**	13.1	7	12.7	7	-0.4	-3
5	India*	11.4	6	11.6	6	0.2	2
6	Paraguay*	3.0	2	3.8	2	0.8	27
7	China*	2.8	1	2.9	2	0.1	4
8	Pakistan*	3.0	2	2.8	1	-0.2	-7
9	South Africa*	2.7	1	2.7	1	0	0
10	Uruguay*	1.1	1	1.3	1	0.2	18
11	Bolivia*	1.3	1	1.3	1	0	0
12	*Australia**	0.9	<1	0.8	<1	-0.1	-11
13	Philippines*	0.6	<1	0.6	<1	0	0
14	Myanmar*	0.3	<1	0.3	<1	0	0
15	Sudan*	0.2	<1	0.2	<1	0	27
16	Mexico*	0.1	<1	0.2	<1	0.1	100
17	*Spain**	0.1	<1	0.1	<1	0	0
18	Colombia*	0.1	<1	0.1	<1	<0.1	0
19	Vietnam	<0.1	<1	<0.1	<1	<0.1	0
20	Honduras	<0.1	<1	<0.1	<1	<0.1	0
21	Chile	<0.1	<1	<0.1	<1	<0.1	0
22	Portugal	<0.1	<1	<0.1	<1	<0.1	0
23	Bangladesh	<0.1	<1	<0.1	<1	<0.1	0
24	Costa Rica	<0.1	<1	<0.1	<1	<0.1	0
25	Indonesia	--	--	<0.1	<1	--	--
26	eSwatini	--	--	<0.1	<1	--	--
	Total	**189.8**	**100**	**191.7**	**100**	**1.9**	**1.0**

* Biotech mega-countries growing 50,000 hectares or more
** Rounded-off to the nearest hundred thousand or more

2.2 Stomatic Embryogenesis and Research

Past several decades showed incredible progress of biotechnology and public interest in GM crops. Efficient production of genetically engineered crops and further high profitability of major commercial crops boosted development of genetic engineering. Every transgenic plant engineering has several important stages, one of them is expressing transgene and further possibility to form a fertile plant using tissue culture techniques. Most of the elite commercial crops are recalcitrant and extremely difficult for tissue culture manipulations. There have been numerous studies on improving regeneration capacity and one of the promising approaches is to use morphogenic genes to help plant regeneration. All approaches try to regulate morphogenesis to develop plant organs by involving gene networks (Gordon-Kamm et al., 2019).

De novo plant regeneration can occur through somatic embryogenesis or organogenesis in plants using exogenous plant hormones. The same situation is with cotton crop. Genetic transformation is very long, labor-intensive, cultures are difficult to maintain, seeds tend to be contaminated. Moreover, genotype dependent transformation drastically limits choices to commercially irrelevant genotypes (Mayer et al., 1993).

Embryogenesis in flowering plants is a process that develops organization of initial body structure as showed by the seedling; new organs and structures are formed from meristematic parts of shoot and root in the stage of postembryonic growth. Apical basal pattern forms meristems and later on additional structures are being formed. Elements such as hypocotyls, radicle and cotyledons do not accord to postembryonic phase. The second pattern is formed perpendicular to polarity axis. One of the useful ways to understand body organization in embryogenesis is usage of mutants. Genetic approaches are helping to give an insight into processes of embryogenesis in plants and there have been studies of mutant screens in plants like Arabidopsis, maize, cotton, rice etc., which helped to identify processes of early development such as first asymmetric division of zygote (single cell) (Mayer et al., 1993). This genetic dissection can demonstrate a starting point of developmental processes. Unique mutant phenotypes are recognizing relevant genes; however, connection of gene activity and mutant phenotypes are needed to be arranged (Mayer et al., 1993).

In several reviews of morphogenic genes, problems of regeneration of transformed plants were discussed. Transformation of most of the crops remain a big challenge due to inefficient tissue culture protocols, difficult optimization of regeneration protocols, recalcitrant nature of plants, extremely low transformation efficiency. For many decades, this problem was addressed by techniques of tissue culture that can enhance regeneration using somatic embryogenesis or meristem. Identification and appropriate application of morphogenic genes can be a useful tool to overcome these problems. Studies were conducted on monocots and dicots discovering effect of morphogenic genes on stimulation of growth. Nevertheless, side effects of this kind of constructs were seen as pleiotropic phenotypes. Elimination and restricting of pleiotropic effects have become a main issue for different researchers. Some of the applications that can control ectopic expression are tissue specific and inducible promoters, deletion of morphogenic genes and transient expression. Reviews collected work that highlights overexpression of morphogenic genes in different crops (Figure 2.5) (Kulinska-Lukaszek et al., 2012; Yavuz et al., 2020).

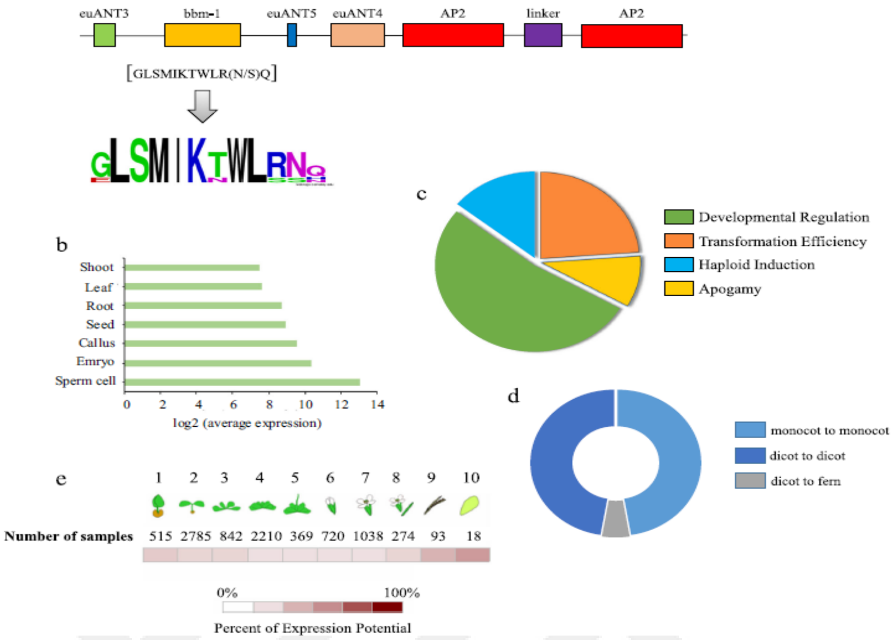

Figure 2.5. Amino acid sequence in bbm-1 motif, *BBM* gene domains (a), *BBM* expression in Arabidopsis (b), main four categories of *BBM* classification based on Research (c), *BBM* studies classification (d) and *BBM* expression (e) (Yavuz etal., 2020)

Morphogenesis is a process of organized development of plant organs, tissues that involves complex gene networks to promote or mitigate developmental processes. Ever expanding gene number was found by studying the processes of somatic embryogenesis and by ectopically expressing morphogenic genes (Kulinska-Lukaszek et al., 2012). Moreover, non-plant genes are being used to improve regeneration capacity and regeneration rates. The list of genes is shown on table 2.4.

Table 2.4. Non-plant genes used for increasing regeneration capacity (Kulinska-Lukaszek et al., 2012).

Genes	Names	reference
Agrobacterium tumour-inducing genes	1-isopentyl transferase or ipt gene	Smigocki and Owens, 1998
	2 rolB	Otten, 2018
	3 rolC	Casanova et al., 2004
	4 6B	Otten, 2018
	5 tzs	Roeckel et al., 1998

Except morpohogenic genes giving insight into plant developing systems, cultures of microspore derived embryos are being studied as a model practice to investigate totipotency of cells to learn more about plant embryogenesis. Work of Joosen et al. (2007), showed comparison of rapeseed proteome and transcriptome microspore derived embryos from initial stage to heart stage of embryos. Two microspore derived systems were studied such as suspensor lacking unorganized cultures, and cultures with suspensors having the same developmental pathway as zygotic embryos. Microspore embryos carrying suspensor like structures had better histodifferentiation showing better role of suspensors in embryo development. Gel electrophoresis, protein sequencing, DNA array analyses were performed of two types of Microspore-derived embryos MDEs. Research of 220 potential markers of embryo development and 32 embryos overexpressed protein positions showed significant role of them in metabolism of ascorbate, glycolysis, synthesis of proteins during embryo development. Identification of 135 robust markers suggested coregulation of them during MDE development at protein and gene levels. Expression rates of conventional globular phase microspore embryos were compared with suspensor bearing embryos revealing genes that may improve histodifferentiation in suspensor embryo structures. The work showed collection of embryo early genes and proteins that may help in marker discovering and in studies of gene function (Joosen et al., 2007).

Except morphogenic genes, early response auxin genes, modifications in suspensor structures, rearrangement in histone deacetylases also affect cell totipotency. Pollen grains or male gametophyte's function is in delivering male gametes to embryo. Male gamete's role finish after double fertilization. These pollen grains are widely used in haploid production although the method is difficult. The work of Li et al. (2014), demonstrated that histone deacetylases regulate the shift to haploid embryogenesis. Repressing the work of histone deacetylases by trichostatin A in rapeseed pollen grains can guide to a significant rise in cells that shift pollens to embryogenesis. Rapeseed haploid induction is possible with application of high temperatures; however, embryogenesis is not dependent temperature stress applications. Arabidopsis pollen grains are recalcitrant to development of haploid embryos in culture but organize clusters in response to trichostatin A treatments. HDA17 is a histone deacetylase protein that participates in this process. Trichostatin A applications on pollen grains relate to H3 and H4 histones hyperacetylation. The work suggested that pollen grain totipotency is controlled by histone deacetylases mechanism, and that stresses applied to start development of haploid embryos effect this mechanism (Li et al., 2014).

Superiority of post embryonic development is outcome of appropriate reaction to environmental factors, which is important in plant growth and adaptation. Adaptation to environmental stimuli includes not only processes of physiology such as adaptation to soil salinity (Baxter et al., 2010), but as well as to changes in morphology, including escape form shade that promotes excessive elongation (Kami et al., 2010). Another limitation of plants is relatively inflexible cell walls which consist of cellulose mostly, attaching to connecting pectin. Cell migrations are restricted due to this scaffold (Sánchez-Rodríguez et al., 2010). Limitation of movement is one of the reasons of why cell differentiation is governed by position, not lineage. Due to situation of cell frame, developmental processes need to be managed between cells around and morphogenesis can be enlarged by integrally controlling local developmental rates (Van den Berg et al., 1995).

This differential modulation mostly situated in organ development, which based on two main stages – cells proliferation and expansion. When the proliferation of meristematic cell's is finished, two main stages of cell development start to organize and shape cell's molecular identity that eventually forms organs and tissues. Growth of plants depends equally on cell division amount and elongation direction. And all developmental processes are managed by phytohormones pathways (Depuydt and Hardtke, 2011).

Hecht et al. (2011), reported isolation of *SERK1* gene from Arabidopsis and demonstrated gene role in beginning of somatic embryogenesis. It was revealed that *SERK1* gene is highly activated during early SE. Megasporogenesis is the first stage where *AtSERK1* is expressed in growing ovules. The gene expression can be seen in embryo sac before the end of fertilization as well. Moreover, it was shown that embryo cells have strong expression patter until heart shaped stage of embryos. Furthermore, pattern was not detected in developing seed or embryo. Vascular tissue of mature plant has low pattern of the gene. Introduction of *35S:AtSERK* did not give any different phenotype. However, the change was detected in improved SE rates. Therefore, overexpressed level is enough to show improved embryogenic performance (Hecht et al., 2001).

Boutilier et al. (2002), in the work regarded to molecular pathways described one of the embryogenesis pathways in rapeseed. Upregulated genes during SE were identified using subtractive hybridization in immature microspore cultures of rapeseed. *BBM* gene was found to be expressed in growing seeds and embryo cultures. Overexpression of this gene in rapeseed showed spontaneous embryo formation and led to appearance of cotyledonary structures. Moreover, transformed seedlings had pleiotropic phenotypes such as alteration in morphology of leaves and flowers, regeneration on hormone free medias and neoplastic growth. It was suggested that *BBM* gene has regulatory function in cell proliferation during SE (Boutilier et al., 2002).

The work of Awasthi et al. (2017), showed identification of crucial genes needed for SE in genome database of banana plant. The genes such as *BABY BOOM (BBM), WUSCHEL (WUS), LEAFY COTYLEDON (LEC), LEAFY COTYLEDON LIKE (LIL), VIVIPAROUS1 (VP1), CUP SHAPED COTYLEDONS (CUC), BOLITA (BOL) and AGAMOUS LIKE (AGL)* were analyzed for chromosomal and subcellular localizations, structural modifications. Conserved domains were identified in abovementioned TFs through protein sequencing. Most of the monocots share close evolutionary pathways of SE TFs that was showed by Phylogenetic tree. The work showed expression profiles of 18 genes in different callus stages such as embryonic embryo stage, non-embryogenic stage and in cell suspensions of banana cultures. Induction medium containing 2,4-D hormone showed higher expression of several genes – *BBM1-2*, *WUS2* and *VP1*. The work proposed 2,4-D as inducer of certain genes in SE. Cell suspension cultures in embryogenic stage had higher expression profiles of *BBM2* and *WUS2* genes compared to profiles of genes in non-embryogenic stage suspensions. Moreover, VP1 gene was highly expressed during both stages of callus development, whereas *LEC2* gene had higher rate in NE stage. Results of research propose that *LEC2* gene is crucial during NE callus stage. The work also mentioned that *BBM* and *WUS* genes can be used as SE molecular marker in banana plants (Awasthi et al., 2017).

2.2.1 Hormonal pathway involved in somatic embryogenesis

Plant ontogeny is uniquely flexible that is driven by hormone pathways. These pathways regulate intrinsic growth pathways, transport environmental processes. Several groups of phytohormones exist, and some of the central regulators of developmental programs are auxins, gibberellins and brassinosteroids. Cell elongation notable needs hormone regulation pathways. Modulation of developmental phenomena's is done by several classes of hormones that synergistically co regulate proposing redundancy or cross regulation among various pathways. The exact regulation is not clear suggesting different views such as targeting one and specific module of transcriptome or dividing modules that promote growth. However, extremely low number of molecular regulators of exact crosstalk is found, transcription leveled cross-regulatory correlations are collecting evidence for union of hormone pathways, for example the process of root meristem growing. Each year the library of *Viridiplantae* (the green lineage) genome sequences is growing making available the study of phytohormones evolution history. The green lineage can help to reveal numerous relations that are happening among hormone groups. Some of the available studies propose that abovementioned three hormones developed signaling referring to arisen complexity of morphology and most probably increasing flexibility. Biggest quality of plant growth is possibility of forming postembryonic plant body. This differs animals from plants that form body organs during embryogenesis. Seedling owns individual stem cells in meristematic regions of root, shoots which shape most of the body organs in plants post-embryonically (Depuydt and Hardtke, 2011).

Hormonal pathways are building remarkable developmental programs that include eight characterized plant hormone groups. Plant hormones are playing central role for elongation of cells and overall, in plant growth. Cross-regulatory relations of these hormones in plant development have been studied and synergistic work is suggesting different growth phenomena. These hormones are connected to each other by different manners, controlling every stage of life development. Since the last century there has been a dramatic progress in understanding hormone pathways. Most of the hormones are well studied, whereas some are still emerging. Hormone levels are not stable and differ based on environmental conditions. It was established that some hormone groups such as auxin are essential in different aspects of plant growth, whereas some groups are necessary in certain conditions, such us defense of plants. Dramatic change was observed when receptors of most hormones were identified. Hormone response raised several themes such as difference of plant hormone receptors from animals, important role of protein degradation in hormone signaling pathway, change in transcription because of hormone signaling and multiple levels of hormone interaction (Santner and Estelle, 2009).

Auxin is one of the major plant hormones that participate in every stage of plant development. Synthesized auxin is distributed via cell-to-cell transportation system (Vieten et al., 2009) and carriers of cellular auxin help to form auxin maxima (Santner et al., 2009). Local accumulation of auxin is essential for leaf initiation and more. Transport proteins that asymmetrically localized within cell walls determine auxin direction. Two large transcription factor families regulate auxin-regulated transcription. These TF families are proteins of auxin/indole-3-acetic acid (Aux/IAA) and the AUXIN RESPONSE FACTORs (ARFs) (Santner et al., 2009). Remarkably, proteins of Aux/IAA are short-termed and ubiquitin-proteasome pathway is needed for their degradation (Mockaitis and Estelle, 2008).

Figure 2.6 illustrates hormonal crosstalk with genes that are involved in SE. Increase of endogenous IAA leads to expanded expression profiles of several genes that encode tryptophan aminotransferase (Ayil-Gutiérrez et al. 2013). It was established that *SERK1* gene has ability to directly activate *BBM* gene and indirectly may affect hormonal pathway of three genes – *BBM*, *AGL15*, *WUS* (Pérez-Pascual et al. 2018). However, one of the morphogenic genes – *LEC1* was found to be downregulated when *SERK1* has increased level in *Coffea canephora* (Pérez-Pascual et al. 2018).

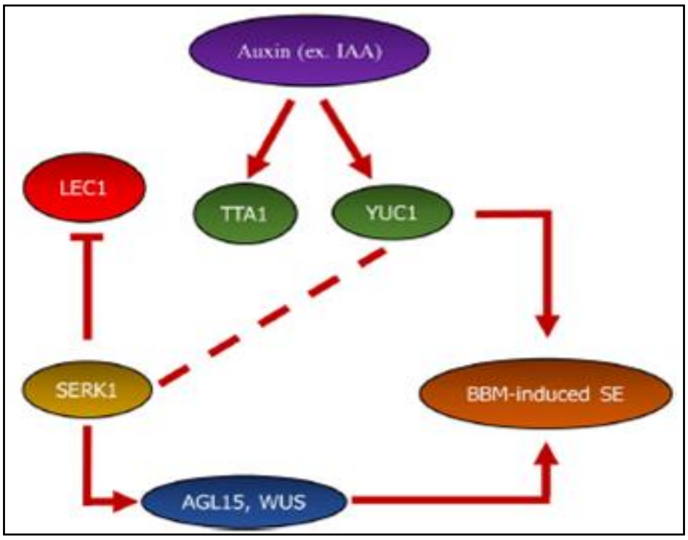

Figure 2.6. Auxin signalling pathway and possible *BBM* up regulation (Yavuz et al., 2020)

Understanding of hormone molecular pathways (Figure 2.6) can help to manipulate phytohormones to regulate developmental processes of plants. Components of downstream signaling are being studied that help to understand how pathways are working (Santner and Estelle, 2009).

Most of the auxin early response genes work as an important part of auxin signaling pathway and control different processes. In a study of Sun et al. (2019), auxin early response genes in upland cotton were analyzed through genome wide identification and comprehensive analysis was conducted (Sun et al., 2019). In the work were identified genes as shown in the Table 2.5.

Table 2.5. Auxin early response genes

Order	Name	Quantity
1	ARFs (auxin response factors)	71
2	Aux/IAA (Auxin/Indole-3-Acetic Acid)	86
3	GH3 (Gretchen Hagen3)	63
4	SAUR (small auxin upregulated RNA)	194

Moreover, phylogenetic analysis showed that families of SAUR, ARF and GH3 were possibly subject to considerable evolutionary division between *Gossypium hirsutum and Arabidopsis thaliana.* On the other hand, family of Aux/IAA genes were evolutionary conserved. Expression profiles revealed that these gene families have had considerable participation in assessment of callus embryogenic competence in upland cotton. The work provided RNA sequencing of important stages of SE in totipotent and recalcitrant cultivars of *Gossypium hirsutum,* whereas embryogenic and non-embryogenic callus were sampled and sequenced using Illumina.

This study also revealed that genes of Aux/IAA family are possessing higher expression levels in non-embryogenic callus in totipotent cultivar compared to recalcitrant cultivar. Tight co expression of auxin early response genes with somatic embryogenesis genes was observed showing that SE is regulated by these gene family interaction (Sun et al., 2019).

Auxin response in cells is mediated by receptors that can incorporate with TF domains. Receptor TIR1 (TRANSPORT INHIBITOR RESPONSE 1) (Dharmasiri et al., 2005) and homologs of it are fundamental parts of ubiquitin ligases that can link with AUX/IAA family (Dharmasiri et al., 2005; Gray et al., 2001). Auxin is enhancing connection of receptors to proteins of AUX/IAA family (Tan et al., 2007), issuing ubiquitination. Moreover, AUX/IAA family can create protein interactions due to situated domain. Further AUX/IAA degradation is important for auxin signaling because it permits target genes activation by activating auxin response factors (Szemenyei et al., 2008; Guilfoyle and Hagen, 2007).

The work of Robert et al. (2015), showed key regulators of auxin efflux that plays important role in describing embryo axis and further plant development. Several works defined role of PIN transporters, that are carrying auxin efflux throughout shoot and root definition. However, influx carriers such as AUX1 and LIKE-AUX1 proteins are not studied well for their specific roles. This research showed necessity of AUX1 and LAX in formation of root and shoot poles on example of MDEs. Moreover, the work gives information about ARF5 and its loop with auxin transport. These carriers such as PIN that participate in auxin efflux with auxin influx regulators AUX and LAX are playing crucial role in transport of auxin to the root tips. Results received during this research indicate that cell specification based on auxin needs proper transport of auxin mediated by mechanisms of influx and efflux (Robert et al., 2015).

In a work dedicated to ARFs, that are part of early auxin genes, it was shown that these factors bind to elements of auxin response. The work provided analysis of suppression and activation functions/capabilities of ARFs, whereas it was found that four ARFs from nine possess transcription activating features (Ulmasov et al., 1999).

Most of the available data proposes that signaling pathway of auxin is the oldest, continued by gibberellin and followed by brassinosteroids (Depuydt and Hardtke, 2011). Regarding results of experiments on mutants with disarranged phytohormones biosynthesis or recognition, four groups are accepted as significant growth regulators. They are auxin, cytokinin, brassinosteroids and gibberellins. It was established that cell proliferation is done by cytokinin, elongation by gibberellins, whereas auxins are participating in both processes. Brassinosteroids can have role in elongation as well as in proliferation. Interestingly, these four phytohormones can regulate alone as well as in crosstalk (Santner et al., 2009; Hardtke et al., 2007).

2.2.2 AP2/ERF transcription factor family

One of the biggest gene families that are unique in the plant world is a family of morphogenic genes. *BBM* is one of the numerous genes in AP2/ERF transcription factor family that involves different roles in plant growth and development. Up to date scientists are working with this gene discovering aspects of regulation, co expression and development. As it is showed in the table, *BBM* gene is quite famous topic of research (figure 2.6) (Yavuz et al., 2020).

LAFL genes network unites several genes that has B3 domain in common. These genes include *FUS3*, *ABI3*, *LEC1/LEC2* (Figure 2.7) (Jia et al. 2013). *BBM* triggers SE and this is quantitatively regulated by *LEC2* and *ABI3* genes (Horstman et al., 2017; Wójcik et al., 2020; Wójcikowska et al., 2020; Kumar and Jha, 2020).

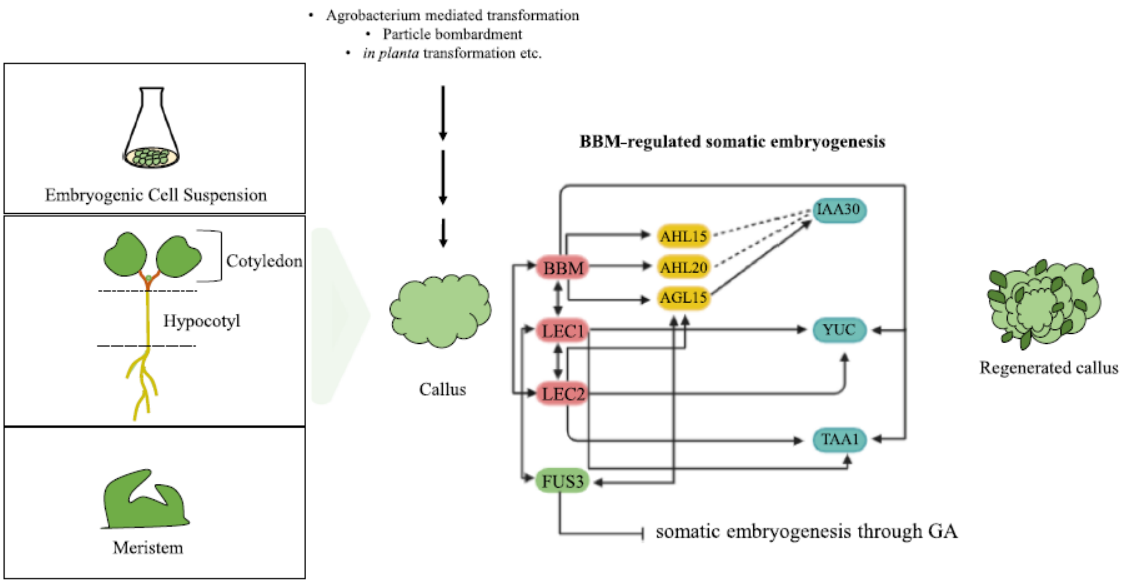

Figure 2.7. Role of *BBM* gene in SE (Yavuz et al., 2020)

Modern conditions of environment are highly undesirable with changing weather conditions, less water availability, salt distribution and soil erosion. In order to protect themselves, plants are developing changes that are able to face abiotic stresses. Abiotic stresses are activating special network system of signaling that is regulated by transcription factors. These transcription factors regulate special stress responsive genes (Mizoi et al., 2012). One of this transcription factors are AP2/ERF family that is plant specific and has conserved DNA binding domain. The family includes four crucial subfamilies such as ERF, AP2, RAV and DREB (Sakuma et al., 2002).

This TF family has wide functions starting from regulating developmental processes finishing by response network to stresses. DREBs are one of the proteins of AP2/ERF family that activate stress genes by binding to the cis-acting elements. DREB subfamily genes are being studied and showed that they are crucial elements of plant stress reaction. Moreover, ERF members are capable of binding to ethylene-responsive element and regulate plant reaction to abiotic stress (Mizoi et al., 2012).

Transcription factor family of AP2/ERF includes numerous uncharacterized genes. There has been research (Nole-Wilson et al., 2005) covering Phylogenetic trees of 18 AP2 subgroup genes. Seven genes of Arabidopsis thaliana showed similar functions as *AIN* gene that participates in flower development. Moreover, *AIL*-like genes showed expression patterns in other plant tissues but mostly in developing young tissues. In situ hybridization was used to characterize spatial distribution of *AIL5, AIL6, AIL7* genes and all of them are situated in flowers. *AIL5* gene overexpression resulted in bigger flower organs. Results of work showed consistent expression pattern of *AIL* genes is present in dividing meristematic regions (Nole-Wilson et al., 2005).

Gene expression control can be achieved by regulators such as transcription factors and microRNAs (Pabo and Sauer, 1992). Transcription factors are controlling genes and it is determined by these protein DNA binding domains. The domains are opening a way for binding TF to a special DNA motif. Different approaches have been used to identify regulatory motifs such as mutation of promoter sequences, expression cloning, whole genome sequencing and many others (Holstege and Clevers, 2006).

Genome wide approaches are important for explaining TFs; ChIP-based techniques are giving more direct results although specific developmental stage is limiting this approach. Bioinformatic tools for predicting regulatory regions work efficient as well, but activity testing should be done.

EEL (enhancer element locator) algorithm approach assumes that binding sites are conserved relaying on orthologous regulatory regions. These methods need further development and in comparison, to old approaches, they provide better explanation of regulatory systems (Holstege and Clevers, 2006).

2.2.3 *BBM* gene identification

Research reports around the world on *BBM* gene show that it can significantly improve regeneration. In the work provided by Passarinho et al. (2008), ectopic expression of *BBM* gene led to spontaneous cell proliferation of SE, as well as induced organogenesis and callus development. The work showed target genes that are activated by *BBM* ectopic expression in Arabidopsis seedlings. Cycloheximide and analysis of DNA microarray in tandem with *BBM:GR* protein that is post translationally regulated were used to identify target genes. The work has showed that *BBM* is activating numerous uncharacterized genes with potential participation in transcription, cell signalling pathway, protein turnover, cell wall synthesis, etc. Some of the target genes are constituted in meristematic regions. Another group of target genes are being showed to be involved in modifications of cell wall. This research showed that ACTIN DEPOLYMERIZING FACTOR9 (ADF9) protein is encoded by one of the target genes of *BBM*. Moreover, actin marker was used to follow consequences of activating the BBM:GR. Significant changes were observed in ectopic expression of *BBM:GR* that resulted in difference in actin organization. The work concluded that *BBM* is capable of activating sophisticated network associated with developmental processes (Passarinho et al., 2008).

Another work in discovering relations of SE and *BBM* gene expression was performed by Kulinska-Lukaszek et al. (2012), where immature embryos were studied exploring *BBM::GUS* construct. Throughout the culture period of explants, spatio-temporal difference was observed in expression of *BBM* gene. Except distal cotyledonary parts in immature zygotic embryos, *BBM* activity was observed. Moreover, GUS staining showed significant increase in every part of explants with stable expression for a few days followed up with moderate decrease of GUS expression on the 5th day. Activity of *BBM* promoter divided cells, developed somatic embryos and callus. *BBM* expression was absent or poorly visible in non-morphogenetic parts (Kulinska-Lukaszek et al., 2012).

The work of Boutilier et al. (2002), covering molecular mechanisms of embryonic pathway showed more insight into initiation processes. Subtractive hybridization was used to establish target genes during embryo development in Brassica napus immature pollens. One of the discovered upregulated genes is *BABY BOOM* (*BBM*) which is mostly expressed in seeds and growing embryos. Somatic embryos and cotyledon like structures were formed in response to *BBM* ectopic expression in Brassica napus and Arabidopsis thaliana. Moreover, pleiotropic phenotypes were formed such as hormone free plants regeneration, different morphology of leaves and flowers, neoplastic growth. It was suggested that overexpressed *BBM* gene in combination with *BBM* pattern in seeds can promote cell differentiation and cause morphogenesis during SE. This research provided RNA blotting, RT-PCR, in situ hybridization of mRNA to discover spatial and temporal motifs of *BBM* in growing seeds and microspore cultures. It was shown that *BBM* expression is found in the 4-day old embryogenic phase, despite the fact that 4day old non-embryogenic phase doesn't express transcript of the gene. Embryos of globular to cotyledonary stage express *BBM* transcripts. Non-quantative RT-PCR was used to detect mRNA from *Brassica napus* seeds due to difficult detection from total RNA. Patterns observed in seed development and microspore embryos are similar concluding that *BBM* expression can be found in earliest globular embryo development until latest stage of seed development. Analysis of non-seed tissues showed that extremely less amount of *BBM* pattern can be found. Genetic constructs used in this research are listed in Table 2.6 (Boutilier et al., 2002).

Table 2.6. Genetic constructs tested in research of Boutilier et al. (2002)

Construct name	Vector	Gene	Promoter	Specific feature	Strain
35S::BBM	pBINPLUS	*BBM1*	35S sunflower	*Alfalfa mosaic virus* translational enhancer	C58C1pMP90
UBI::BBM	pBINPLUS	*BBM2*	*UbB1 POLYUBIQUITIN* promoter	Lacks first Intron and ATG tr.site	C58C1pMP90

In the work of Srinivsan et al. (2007) heterologous expression of *BBM* gene was used to improve regeneration rates of tobacco. Target genes can be studied by mutants and gain of function research showed that *BBM* gene is sufficient to start spontaneous SE in *Arabidopsis* and *Brassica*. The *35S::BBM* construct showed similar response in tobacco lines with callus initiation, sterility and rumpling of leaves, however spontaneous SE was not observed. Lines with severe ectopic phenotypes are not able to regenerate due to complete sterility. And therefore, the work showed generation of lines with significant misexpression phenotypes by using *BBM:GR* construct. The *BBM:GR* construct was able to obtain shoot and root development, whereas SE was induced using hypocotyls in a cytokinin media. This work showed differences between *Arabidopsis/Brassica* and tobacco lines (Srinivasan et al., 2007).

Lutz et al. (2015) provided research on a system of *BBM* gene that is induced by steroids capable of increases regeneration in leaf explants of Arabidopsis (Lutz et al., 2015). Sikdar et al. (1998), reported that leaf explants in a prolonged culture periods may cause sterility of plants. Prolonged tissue culture period of *Arabidopsis* can cause sterility of plants (Sikdar et al., 1998). Among possible reasons of sterility were named essential application of 2,4-D and as a resulted aneuploidy/polyploidy; extended culture periods that cause somaclonal variation; polyploidy of leaf explants (Galbraith et al., 1991; Melaragno et al., 1993; Zoschke et al., 2007).

To overcome these problems, effective regeneration system was needed to induce cell division, sufficient regeneration capacity and prolongation of diploid state. Preferable explants were shoot apex meristematic cells and embryo cells.

The work of Lutz et al. (2015) tested possibility of obtaining fertile plants by ectopic expression of TFs in a long culture period. Two constructs were developed by fusing glucocorticoid receptor with coding region of *BBM* gene. Upon presence of dexamethasone, which is steroid, *BBM:GR* is entering the nucleus and triggering embryogenesis genes, while in an absence of this steroid, fusion protein is situated in the cytoplasm. Plant culture media containing DEX showed shoot regeneration from leaf explants. Medias without DEX application were able to start flowering and seed development. Prolonged culture periods are possible to obtain fertile plants in leaf explants with application of *BBM:GR* construct (Lutz et al., 2015). It was reported that no difference was observed between transformed and non-transformed *Arabidopsis* plants on media grown without steroid inducer. However, shoot regeneration and embryo formation was observed on leaf explants with application of DEX. Therefore, this technique is able to aid in research with a long tissue culture studies and help in getting fertile explants from Arabidopsis plastid transformation (Lutz et al., 2015).

In order to understand signalling pathway of somatic embryogenesis genes El Ouakfaoui et al. (2010) provided research on AP2 TF family genes, such as *BBM1*, *AIL5*, *PLT2* isolated from embryo cultures of soyabean (*Glycine max*). It was showed that isolated *BBM1* gene among *Arabidopsis*, *Glycine max* and *Brassica napus* are homologous to each other, whereas *Glycine max PLT2* and *AIL5* genes are homologous to *Arabidopsis PLT2* and *AIL5* genes. Over expression of *BBM1* gene caused formation of somatic embryos and induced further development of pleiotropic phenotypes on post germinative development. Moreover, sequence comparisons were performed and there were motifs that were outside of AP2 domain. Cooperative action of motifs was revealed (El Ouakfaoui et al., 2010).

In a work of Omidvar et al. (2013), another member from AP2/ERF TF family was isolated and studied. Probe was designed based on tandem repeats of ethylene-responsive element and hybrid yeast system. Oil palm was used as a source for ERE binding protein. Based on phylogenetic analysis and alignment, this protein was classified to one of subfamilies of AP2/ERF, to DREB/CBF. Through EMSA it was revealed that *EgEREBP* is capable of binding to ERE as well as to DRE. Expression of *EgEREBP* gene is activated under application of different hormones that was established by RT-PCR analysis (Omidvar et al., 2013).

One of the works conducted is sweet pepper genetic transformation that was mediated by BBM TF. Genetic editing of economically important and nutritionally rich crop can raise community-based resource for functional genetic studies of this crop. Sweet pepper is known to be recalcitrant with low levels of recovery in *Agrobacterium* mediated transformation and protocols of transformation are needed to be revised. Impossibility of sweet pepper to develop cells for transformation and regeneration are the main problems. *BBM* gene was efficiently used for overcoming this problem and temporary gene activation is able to induce cell regeneration and to produce somatic embryos in progeny plants (Heidmann et al., 2011).

Production of doubled haploids is necessary for establishing large scale production of grain cereals and efficient protocol is needed. Most of the breeding programs prefer usage of isolated microspore culture, although the method has several difficulties that stop large scale production. Genes that can manipulate processes of embryogenesis can be one of the successful approaches to overcome this limit. For this reason, *BBM*-like proteins were used from maize amino acid sequences for studying bread wheat. Analysis of amino acid sequences showed that AP2 domains are present as well as bbm-1 motif. On a result of analyses, it was suggested that *TaBBM-gB* can be a pseudogene. Comparison of homologs with other *BBM*-like genes showed similarity and expression was detected in microspores cultures. Homolog of *TaBBM* can be efficient for double haploids production, as well as for better regeneration and transformation in wheat species (Bilichak et al., 2018).

In a process of induced somatic embryogenesis embryos are formed from vegetative cells rather than from fusion of gametes. In a work of Horstman et al. (2017), *BBM*, *LEC1*, *LEC2* genes were showed as key mangers of somatic embryogenesis due to results obtained form ectopic overexpression of genes in *Arabidopsis* with application of growth hormones. However, exact molecular regulation pathways are being studied. This research revealed that *BBM* gene is upstream of *LAFL* gene network that includes *FUS3*, *ABI3*, *LEC1* and *LEC2* genes. In addition to this *BBM* regulated *LAFL* network, the work showed that *BBM* mediated SE depends on dose and context and most of the phenotypes are connected with *LAFL* differential manner (Horstman et al., 2017) Moreover, another *BBM*-like protein *PLT2* was upstream of *LAFL* network. Several constructs were developed as shown on Table 2.7.

Table 2.7. Constructs developed in research of Horstman et al. (2017)

Studied constructs	Mutants studied
35S:BBM-GR	*lec2-1*
35:BBM-GFP-GR	*agl15-3*
BBM:BBM-YFP	*pkl-1*
5S:BBM-GFP	*lec1-2*
LEC1:LEC1-GFP	*fus3-3*

Research provided by Mookkan et al. (2017), showed genetic transformation in maize and sorghum with the help of *BBM* and *WUS2* genes. This study provided complex research on co expression of two morphogenic genes *BBM* and *WUS2* in maize. Moreover, excision system of CRE/lox was used. Constructs of *RAB17M:CRE, NOS At:WUS2, UBIM:BBM, UBIM:GFP, CRE:WUS2:BBM* were checked on recalcitrant maize inbred and sorghum (Mookkan et al., 2017).

Li et al. (2014) showed characterization and analysis of *BBM* gene from two larch species. *BBM* and *PLT* genes share four conserved regions that may suggest similar functions. Moreover, in one larch species expressing *BBM* gene, several cis-acting elements and motifs were identified (Table 2.8).

Table 2.8. Cis-acting elements found in larch species expressing *BBM* gene

<table>
<tr><td rowspan="7">Potential cis-acting elements</td><td colspan="2">Name</td><td>Function</td></tr>
<tr><td colspan="2">TATABOX5</td><td>Functional element</td></tr>
<tr><td colspan="2">ROOTMOTIFTAPOX1</td><td>Specificity of root</td></tr>
<tr><td colspan="2">AUXREPSIAA4</td><td>MYB identification element</td></tr>
<tr><td colspan="2">ARR1AT</td><td>Response to cytokinin</td></tr>
<tr><td colspan="2">GARE1OSREP1</td><td>Response to gibberellin</td></tr>
<tr><td colspan="2">PYRIMIDINEBOXHVEPB1</td><td>Response to abscisic acid</td></tr>
<tr><td colspan="4">Conserved motifs</td></tr>
<tr><td>Gene</td><td>Motif 1</td><td>Motif 2</td><td>BBM motif</td></tr>
<tr><td>LkBBM</td><td>MSSNWLFLSPSHLD</td><td>ILEPKLDFLG</td><td>DNISMLSQN</td></tr>
<tr><td>AtBBM</td><td>MSSNWLFLSPSLESRDW</td><td>IPKLNFLG</td><td>DDSLSMLSQN</td></tr>
<tr><td>PtBBM1</td><td>MSSNWLFLSLHLSDSQDWN</td><td>IQPKLNFLG</td><td>DSSISMLSKN</td></tr>
<tr><td>PtBBM2</td><td>MSSNWLFLSPSLHLSDQDWN</td><td>IQPKLNFLG</td><td>DSSISMLSKS</td></tr>
<tr><td>AtPLT1</td><td>MSSNWLFLSPSLHLNDNQFWN</td><td>MIEPKVDFLG</td><td>NDNSLSVNEES</td></tr>
<tr><td>AtPLT2</td><td>MSSNWLFLSPLHLNDNQWNQEW</td><td>MIEPKVDFLG</td><td>NDNSLTVNEES</td></tr>
<tr><td>PtPLT 1</td><td>MSSNWLFLSPLHLNDNQEWS</td><td>LLEPKVDFLG</td><td>NENSLVVEEN</td></tr>
<tr><td>PtPLT 2</td><td>MSSNWLFLSP</td><td>LLEPKVDFLG</td><td>NDNSLVVEEN</td></tr>
</table>

Specific expression was found in root parts of larch, which shows that *LkBBM* is playing important role in root growth. Transcript level of *LkBBM* was increasing on 8th day of root treatments. The work proposed the usage of *LkBBM* as molecular marker for larch root primordial (Li et al., 2014).

Major monocots are studied for successful transformation, even though that genotypes with poor agronomics are being selected for editing due to ineffective regeneration of elite commercial genotypes. Fortunately, research (Lowe et al., 2016) is showing possibility of successful transformation of non-transformable maize inbred like it was done with recalcitrant maize inbred – PHH5G. Usage of morphogenic transcription factors such as *BBM* and *WUS2* made it possible and recovered 40% of transgenic calli. However, limitations of monocots are still involved with the space required for supplying immature embryos. Research also showed transformation using embryo slices of mature maize seeds and leaves as explants. Moreover, effective stimulation was showed in indica rice, sorghum and sugarcane (Lowe et al., 2016).

Another excellent work demonstrated in dicots where cytokinin formation was observed under gene stimulation (Ebinuma et al., 1997). Unfortunately, attempts of repeating this methodology by numerous scientists are not recorded, but it can be said the attempts were with poor results due to a few reports of transformation using growth-stimulating genes (Lowe et al., 2016).

Another work (Bandupriya and Dunwell, 2012) performed in studying AP2/ERF transcription factor family showed potential in improving somatic embryogenesis in coconut. *35S:CnANT* construct was developed to study functions of *BBM* homologous gene. Screening of transformants was done by herbicide spraying. Pleiotropic effect was seen in transgenic seedlings. This work is showing potential tool for improving regeneration capacity of transformants (Bandupriya and Dunwell, 2012).

Situation in maize genetic transformation changed a little since the first report of immature embryo being suitable for direct transformation (Songstad et al., 1988). Transformation of maize includes immature embryos as explants, callus selection during some time, efficiency mostly vary among genotypes and inbred making maize transformation difficult to work in academic laboratories (Jones et al., 2019).

Research of Yang et al. (2014), showed *BBM* orthologous genes in *Rosa canina* and amplified for further studies. Phylogenetic analysis revealed that *BBM* genes form roses are orthologous to *BBM* genes form soybean and barrel clover. Expression pattern of *BBM* gene from roses was detected in young dividing tissues such as calluses, developing roots, whereas old tissues did not have expression patter of *BBM* gene. Expression of gene was found in nucleus. Two genetic constructs designed for improving embryogenesis were tested. These constructs are *35S::RcBBM1* and *35S::RcBBM2* showed better shoot regeneration, however spontaneous SE was not detected. The work suggests usage of *RcBBMs* for increasing efficiency of shoot development (Yang et al., 2014).

Rupps et al. (2016) in a work covering somatic embryogenesis in larch tree isolated and studied several genes such as *BBM*, *WUS* related *WOX2*, *LEC1* and *SERK*. The genes were compared with other sequences that revealed similarity to angiosperm homologs. Expression pattern was analysed during SE that showed activity of *LEC1* and *WOX2* genes in the beginning of somatic embryogenesis, whereas *BBM* and *SERK* genes are activated later. *LEC1* ectopic expression effects on formation of cotyledons and germination rates. The work suggests usage of genes as molecular markers during SE (Rupps et al., 2016).

Reproduction systems of angiosperms require complex growth of different tissue with multiple genotypes. To reach fertilization, haploid megagametophyte and ovary that is diploid, need to harmonize their growth, after that pollen grains (male gametes) must manage to get ovary sac. After the process if fertilization seed growth needs managed growth of integuments, endosperm, and zygote. Communication of these tissues is done through factors that contribute to signaling and transcription. The work described wide roles for chromatin remodelers in Arabidopsis. *PICKLE* is one of the remodelers and in case of absence abnormal phenotypes are received. Another paralogous gene called *PICKLE RELATED2* has opposite functions to *PICKLE* showing suppression of pickle phenotypes. Interestingly, modifications in size of pickle phenotypes are enough to find embryo expression characteristics in roots of seedlings. The work suggested significant part for chromatin remodelers in reproduction and showed influence of one tissue to other tissues (Carter et al., 2016).

AIL, *AIL*-like transcription factors are significant regulators of cell developing pathways. There have been plenty of works related with *AIL* gene, however, signaling pathway and crosstalk need to be revised. Horstman et al. (2015), showed physical interaction of *BBM* gene and AIL proteins with different HDG TF family members.

HOMEODOMAIN GLABROUS (HDG) members such as *HDG1*, *HDG11* and *HDG12* arrested root and shoot growth because of overexpression. On the contrary, downregulation of these genes leads in cells over proliferation. Results obtained suggest that HDG proteins are participating in cell differentiation. The work also demonstrated regulation co-network of *BBM* and *HDG1*, and a network where expression profiles of *BBM*, *AIL*, *HDG* are regulated by each other of genes. To sum up, results are proposing different roles for *HDGs* as cell differentiators and AIL proteins as cell proliferators. Moreover, it is suggested that functions of these proteins are regulated in transcriptional level (Horstman et al., 2015).

In a work of Passarinho et al. (2008), overexpressed rapeseed *BBM* gene was sufficient to lead spontaneous embryo formation and induce callus formation. Upregulated genes during somatic embryogenesis were analyzed using DNA microarray and *BBM:GR* construct in combination with cycloheximide to see whether *BBM* gene is activating SE. The work showed transcriptional activation of uncharacterized genes that possibly regulate SE. Some of the genes participate in alterations of the cell wall and expressed in meristematic regions. ACTIN DEPOLYMERIZING FACTOR9 (ADF9) is one of the proteins that is encoded by target genes of *BBM*. Effect of *BBM:GR* construct on cytoskeleton was studied using actin marker – GFP:FABD. Activation of *BBM:GR* by dexamethasone promoted drastic difference in organization of actin forming networks that have high rates of turnover. The data of research suggest that network of fundamental pathways is activated by *BBM* gene (Passarinho et al., 2008).

Florez et al. (2015), showed promotion of SE in cacao using *BBM* gene. Low efficiency of propagation through SE and recalcitrant nature of *Theobroma cacao* promoted searching ways to erase limitation of propagation in large scales. Expression of *BBM* gene during embryo formation and development was studied. The study compares expression patter of the gene during somatic and zygotic embryogenesis. It was concluded that *BBM* gene is expressed in higher levels throughout SE. Moreover, the work showed that exogenous hormone applications were not needed to establish SE in cacao and *Arabidopsis* cultures. The strategy is suggesting hormone free technology of cell reprogramming and better SE manipulation. Method is highly beneficial for micropropagation of elite recalcitrant varieties as well as for increasing regeneration capacity of transgenic plants (Florez et al., 2015).

Another outstanding work was done by Khanday et al. (2019) showed effect of *BBM* gene in embryogenesis after fertilization process in rice plant. *BBM* gene is found in sperm cells of *Oryza sativa*. It was proven that *BBM* overexpression is sufficient for the process of parthenogenesis. Appearance of *BBM1* profile is specific for the male allele, but thereafter biparental. Embryo development arrest and abortion are observed as a result of triple knockout of *BBM* genes; however male transported *BBM1* gene is rescuing embryo formation. Observed processes suggest that fertilization requires pluripotency features transported by male genome. This research showed possibility of obtaining clonal progeny that maintains parental heterozygosity. It can be obtained by expression of *BBM1* gene in egg cells, in combination with genome editing to replace mitosis to meiosis (MiMe). Multiple generations of clones maintain traits of asexual propagation making impossible progenies to have increased yields. The work showed viability of asexual propagation and propose possibility of maintaining hybrid vigor by seed propagation (Khanday et al., 2019).

One of the naturally occurring processes of asexual seed formation in flowering plants is apomixes. It avoids meiosis or egg fertilization. Normally, plants that are formed from apomixes are identical genetically to original maternal plant. This process of apomixes can be significantly important for saving vigor from hybrid generations. For example, members of grass family are reproducing by the process of apospory. Apospory can be characterized by apomeiosis and parthenogenesis. Ovary nuclear cells are forming unreduced number of embryo sacs in apomeiosis, whereas parthenogenesis is characterized by development of unreduced egg in the embryo avoiding fertilization. In one the grass family species - *Pennisetum squamulatum*, the process of apospory is segregated as one dominant locus – ASGR, which stands for apospory specific genomic region. The study showed that *PsASGR-BBML* gene is found in unfertilized egg cells that can start parthenogenesis and can induce haploid generation from transgenic sexual *Pennisetum glaucum*. Research also showed reduced parthenogenetic embryos and decreased embryo cell rates as a result of decreasing *PsASGR-BBML* expression profile. The work proposed crucial part of *PsASGR-BBML* in the process of parthenogenesis. Results obtained from experiments suggest usage of construct as a valuable tool for haploid induction (Conner et al., 2015).

Another similar work was provided using *PsASGR-BBML* construct in rice and maize to investigate possibility of establishing haploid lines in the crops. The work aimed to study ability of naturally occurring apomixes on crops. *PsASGR-BBML* is capable of inducing parthenogenesis that leads to establishment of haploid lines. During research two promoters were tested, as well as genomic and cDNA sequences from *PsASGR-BBML*. Mature seeds of rice and maize were successful as an explant for recovering haploid plants. However, *PsASGR-BBML* was unsuccessful in haploid development in Arabidopsis plant. The work suggests further investigations to study complexity of embryo formation and *PsASGR-BBML* characterization (Conner et al., 2017).

Another research investigating promotion of regeneration and increasing transformation efficiency was done involving recalcitrant maize inbred and sorghum lines using co expression of *BBM* and *WUS* genes. Differential use of *BBM* and *WUS* genes with CRE/lox system that is inducing desiccation, was suggested to enable regeneration without selectable marker usage. Expression cassette that was used in the study contains drought inducible promoter that drives CRE that brackets *WUS, CRE* and *BBM* genes. Maize UBI promoter was used to direct GFP expression that enables developmental and transient analysis. Vector transformation illustrated temporary GFP expression and promoted SE in both sorghum and maize explants. Desiccation stress oppression made possible excision of *BBM*, *CRE* and *WUS*, allowing further plant regeneration and *UBIM::GFP* to be maintained. Strong expression of GFP was found in both early and late embryos, pollen, vegetative plant parts and relatively young shoots. PCR and Southern blot were performed in addition to GFP analysis. Indication of positive inheritance of transgenic qualities was done by segregation analysis of progenies. This research showed possibility of extending application of morphogenic genes to solve transformation difficulties (Mookkan et al., 2017).

CHAPTER III

MATERIALS AND METHODS

This research is focused on understanding the role of *BBM* gene in cotton embryogenesis and investigating whether *BBM* gene is activating *LEC1*, *FUS3*, *LEC2* and negative regulator of embryogenesis – *PKL* gene.

3.1 Experimental Materials

3.1.1 Plant materials

In the beginning of research, cotton Coker 312 cultivars were planted in greenhouse and leaves were used as explants for DNA extraction. Coker 312 non-embryogenic calli was used as an explant for RNA extraction (Figure 3.1). Moreover, soybean cultivar was used as an alternative source of *BBM1* gene. Seeds of Coker 312 were received from Aydin Nazilli Research Institute.

Figure 3.1. Cotton DNA and RNA sources, Coker 312 for DNA (left) and Coker 312 calli for RNA (right)

Cotton cultivars were grown for 3 months, regularly watered on soil and perlite mixture (3:1). Cotton calli was grown for 3-4 months on CIM in a growth chamber at 27 ± 1 °C with 16:8 h photoperiod with a monthly subculture.

Furthermore, cotton Coker 312 was used for genetic transformation experiments. Coker 312 has a high embryogenic potential compared to other *Gossypium* genotypes and exhibits broad responses to different growth regulators, classified as a model cultivar (Trolinder and Xhixian, 1989; Trolinder and Goodin, 1988). Before transformation experiments, cotton plants were grown in aseptic conditions (Figure 3.2).

Figure 33.2. *In vitro* propogation of Coker 312 cultivars under tissue culture conditions. Individual tubes used for preventing contamination

Cotton cultivars were grown for 7 days, and only 1 week old plants were used for transformation due to high response and relatively less amount of phenols produced by a plant in this age (Ozyigit et al., 2007). All cotton cultures were grown at 27±21 ºC with a photoperiod of 16/8 h on MS medium containing 1mg/l BAP and 1 mg/l GA_3 hormones to improve germination of seeds (Murashige and Skoog, 1962).

3.1.2 Bacteria strains and plasmid

Competent cells of *E. coli Top10* strain were used as a source for multiplication and further *AGL-1* strain of *A. tumefaciens* was selected as a source for construct transferring. Gateway gene transfer technique was used for *BBM1* gene from soybean with pEarlyGate100 vector, whereas TA cloning was used for *BBM* gene from cotton. Maps of genetic constructs were prepared using SnapGene program.

3.2 Methods

3.2.1 Cotton seed sterilization and tissue culture

Cotton seeds were carefully observed, and healthy ones selected for tissue culture. Prior to surface sterilization seeds were delinted by adding few drops of 100% H_2SO_4 and swirling until full browning. After obtaining deep brown color, seeds were immediately rinsed in tap water several times. Further, all seeds were kept in autoclaved water containing sulfur powder as a fungicide for 30 minutes. All further experiments were performed in aseptic conditions inside laminar. Seeds were sterilized in two ways with 15% H_2O_2 and 5% bleach. In both ways of sterilization with hydrogen peroxide and bleach, seeds were placed into tubes and continuously shook for 13 minutes, later washed with autoclaved water with a few drops of Tween-20 and rinsed three times in autoclaved water. Then seeds were rinsed in 70% alcohol and again washed with water. Seeds were placed on sterile autoclaved tissue paper for drying (Figure 3.3):

Figure 3.3. Steps of cotton seed sterilization, pre-treatment of seeds in fungicide (1), treatment with 15% hydrogen peroxide (2), treatment with tween-20 (3), washing in autoclaved water (4), seed drying (5) and seed germination (6)

3.2.2 DNA and RNA extraction

Cotton and soybean RNA extraction was performed by Trizol method. Prior to RNA extraction, mortals, pestles, tips, Eppendorf tubes were treated with DEPC water and autoclaved 40 minutes at 121℃ and dried in the oven overnight at 70℃. Plant samples were grinded with the help of mortal and pestle adding liquid nitrogen. Each sample of 0.2 g was treated with 1.5 ml of TRI Reagent® (Sigma-Aldrich, T9424). Vortexing was performed to homogenize samples and left for 10 minutes incubation at room temperature. Centrifuge at 14,000 rpm and 4C was used to separate aliquots into two phases. After centrifuging, obtained supernatant was transferred into clean new Eppendorf tube and chloroform of 400 µl was put. Next, shook by hand for 15 seconds and centrifuged for 15 minutes at 14,000 rpm and 4C. Obtained supernatant was transferred to a clean tube. Supernatant was treated with 500 µl of ice-cold isopropanol alcohol and left for incubation at room temperature. Further, it was again centrifuged at 11,000 rpm and 4C for ten minutes. Liquid phase was removed from tube and pellet was treated by 1 ml of 70% EtOH. Tubes were centrifuged at 9,000 rpm at 4C for five minutes. Next, liquid phase was discarded from the tube and pellets were dried using hood for approximately 10 minutes. Pellets were dissolved by adding 50 µl of DEPC water. Next step of genomic DNA removal was performed. RNase free tube was used for further reactions.

Table 3.1. Removal of genomic DNA from RNA samples

RNA	1 µg
10X Reaction buffer with magnesium chloride	1µl
DNase I, RNase free	1µl (1U)
DEPC water	To 10 µl

All components were added into the tube and incubated for 30 minutes at 37C. Further, 1 µl 50 mM EDTA was put inside reaction tube and incubation at 65C for ten minutes was performed. NanoDrop spectrophotometer was used to check quality of obtained RNA. Prior to gel electrophoresis that was used to observe RNA integrity, gel tank was soaked in 5% H_20_2 for thirty minutes and rinsed with DEPC water. For preparing gel 1% agarose gel was added and boiled in 0.5x TBE (Trisborate EDTA) buffer. Next, ethidium bromide was added into the flask after solution had been cooled. Solution was poured into the gel tank with combs without making any bubbles that may affect integration. After 30 minutes of solidification, all samples of RNA were loaded carefully and in the end DNA ladder was added. Finally, after one hour run at 90V, samples were observed under the UV light.

3.2.3 cDNA synthesis

First strand cDNA was obtained from converting RNA samples with the help of Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit (Cat. No. K1621).

Table 3.2. The content of cDNA synthesis reaction

Reaction	Amount	Concentration
RNA	6.5 µl	~2000 ng
Oligo(dT)18 Primer	3.0 µl	0.75 µM
5X reaction buffer	4.0 µl	1X
RiboLock RNase inhibitor (20U/ µl)	0.5 µl	10 U
dNTP (10 mM)	2.0 µl	1 mM
Revert Aid Reverse Transcriptase (200U)	1.0 µl	200 U
DEPC Water	3.0 µl	
Total	20 µl	

Reaction of chemicals listed in the abovementioned table was prepared and the mixture was left on incubation for one hour at 37 °C and ten minutes at 70 °C in PCR machine and cDNA synthesis were finished.

3.2.4 Primer design for insertion of the *BBM* gene into the vector

NCBI primer design program was used to design specific primers for cotton and soybean. *GhBBM*-F and *GhBBM*-R were designed for TA cloning, whilst *GmBBM1*-F and *GmBBM1*-R for Gateway cloning. Both primers have *att*B1 and *att*B2 attachment sites (Table 3.3).

Table 3.3. Primers designed for Gateway cloning; M13, *GmBBM1* primers were used in BP reaction, *npt*II primer pair was used in LR reaction

Name	Forward	Reverse
GhBBM	5'-GGGGACAAGTTTGTACAASAAGCGCAGGCTTCATGAGTTCACAAAACATTGATG-3'	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTGTCACTCCATTTCCAGTTTTTCC-3'
GmBBM1	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGGGTCTATGAATTTGTT-3'	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTATTCATGCAATAAAGAGAAGGG-3'
M13	5'-GTAAAACGACGGCCAGT-3'	5'-CAGGAAACAGCTATGAC-3'
*npt*II	5'-TTGCTCCTGCCGAGAAAG-3'	5'-GAAGGCGATAGAAGGCGA-3'
bar	5'-GCACCATCGTCAACCACTA-3'	5'-ACAGCGACCACGCTCTTGAA-3'

3.2.5 Amplification of *BBM* gene from soybean cDNA and cotton DNA by PCR

Cotton DNA and soybean cDNA were used as templates for amplification of BBM gene by Phusion® High Fidelity DNA Polymerase. Gradient PCR was used to optimize temperatures in the range of 44 and 64°C. PCR components and temperature cycles are given in the table below (Table 3.4).

Table 3.4. PCR components used for gene amplification

Components	1x
Forward Primer 10 mM	1 µl
Reverse Primer 10mM	1 µl
5X Phusion™ Buffer 1X	4 µl
dNTPs 10mM	0,4 µl
DMSO 10mM (3%)	0,6µl
Phusion® High Fidelity DNA Polymerase (Thermo Scientific) 0.02 U/µL	0,2 µl
H20	7,8 µl
Template 20-100 ng	5µl

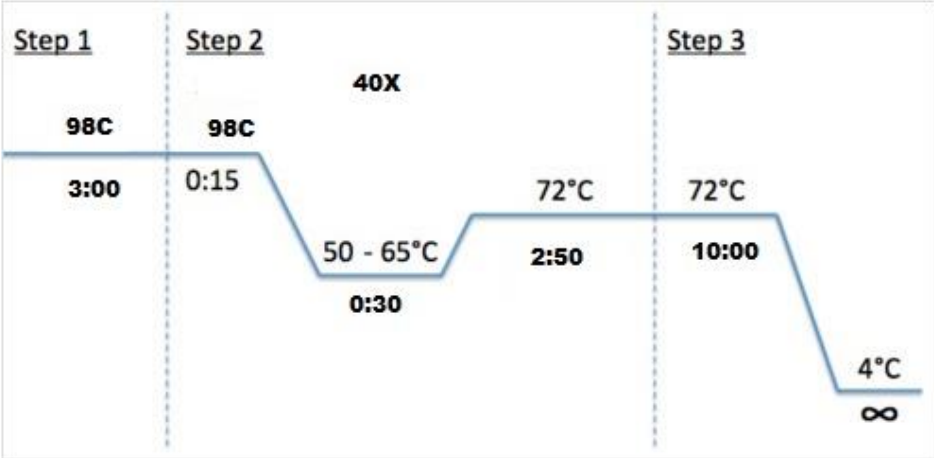

Figure 3.4. PCR cycling conditions for amplification of *BBM* and *BBM1* genes by gateway primers

3.2.6 Preparation of agarose gel electrophoresis and confirmation of amplified PCR fragment

Gel electrophoresis was used to confirm PCR results using 0.5% TBE buffer. Agarose of 1% was heated in microwave oven and ethidium bromide was added. Solution was poured into the gel tank and waited until complete solidification. PCR samples were loaded into the walls and run for one hour at 90V. Later, PCR results were observed under UV light.

3.2.7 Purification of *BBM* gene fragment from agarose gel

BBM1 gene was amplified with Phusion® DNA polymerase for Gateway method, while for BBM gene Taq polymerase was used in TA cloning. Amplified fragments of genes were purified from agarose gel with *EasyPure*® Quick Gel Extraction Kit (Transbiotech) using blade in a required way. Thin layers of exact bands were weighted and transferred into 1.5 ml Eppendorf tube. Gel solubilization buffer was added into the gel slice in proportion of 3:1 and incubated at 55C for ten minutes until gel is completely dissolved. During the process of dissolving, the tube was vortexed every two minutes. Next step was adding isopropanol to the dissolved solution. Solution was transferred to the spin column after temperature of solution had decreased. Incubation for one minute in room temperature was done and one minute centrifuging at 10,000×g was performed. Flow through was discarded and 650 µl of Wash Buffer was added and one minute centrifuging was performed. Flow through was discarded and empty column was centrifuged at 10,000×g for one minute and spin column was transferred into a new Eppendorf tube. Preheated at 65ºC elution buffer of 30 µl was added to the center of column and centrifuged one minute. Flow through was added into the spin column and centrifuged once more at 10,000×g. Eluted gel slice was stored at -20ºC.

3.3 Subcloning of PCR Product by TA Cloning Method

TA cloning was selected as a method of subcloning amplified gene fragment. For this purpose, bacterial expression vector pDrive vector (QIAGEN PCR Cloning Kit (Catalog no. 231124)) was selected. Ligation was prepared as in the following Table 3.5.

Table 3.5. Ligation mixture for TA cloning

Reaction	**Amount**
pDrive Cloning Vector (50 ng/µl)	1.0 µl
PCR product (30 ng/ µl)	3.0 µl
Distilled water	1.0 µl
Ligation Master Mix, 2x	5.0 µl
Total	10 µl

Ligation Master Mix was used at last and transferred at -20 °C immediately. Incubation of ligation reaction was done twelve hours at 16 °C and continued overnight at 4 °C.

3.3.1 Transformation of ligated product to *Top-10 E. coli* components cells

3.3.1.1 Preparation of Top-10 *E.coli* component cells

Competent cells were prepared according to the here below protocol:

Strain of *E. coli Top-10* was grown with kanamycin antibiotic before preparation of competent cells at 37 °C overnight and streaking was done to get single colonies. Freshly grown single colony was selected and inoculated in a tube containing 10 ml of liquid LB media without usage of antibiotic. Next, culture was inoculated overnight at 37 °C. After confirming a good growth, 1 ml of culture was taken for a flask containing

100 ml of liquid media. In four hours, cultures were grown and transferred into two 50 ml tubes. The tubes were kept on ice for twenty minutes and centrifuged at 3,000 g for ten minutes at 4 °C. After receiving precipitation of cells, media was discarded, and cells were suspended in $CaCl_2$ further incubation on ice was performed. After incubation, mixture was centrifuged at 3,000 g for ten minutes at 4 °C. After throwing supernatant precipitation was resuspended using ice cold 0.1 M $CaCl_2$ having 15% glycerol. Obtained mixture was divided into 50 µl aliquots and poured into tubes. All tubes were immediately transferred to ice. Prepared cells were kept in -80 °C freezer.

3.3.1.2 *E. coli* transformation of ligated product

Competent cells from -80 °C were incubated on ice for ten minutes and 5 µl ligation reaction was added into the incubated cells. After adding, gentle tapping was done by hands and mixture of cells and ligation reaction were incubated for thirty minutes on ice. After that, heat shock was given to the incubated reaction at 42°C for one minute. Tube was immediately put on ice and chilled for three minutes. Before one hour incubation at 37°C, LB medium of 450 µl was added to the tube. Spreader and ampicillin plates were prepared and left on UV before spreading bacterial mixture. After one hour of incubation, grown bacterial mixture was spread on a plate containing 100mg/l of ampicillin and after complete drying transferred to the 37°C incubator overnight.

3.3.1.3 Preparation of ampicillin antibiotic solid LB (Lauria-Bertani) medium

Solid LB medium was prepared adding 28 g of Nutrient Agar to one-liter distilled water and mixed until complete dissolving of powder. Solution was autoclaved at 121°C for 20 minutes and after cooling down until 50°C, added 1 ml of ampicillin in aseptic conditions and poured into plastic Petri plates.

3.3.2 Confirmation of pDRIVE plasmid containing *BBM* gene

Ten colonies from all received were screened for positive result by colony PCR using gene specific primers. All colonies were added by a sterile tip directly to the master mix prepared for PCR.

Table 3.6. Colony PCR content

Content	Amount
PCR Master Mix (2×) (contains Taq Polymerase and dNTP)	7 µl
M13-F Primer (10mM)	0.5 µl
M13-R Primer (10mM)	0.5 µl
Water	12 µl
Total	20 µl

Clones that were tested positive were screened by PCR using gene specific primers (*BBM*-F and *BBM*-R). Once more confirmed clones were inoculated in ampilicillin LB broth and left in water bath at 37C overnight. After getting well grown bacterial inoculation, plasmid extraction was performed by Thermo Scientific plasmid kit (#K0503). Bacterial inoculation was centrifuged at 14000 rpm for 10 minutes in order to get bacterial precipitation. Resuspension solution of 250 µl was used for suspending cells. Received cell suspension was transferred into a microcentrifuge tube and 250 µl Lysis Solution was poured in solution. Tubes were inverted by hands 4-6 times and viscous solution was obtained. Neutralization Solution of 350 µl was poured into the tube and quickly inverted several times. Bacterial lysate turned into a cloudy solution and centrifuged for five minutes. Obtained supernatant was carefully added into the new GeneJET spin column. Stage of adding 500 µl Wash Buffer and centrifuging for one minute was done for two times. Obtained flow-through was removed and empty column was centrifuged for one minute. Next, columns were transferred into new labeled tubes and heated 40 µl elution buffer was poured right to the center of membrane. Tubes with elution buffer were incubated for two minutes and centrifuged for two minutes. Columns were removed and obtained plasmids were stored at -20°C. Concentrations were checked by running gel for 50 minutes.

3.3.2.1 Confirmation of clones by restriction analysis

After plasmid extraction, positive clones were tested by restriction analysis. For this reason, following was used.

Table 3.7. Restriction analysis reaction components

1	Plasmid (100ng/ul)	2 µl
2	FD Buffer (Thermo)	2 µl
3	*BamH*I (Thermo)	0.5 µl
4	*Sac*I (Thermo)	0.5 µl
5	H_2O	15 µl
6	Total	20 µl

After preparing reaction mixture, incubation was done for thirty minutes at 37 °C and heat inactivation was done by giving 37 °C for ten minutes.

3.4 Cloning of *BBM1* Gene to Expression Vector (pEarleyGate100)

3.4.1 Amplification of *BBM1* gene by gateway primers (*BBM1*-GTF and *BBM1*-GTR)

Soybean cultivar cDNAs were used to amplify *BBM1* target gene. Phusion® Polymerase was used to amplify target gene by PCR with optimized proper annealing temperature of °C by designed primers. Gel electrophoresis of 1% in buffer of 0.5% TBE (Trisborate EDTA) was used to screen results. When proper band was received, it was carefully cut form the gel under UV light and put to the microcentrifuge tube. Next, each tube was weighted for fragment gel purification using *EasyPure*® Quick Gel Extraction Kit (Transbiotech).

3.4.2 Gateway cloning of *BBM1* gene

To transfer *BBM1* gene to the plant, gateway cloning technique was selected. The Gateway Cloning technique was done in two stages.

3.4.3 First stage: BP reaction

BP reaction was done based on Table 3.8

Table 3.8. BP reaction components

BP Reaction	Amount	Control Reaction	Amount
attB-PCR product (25 ng)	4 µl	pEXP7-tet Positive Control (50 ng/ µl)	2 µl
pDONR221™ vector (150 ng)	1 µl	pDONR221™ vector (150 ng)	1 µl
5X BP Clonase™	2 µl	5X BP Clonase™	2 µl
TE Buffer	3 µl	TE Buffer	5 µl
Total	10 µl	Total	10 µl

BP reaction was done using Invitrogen BP Clone II enzyme. Moreover, pEXP7-tet was performed to have positive test control. Components of BP reaction except enzyme were melted on ice and centrifuged for better mixing. Reaction mixture was performed for one hour at 25°C. Mixtures were treated with 1 µl Proteinase K to terminate reaction. Further step was incubation at 37°C for ten minutes.

3.4.3.1 Transformation of BP reaction mixture to the Top-10 *E. coli* components cells

Competent cells of *E. coli* strain Top-10 were used for BP reaction. Cells were chilled on ice for ten minutes before adding 1 µl of BP reaction mixture and incubated on ice for thirty minutes. Next, heat shock was given applying 42°C for fifty seconds. Cells were chilled on ice for three minutes more. LB broth of 450 µl was added into the cells. Incubation was done for one hour at 37°C in special microcentrifuge incubator. Grown cells were spread on LB plates containing 50 µg/mL of kanamycin antibiotic until complete drying. Plates were incubated at 37ºC overnight.

3.4.3.2 Preparation of antibiotic solid LB (Lauria-Bertani) medium

Nutrient agar of 28 g was weighted and added to 1 L of distilled water and autoclaved at 121 °C for 20 minutes. After cooling down until 50°C, 1 ml of kanamycin was added and poured to plastic plates.

3.4.3.3 Confirmation of *pDNR221* plasmid containing *BBM1* gene

After receiving plates full of grown colonies, ten were selected for screening by colony PCR using gene specific primers and *BBM1* gel elution sample was used as a positive control. Protocol is given on Table 3.9.

Table 3.9. Colony PCR reaction content

PCR Master Mix (2×) (contains Taq Polymerase and dNTP)	7 µl
BBM1-GTF (10mM)	0.5 µl
BBM1-GTR (10mM)	0.5 µl
Colony	
Water	12 µl
Total	20 µl

Positive clones were selected for further work and grown in 10 ml tube LB broth with 10 µl kanamycin in water bath shaker at 37 ºC overnight.

Plasmid extraction was performed another day using Thermo Scientific plasmid extraction kit (#K0503). Plasmid concentration was checked by running it on gel. Extracted plasmids were used for PCR with M13-F and *BBM1*-R primers.

3.4.4 The second step: LR reaction

LR reaction was done by adding entry clone to the reaction mixture containing LR clonase. Rapid cloning was obtained due to usage of pEarleyGate100 expression vector (Earley et al.,2006).

LR reaction was performed according to Table 3.10

Table 3.10. LR Reaction components

LR Reaction	Amount	Control Reaction	Amount
Entry Clone (50 ng)	2 µl	pENTR-gus (50 ng)	2 µl
Destination Vector (pEarleyGate100) (200 ng)	1 µl	Destination Vector (pEarleyGate100) (200 ng)	1 µl
5X LR Clonase™	2 µl	5X LR Clonase™	2 µl
TE Buffer	5 µl	TE Buffer	5 µl
Total	10 µl	Total	10 µl

LR reaction positive clone was pENTR-gus. Invitrogen LR Clone II enzyme was added last to the reaction mixture and incubation for one hour at 25°C was done. Proteinase K of 1 µl was used for termination of reaction. Ten minutes incubation if samples were done at 37°C. Reaction mixtures were transferred into the competent cells of Top-10 strain. Afrer one day incubation, colonies were screened for positive samples. Several colonies were selected as positive and incubated for inoculation in LB broth. The following day, grown inoculation was used for plasmid extraction by Thermo Scientific plasmid extraction kit (#K0503). After the measurement of plasmid concentrations by gel electrophoresis, plasmids were transfered to the Agrobacterium components cells (*LB4404*).

3.4.4.1 Preparation of *Agrobacterium* competent cell (*LB4404*)

Competent cells of *Agrobacterium LB4404* were prepared prior to experiment. Glycerol stocks of cells were kept on ice for 10 minutes and 10 µl of cells were inoculated in 10 ml LB broth containing 10 µl of rifampicin and incubated at at 28°C in water bath overnight. The following day, grown inoculated bacteria was used for streaking on kanamycin plate, the following day, freshly grown colony was picked and inoculated at 28°C in water bath. After getting well grown inocula, 2 ml was taken for growing in 100 ml flask. After 3 hours of growing, cells were divided into 50 ml tubes and left for chilling on ice. Centrifuging was done at 4000 rpm for ten minutes at at 4°C. Precipitated cells were resuspended using sterile cold water of 50 mL. Centrifuging as previous was done. Precipitated cells were resuspended in 25 ml cold sterile water. Another wash was given in 10% glycerol cold water. After another wash, cells were resuspended in 1 ml filtered 10% glycerol. All obtained cells were divided into aliquots and stored at -80 °C.

3.4.4.2 Transformation of *BBM1*-pEarleyGate100 plasmid in Agrobacterium (*LBA4404*)

Bacterial strain *LB4404* was taken for *BBM1*-pEarleyGate100 plasmid. Ligation product of 2 µl was used to transform in competent cells. After adding ligated product, it was gently tapped and left for chilling on ice for thirty minutes. Chilled cells were poured into clean dry and sterile cuvettes. Next, electroporation was done at 2.4kV voltage and immediately 450 µl was added into cells. After that, incubation at 28ºC for three hours was done. Meanwhile, spreader was washed in ethanol and left on UV for twenty minutes. After incubation, cells were spread on kanamycin (50 µg/mL) plates. Incubation at 28ºC was done. The next day, colonies were screened using colony PCR by gene specific and plasmid primers. Positive colonies were selected for inoculation in LB broth at 28ºC overnight. Plasmids were extracted from freshly grown bacteria and run on gel for concentration. Later, glycerol stocks of positive clones were prepared and stored at -80ºC.

3.5 Transformation of *35S-BnBBM1*-pBINPLUS and *UBI-BnBBM1*-tNOS-pBINPLUS in *Agrobacterium* (*AGL*)

Transformation of two constructs received from Wageningen Plant Research Institute from Dr. Kim Boutilier was done using *AGL* strain. Constructs of *35S-BnBBM1* in pBINPLUS and *UBI-BnBBM1*-tNOS in pBINPLUS were received in FTA card. Cards were eluted with 50 µl of sterile water and incubated for 30 minutes. Further, Top-10 strain was used for *E. coli* transformation. After getting positive clones confirmed by *npt*II primers, plasmids were extracted from clones. Extracted plasmids were used for *Agrobacterium* transformation.

3.5.1 Transfer of *BBM1* to cotton plants by *Agrobacterium*-mediated gene transformation method and propagation of cotton plants in tissue culture conditions

Cotton Coker 312 was grown in MS-0 nutrient medium magenta boxes and tubes to propagate sterile plants. Cotton hypocotyl and leaves were used as sources for transformation. Each Petri plate was divided into groups by five hypocotyl and five leaves. Explants were kept at 27 ± 2ºC with 16/8 h photoperiod in fluorescent growth chambers. Each magenta box contained MS medium with content described in Table 3.11

Table 3.11. Content of nutrients in MS medium (Murashige and Skoog, 1962)

Nutrients	Ingredients	Amount (mg/l)
Macro nutrients	NH_4NO_3	1650.00
	KNO_3	1900.00
	$CaCl_2.2H_20$	440.00
	$MgSO_4.7H_2O$	370.00
	KH_2PO	170.00
Micro nutrients	KI	0.83
	H_3BO_3	6.20
	$MnSO_4.4H_2O$	22.30
	$ZnSO_4.7H_2O$	8.60
	$Na_2MoO_4.2\ H_2O$	0.25
	$CuSO_4.5H_2O$	0.025
	$FeSO_4.7\ H_2O$	27.80
	$Na_2EDTA.7H_2O$	37.30
Vitamins	Myo-Inositol	100
	Nicotinic Acid (free acid)	0.50
	Pyridoxine-HCl	0.50
	Thiamine-HCl	0.10
Amino acid	Glycine	2.00

Table 3.12. Media composition for 1 L of CIM

Agar	7.0 g
MS-Salt	4.4 g
Glucose	30 g
pH	5.8

3.5.2 Preparation of bacterial inoculation

Bacterial strain of *LB4404* harboring *BBM1* gene was used for *Agrobacterium tumefaciens* genetic transformation of cotton plants. Bacteria was streaked on a fresh kanamycin plate and grown overnight in incubator. Single colony was taken for inoculation in liquid nutrient broth containing 10 µl of antibiotic. Freshly grown at 28 ºC bacterial inoculation was used for infiltration plant tissues.

3.5.3 Transfer of *BBM1* to plants

3.5.3.1 Inoculation of explants in *Agrobacterium* culture

Cotton hypocotyl and leaves were placed on plates by 5 pieces. Prior to tissue culture experiments all plates, forceps and scissors, water as well as tissue paper and filter papers were autoclaved. Explants after cutting in liquid MS-0 medium were infiltrated in bacterial culture harbouring pEarleyGate100-*BBM1* and pBINPLUS-*BBM1* and empty pEarleyGate100 constructs. Each infiltration lasted thirty minutes with slight mixing. After thirty minutes infiltration explants were gently shacked for removing excess water and placed onto co-cultivation medium containing 100 mM of acetosyringone, each co-cultivation plate was incubated for two days in growing chamber with photoperiod of 16/8 hours. After 2 days of co-cultivation, explants were taken on double autoclaved water. After that, explants were put on water containing sulcid for 10 minutes to remove remaining excess bacteria. Completely dried plants on filter paper and tissue paper were taken on CIM medium. Each Petri plate contained five explants. Plates were stretched and transferred to a growing chamber with photoperiod of 16/8 hours.

3.5.3.2 Callus induction medium (CIM)

Callus formation and selection in explants were observed in CIM medium containing different plant hormones (Table 3.13). Sulcid was used for killing bacteria in medium and protecting explants from further infiltration. Kanamycin and glufosinate were used as selection antibiotics.

Table 3.13. Contents of CIM0 medium

Ingredients	Amount per L
MS salts	4.4 g
Plant agar	7 g
Glucose	30 g
2,4 D	1 ml
Kinetin	100 µl
Ascorbic acid	250 µl
Myo-inositol	400 µl
Sulcid	1 ml
Silver nitrate	500 µl
Glufosinate/kanamycin	10 µl/125 µl

All ingredients except salts, agar and glucose were filter sterilized and added into medium after autoclaving, pH was adjusted to 5,8 before adding agar into the solution.

3.5.3.3 Transfer and selection on CIM

First signs of callus formation were detected in some plants growing on CIM after 14 days of incubation. Although, 14 days was the fastest duration needed for induction compared to other plants. Mostly, it took 20 days to show callus formation. Callus formed on medium containing 10 µl/l of glufosinate and 125 µl/l of kanamycin, whereas higher amounts of selection antibiotics killed explants after three weeks of incubation. Sulcid of 1 ml/l was found to be optimum for CIM. After, explants were transferred in CIM containing 500 µl/l sulcid.

3.6 Analysis of Regeneration Efficiency

Constructs of *35S:BnBBM*, *35S:GmBBM*, *UBI:BBM*, empty vector were checked for regeneration efficiency by explants type. Analysis of variance and regeneration rates among constructs and explants were compared.

3.7 Confirmation of Transgenic Plants by Molecular Analyses

Analysis of qRT-PCR was provided to determine the expression of *BBM1* gene in obtained callus. Steps of this analysis are provided next.

3.7.1 Total RNA extraction and cDNA synthesis from callus

Before all experiments, RNA extraction from frozen callus was performed. Cotton callus were used as a source. Totally 47 calli were selected for RNA extraction, and 22 for DNase treatment. Out of 22 explants, 14 were selected for cDNA synthesis. Trizol method was used for extracting RNA. Concentration and purity of RNA were checked by gel electrophoresis and NanoDrop.

Transbiotech *EasyScript*® First-Strand cDNA Synthesis SuperMix was used to synthesize cDNA (Cat no AE301), (Table 3.14).

Table 3.14. First-strand cDNA synthesis reaction components

Components	Volume
Total RNA/, RNA	0.1 ng-5 µg/10 pg-500 ng
Anchored Oligo(dT)18 Primer (0.5 µg/µl)	1 µl
or Random Primer(N9) (0.1 µg/µl)	1 µl
Or GSP	2 pmol
2xES Reaction Mix	10 µl
EasyScript® RT/R1 Enzyme Mix	1 µl
RNase – free Water	To 20 µl

Anchored oligo(dT)18 primer was used for ensuring higher efficiency and incubated at 42 ºC for 30 minutes, after enzymes were inactivated at 85 ºC for 5 seconds.

3.7.2 PCR assays of putative transgenic callus

Relatively better 14 DNase treated RNA callus samples were selected for cDNA synthesis. Further PCRs were performed with diluted 1:10 ration cDNAs for test with *bar*, *npt*II and gene specific primers. Totally 14 samples were selected, from which three are from *35S:BnBBM*, six *35S:GmBBM*, one empty vector sample, one control *UBI:BBM* constructs, two cotton *UBI:BBM* construct, one control *35S:GmBBM*.

Table 3.15. The content of PCR reaction for putative transgenic samples

Component	Volume
Nuclease free water	11.6 µl
Buffer	2 µl
F Primer *bar*/ *npt*II/gene specific (10 µM)	0.5 µl
R Primer *bar*/ *npt*II/gene specific (10 µM)	0.5 µl
Taq Polymerase Thermo Scientific	0.2 µl
dNTPs (10 µM)	0.2 µl
Diluted cDNA (1:10)	5 µl

The PCR content and program for *bar* and *npt*II were same and illustrated in Figure 3.5 and Table 3.15.

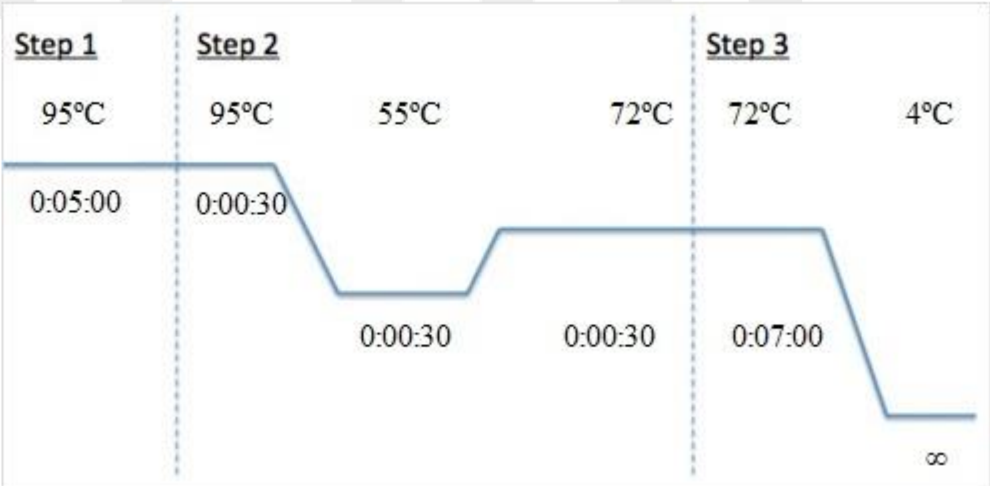

Figure 3.5. PCR program used for amplification by *bar* and *npt*II primers

3.7.3 Calculation of transformation efficiency

Data of callus survived after 2.5 months of selection were calculated for transformation efficiency. Formula of Mean Percent Transformation Efficiency (%MTE) was used:

%MTE= Number of transgenic plants regenerated/Total number of all explants used*100

3.7.4 qRT-PCR analysis

Analysis of qRT-PCR was performed to determine expression of *BBM1* gene under ubiquittin and 35S promoters. All samples used for qRT-PCR analysis were performed as a duplicate and the *18S rRNA* gene was used as a reference gene for normalization. The protocol used for qRT-PCR analysis is given in Table 3.17. The Rotor-Gene Q (QIAGEN) device and software were used for performing gene expression analyses. Gene expression variation was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The primer list, protocol and temperature conditions of the qRT-PCR are illustrated in the Table 3.16, Table 3.17 and Table 3.18, respectively.

Table 3.16. Primers used in qRT-PCR

Name	Forward	Reverse
LEC1	5'-CGCCAACTCAAACACCATGC-3'	5'-ATTTTGGCATGCGTCGGAAG-3'
LEC2	5'-GCTCTGTTGAACCTGATGGC-3	5'-TCGGTTTCCATGAGGGTTGT-3'
BBM	5'-CCTTACTCACGGAGCCAGTA-3'	5'-TTAGTCTCGACTTGTGGTGGT-3'
PKL	5'-CGTTACTTTCCTCTTGCCCCCA-3'	5'-CTCTCAACCAAGCTACTCATCTTCG-3'
FUS3	5'-CGTAAGCTCCCTCCGAAGAA-3'	5'-TCGGCCAAAACCTGTACTTGA-3'
18S rRNA	5'-GGGCATTCGTATTTCATAGTCAGAG-3'	5'-CGGTTCTTGATTAATGAAAACATCCT -3'

Table 3.17. The content of qRT-PCR reaction set up

Content	Amount (µl)
BIO-RAD SYBR® Green Master Mix	5.0
F Primer (10 µM)	0.25
R Primer (10 µM)	0.25
Nuclease free water	2.0
cDNA (1:10)	2.5

Table 3.18. Temperature conditions of qRT-PCR

Step/Segment		Function	Temperate	Duration	Cycle
Initial Denaturation		Taq Polymerase activation	95ºC	15 min	1
Amplification	Denaturation	DNA denaturation	95ºC	10 sec	40
	Annealing	Primer binding	55 ºC	15 sec	
	Extension	New DNA strand synthesis	72ºC	20 sec	
Melting		Melting	72ºC to 99ºC	90 sec	1

Analysis of Variance (ANOVA) was used for estimating statistical difference between the means of the groups which was performed using Statistical Package for the Social Sciences (SPSS) program. The one-way ANOVA test is used to see how independent variable affect in combination on the dependent variable. In order to perform the test several assumptions were made. Observations should be independent and normal distribution of values of dependent variables should be followed. Pr(>F) is the p-value that shows hypothesis of F-test in no difference conditions of null hypothesis. Statistically, the p-value lower than 0.05 is considered significant. In the tests of between-subjects effects for qRT-PCR gene expression rates it was detected those p-values are higher indicating no significance between treatments. ANOVA tests how significant or insignificant are the parameters, but not the different levels. Therefore, post-hoc tests were performed to see exactly which levels are actually different. The Tukey's Honestly-Significant-Difference (TukeyHSD) test was performed to see which groups are different among each other.

CHAPTER IV

RESULTS

4.1 Amplification and Cloning of *BBM* Gene

Following steps were performed to amplify and clone *BBM1* gene

4.1.1 Amplification of *BBM* gene

Cotton Coker 312 cultivar was used for DNA extraction and amplified using gradient PCR. Temperature range from 44ºC to 64ºC was tried and later, 55ºC was selected as amplification temperature. All amplified PCR products were cut and gel eluted with *EasyPure*® Quick Gel Extraction Kit (#EG101-01). Then, eluted fragments (2,3) were used for cloning purposes.

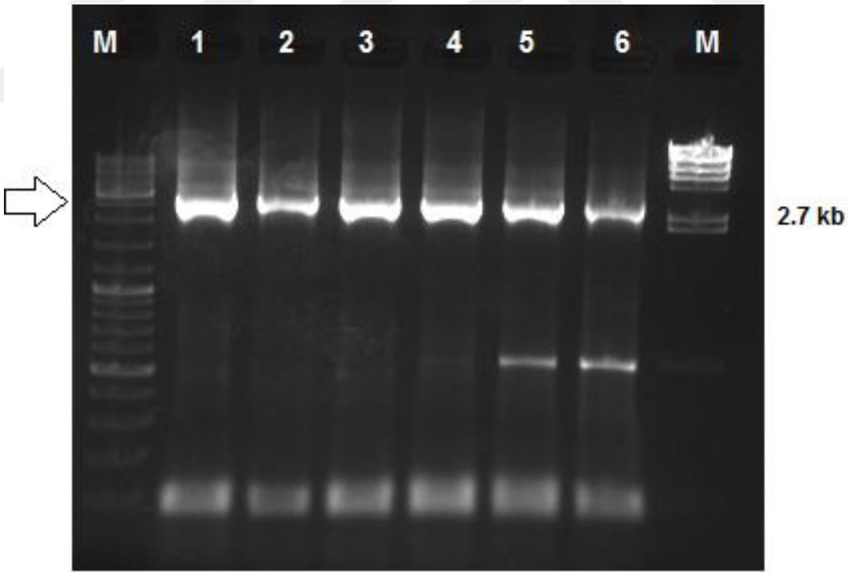

Figure 4.1. Amplification of *BBM* gene by gradient PCR, Lane M: GeneRuler Mix DNA Ladder (Thermo Scientific), Lane 1-2-3-4-5-6: *BBM* gene from cotton Coker 312, amplified at 44° C, 48° C, 52 ° C, 56 ° C, 60° C, 64° C, respectively

4.1.2 Transformation of *BBM into the* pDRIVE plasmid in *E. coli* (Top10)

Ligation reaction was performed using TA cloning kit and ligated product was transformed in competent cells of *E. coli* Top-10 strain using proper transformation protocol for *E. coli* transformation. Obtained colonies were tested using M13 primers, where 3 kb size was expected (figure 4.2). Further, several colonies (2, 3, 8, 10, 11, 12, 14) were tested using gene specific primers, where 2.7 kb was expected (figure 4.3). Colonies (10,11) were inoculated and checked using restriction digestion.

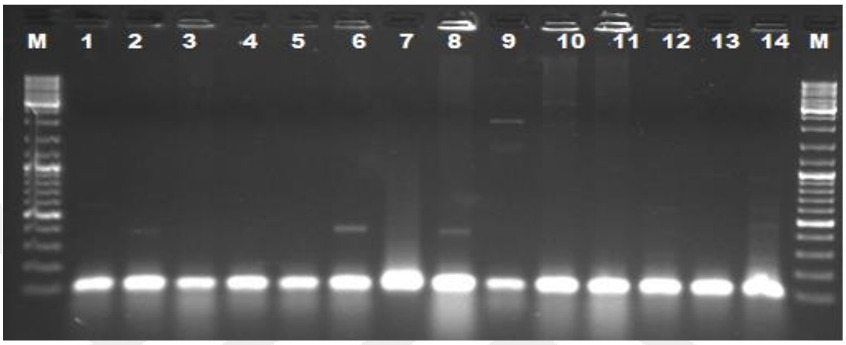

Figure 4.2. Colony PCR assay for the confirmation of TA cloning by using M13 primers, Lane M: GeneRuler Mix DNA Ladder (Thermo Scientific), Lane 1-14 colonies. Expected band size 3 kb

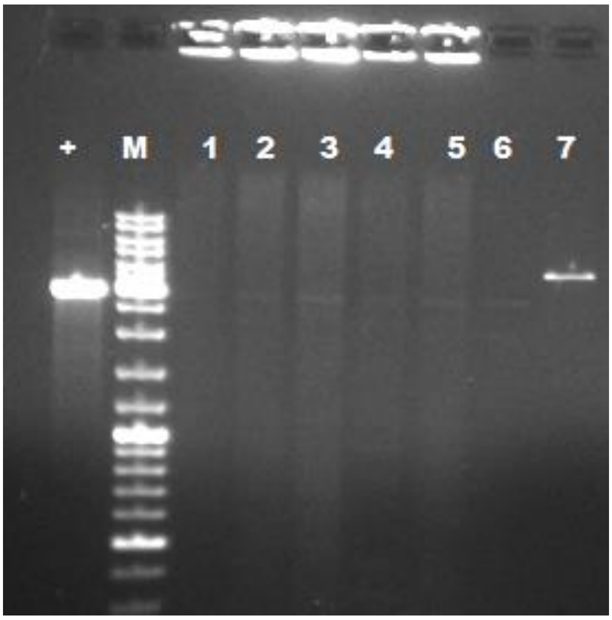

Figure 4.3. Colony PCR assay for the confirmation of TA cloning by using gene specific primers, (BBM-F and BBM-R), M: GeneRuler Mix DNA Ladder (Thermo Scientific), Lane +: positive control, Lane 1-7: results with gene specific primers, (BBM-F and BBM-R). Expected band size 2.7 kb

4.1.3 Confirmation of clones by restriction digestion

Samples selected from colony PCR (colonies 10, 11) were inoculated and plasmids were extracted. Extracted plasmids were run on gel to visualize concentrations (figure 4.4 A). Further, plasmids were checked by restriction digestion using *EcoR*1 in different concentrations of 0.5 µl and 1 µl (figure 4.4 B, 1 and 2 lane, respectively), where size of 2.7 kb was expected (figure 4.4 B).

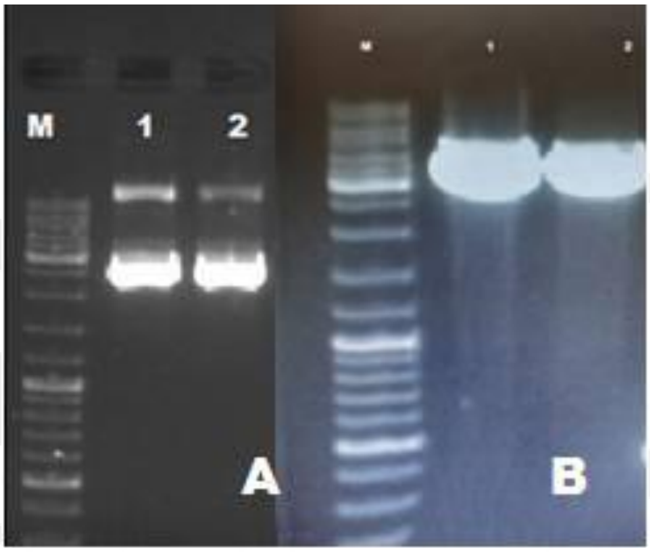

Figure 4.4. Restriction Digestion Analysis of BBM-pDRIVE plasmid, M: GeneRuler Mix DNA Ladder (Thermo Scientific), (A)Lane 1,2: Undigested plasmid. (B) Lane 1, 2: digested plasmid by *EcoRI*. Expected band size 3 kb

After getting wrong sized bands of more than 5 kb, we supposed that *EcoR*I is not recognizing the restriction site or there are no required sites of digestion specific to the *EcoR*I. Therefore, we tried to cut *BBM*-pDRIVE plasmid with *Kpn* and *Hind*III, *BamH*I and *Xba*I. All enzymes were used in amount of 0.3 µl each. Expected sizes of digestion are mentioned in the capture section of figures (Figure 4.5).

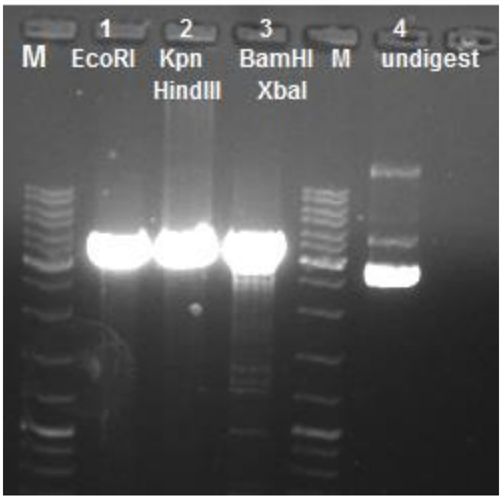

Figure 4.5. Restriction Digestion Analysis of *BBM*-pDRIVE plasmid, M: GeneRuler Mix DNA Ladder (Thermo Scientific), Lane 1: digested plasmid with *EcoR*1(2716), Lane 2: digested plasmid with *Kpn* and *Hind*III (2766 bp), Lane 3: digested plasmid by *Bamh*I and *Xba* (2758), Lane 4: Undigested plasmid

Trobleshooting of restriction digestion can probably occur due to disuse DNA bands, incomplete digestion and unexpected cleavage. Different methods were tried to eliminate the problem of unexpected recognition sites in the ligated product. Cloning strategies were checked, target gene was amplified using high fidelity DNA polymerase, different restriction enzymes were tested. Failed results show that target gene was not incorporated into the vector. Therefore, it was decided to check ligation of the target gene using another vector. After this, ligation reaction using pBIN61 vector followed by *E. coli* transformation was performed (Figure 4.6). Colonies were tested with M13 and gene specific primers. After, positive clones were inoculated and plasmids were extracted. Extracted plasmids were tested by PCR with gene specific primers. However, despite the fact of positive clones from PCR test using different primer sets, and restriction analysis that showed expected 2.7 kb, inoculated and plasmid extracted clones were 3 kb in size after running on gel for the concentration check. We concluded that cleaved restricted band from the restriction digestion might be the cause of unexpected recognition and as a result obtaining wrong bands.

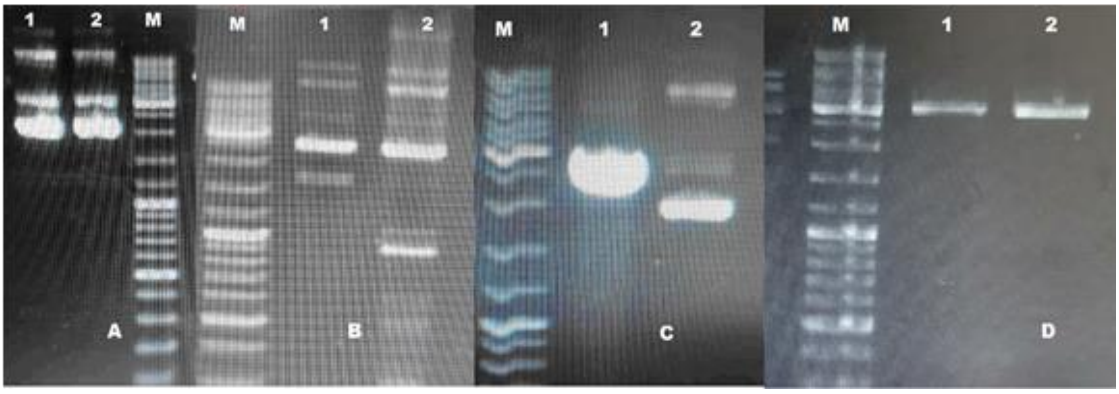

Figure 4.6. PCR results obtained from ligation with pBIN vector. Lane 1,2 extracted plasmids (a), Lane 1: control, Lane 2: amplified plasmid with gene specific primers (*BBM*-F and *BBM*-R) for the confirmation of TA cloning (b), Lane 1: Digested plasmid with *EcoR*I, Lane 2: control (c) and Lane 1,2 gel eluted fragments of digested plasmid (d). M: GeneRuler Mix DNA Ladder (Thermo Scientific)

Hereafter, after being unsuccessful in TA cloning, GateWay method was preferred.

4.2 Gateway Cloning of *BBM1* gene

Gateway cloning method of gene delivering was selected for its high efficiency compared to conventional technique.

4.2.1 Amplification of BBM1 gene by gateway primers

BBM1 target gene was amplified from cDNA soybean cultivar Nova of 2.1 kb size using Phusion® High-Fidelity DNA Polymerase (Figure 4.7).

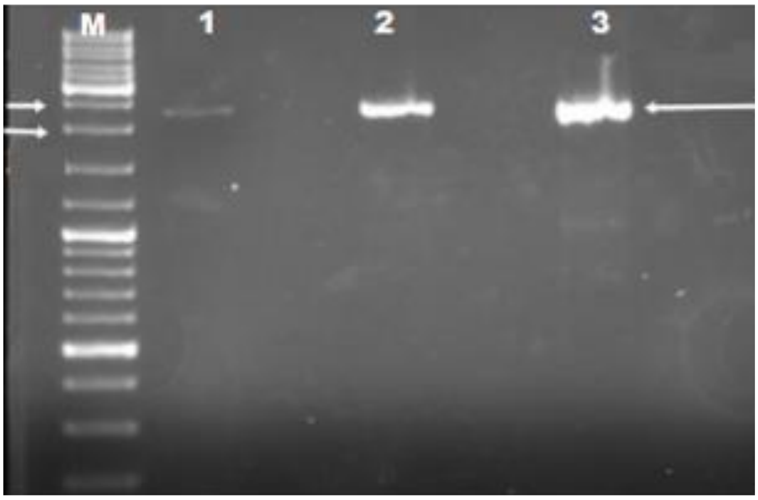

Figure 4.7. Amplification of *BBM1* gene by PCR, Lane M: GeneRuler Mix DNA Ladder (Thermo Scientific), Lane 1-2-3: *BBM1* gene from Arisoy, Nova and Nova, respectively, amplified at 52° C

4.2.2 First step: BP reaction

4.2.2.1 Transformation of *BBM1* into the pDONR221 plasmid in E. coli (Top10)

When BP reaction was completed, reaction mixture was transformed into competent cells of *E. coli* strain *Top10* and incubated overnight. Resulted colonies were tested by colony PCR assay and tested with F-M13 in pair with R-*BBM*; with gene specific primers. Expected size of positive colonies was 2.1 kb. Out of 10 colonies 7 colonies were tested positive from PCR using gene specific primers. Three colonies (1,6,7) were inoculated and plasmids extracted. But before, three colonies were double checked by gene specific primers in one PCR, and in another tested by F-M13 in pair with R-*BBM* primers (figure 4.8).

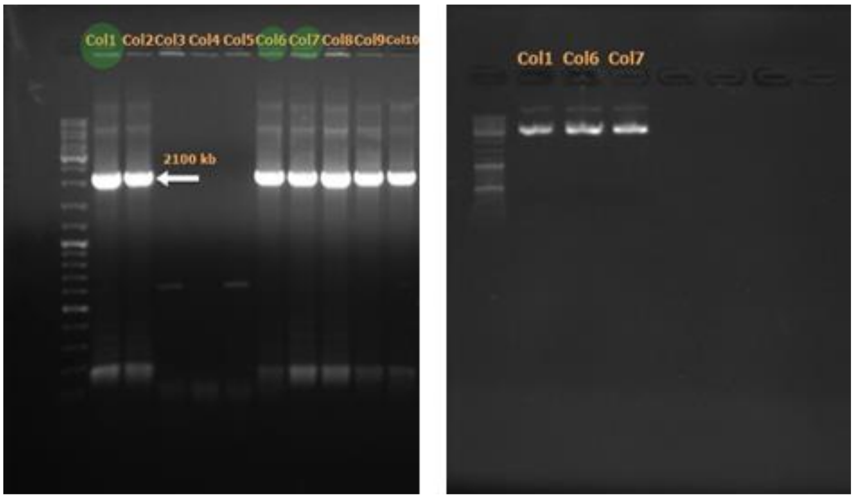

Figure 4.8. Colony PCR assay for the confirmation of BP reaction using gene specific primers, Lane M: GeneRuler Mix DNA Ladder (Thermo Scientific), lane 1-10 colonies (a) and gel eluted fragments of *BBM1* gene (b), Lane 1-3 selected colonies

4.2.3 Confirmation of clones by restriction digestion

Three (1,6,7) colonies that were tested positive by PCR, were checked by restriction digestion. The figure 4.9 B illustrates restriction digestion analysis of *BBM1* gene using *EcoRV* (within plasmid) and *HindIII* (within gene), where expected sizes were 2.1 kb.

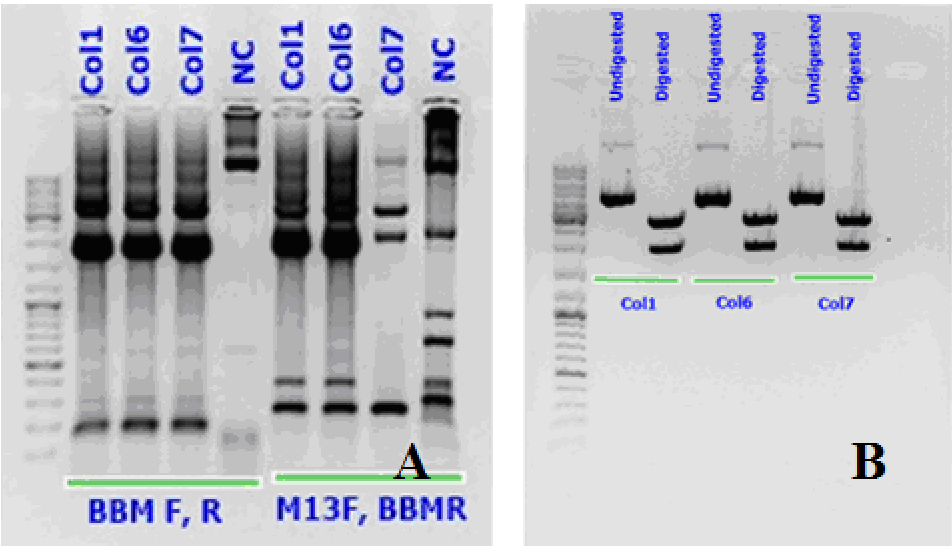

Figure 4.9. Colony PCR assay for the confirmation of BP reaction. PCR with gene specific *BBM1*-F and M13-R primers. Lane 1,2,3,5,6,8: colonies -: negative control (a) and Lane 1,3,5 undigested plasmids, Lane 2,4,6 digested plasmids with *Eco*RV and *Hind*III (b). Lane: M GeneRuler Mix DNA Ladder (Thermo Scientific)

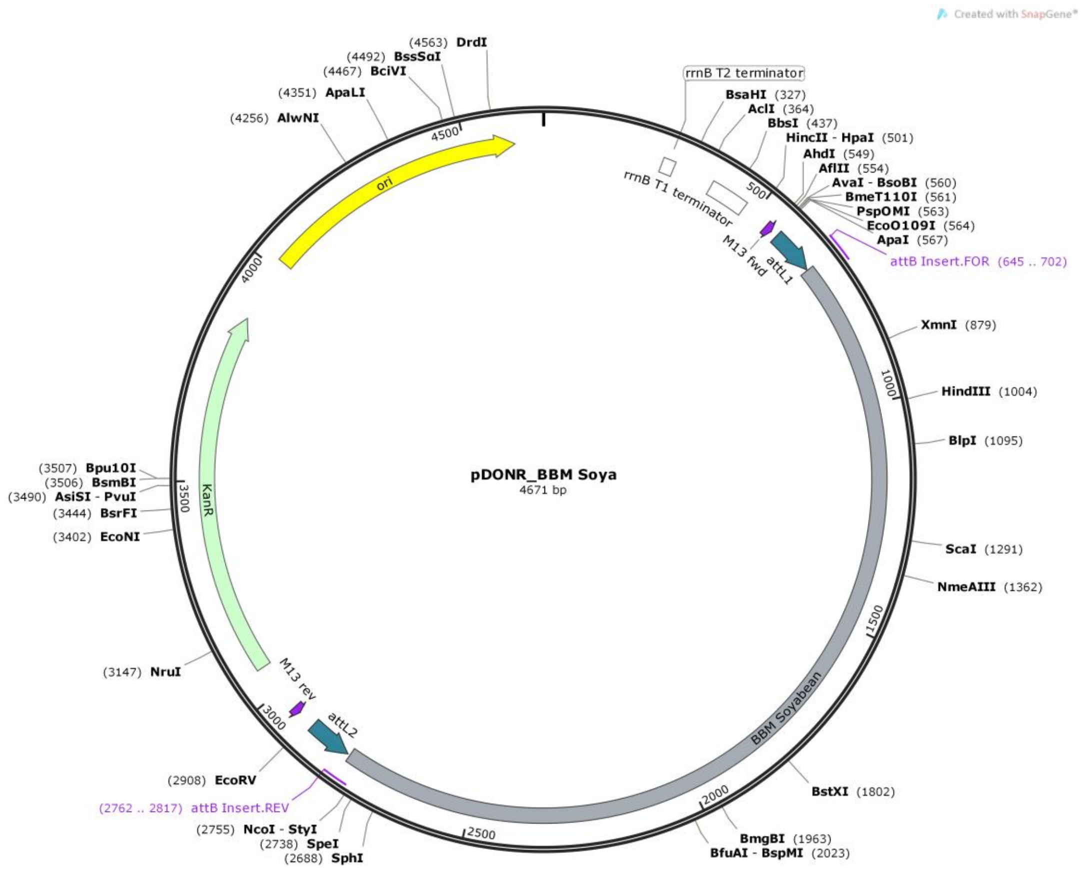

Figure 4.10. Schmatic representation of entry clone pDONR221-*BBM1*, the map was constructed using SnapGene program

Figure 4.10 illustrates generated entry clone pDONR-*GmBBM* construct after completing BP reaction.

4.2.4 LR reaction

4.2.4.1 Transformation of *BBM1 gene* into the pEarleyGate100 plasmid

For LR reaction of GateWay cloning, colony number 6 was selected. *E.coli* transformation was performed using *Top-10* strain. Resulted colonies were tested using gene specific primers, where band of 2.1 was expected (Figure 4.11). Seven (1,3,5,6,7,8, 9) colonies were plasmid extracted and amplified using gene specific primers. After generating an expression clone (Figure 4.12), *Agrobacterium* transformation was performed.

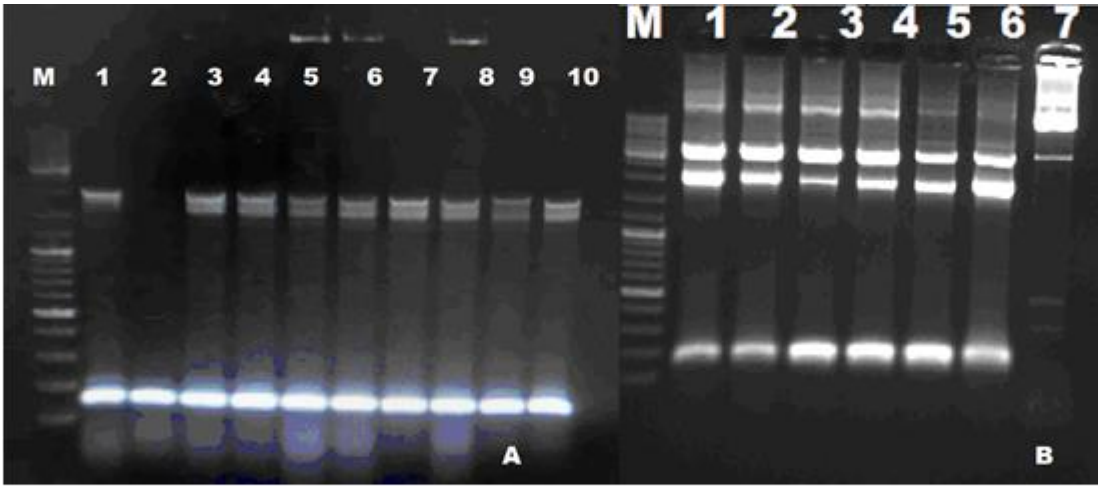

Figure 4.11. Colony PCR assay for the confirmation of LR gateway reaction. With gene specific primers, Lane 1-10 colonies (a) and Lane 1-7: colonies, gel eluted fragments of *BBM1* gene amplified with gateway gene specific primers (b), Lane M: GeneRuler Mix DNA Ladder (Thermo Scientific)

Generated expression vector pEarleyGate100-*GmBBM* construct after completing LR reaction (Figure 4.12).

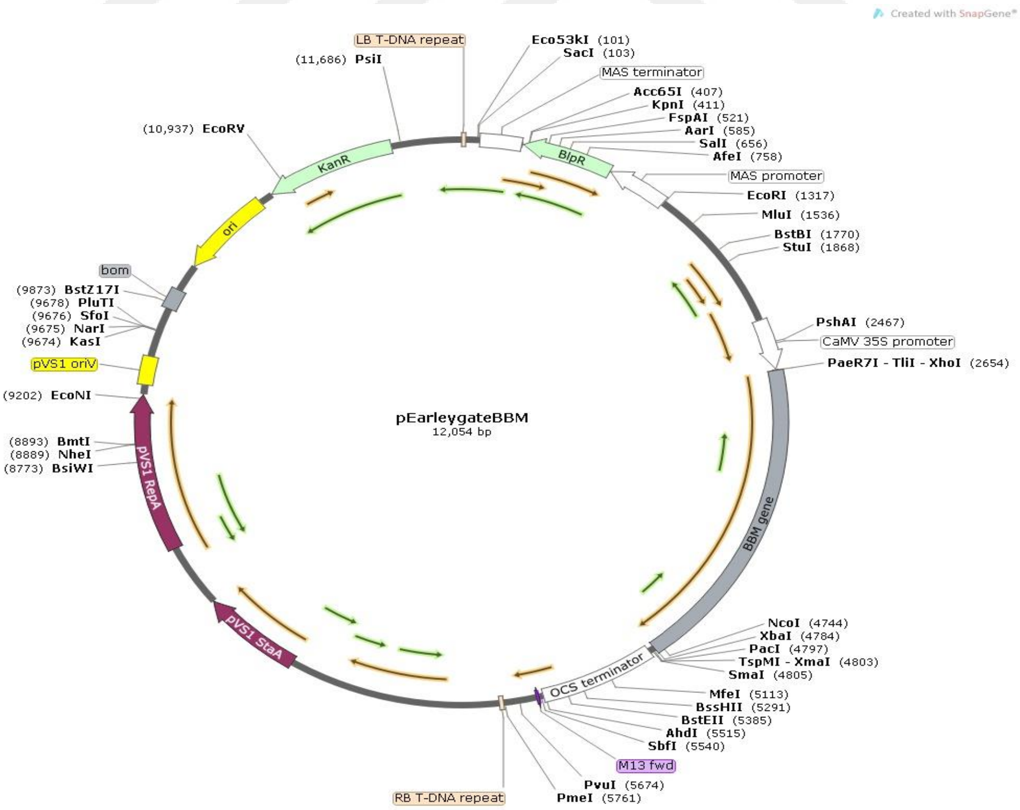

Figure 4.12. Schematic representation of expression clone, pEarleyGate100-BBM1, the map was constructed using SnapGene. The vector contained kanamycin for bacterial selection and Bar gene for plant selection that encodes resistance against glufosinate.

4.2.5 Transformation of *BBM1* gene and empty plasmid into *Agrobacterium* (*AGL-1*)

Positive clones (2,3 figure 4.11 A) from *E. coli* transformation were inoculated and their plasmids were extracted. Further, extracted plasmids were used for Agrobacterium transformation. After 2 days of incubation at 37ºC, 11 colonies were screened for detecting positive clones by PCR using gene specific primers where the control sample was empty pEarleyGate100 vector (Figure 4.13). Out of 11 colonies 10 were positive.

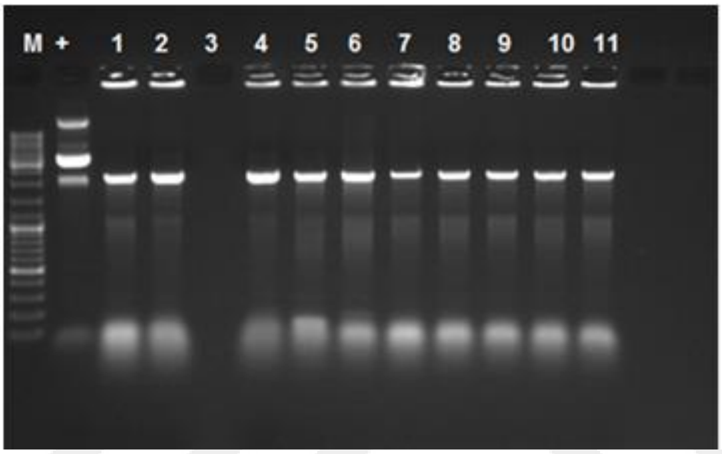

Figure 4.13. PCR assay for the confirmation of *35S:BBM* (*Glycine max*) Agrobacterium transformation, Lane M: GeneRuler Mix DNA Ladder (Thermo Scientific), Lane +: Empty pEarleyGate 100 vector, Lane -: negative control, Lane1-10: DNA results with gene specific primers

Empty pEarleyGate100 vector was transformed into *AGL* cells in order to have empty vector control for detecting effect of vector on regeneration of cotton calli.

Figure 4.14. Colony PCR assay for the confirmation of empty plasmid pErleyGate10Z. Lane 1-12: colonies, Lane M: GeneRuler Mix DNA Ladder (Thermo Scientific)

4.2.6 Transformation of *35S:BnBBM* and *UBI:BBM* constructs to Agrobacterium (*AGL-1*)

Constructs from Flinders Technology Associates (FTA) cards were transferred to *E. coli* and extracted plasmids were transferred into *AGL-1* cells. Detection of positive clones was performed by PCR using *npt*II primers, where the control sample was *35S:BBM E. coli* extracted plasmid (Figure 4.15). Expected band size was 473 bp for the positive clones of *35S:BBM* construct. *UBI:BBM* construct after *Agrobacterium* transformation was tested using *npt*II primers, where the extracted band was 473 bp (Figure 4.16).

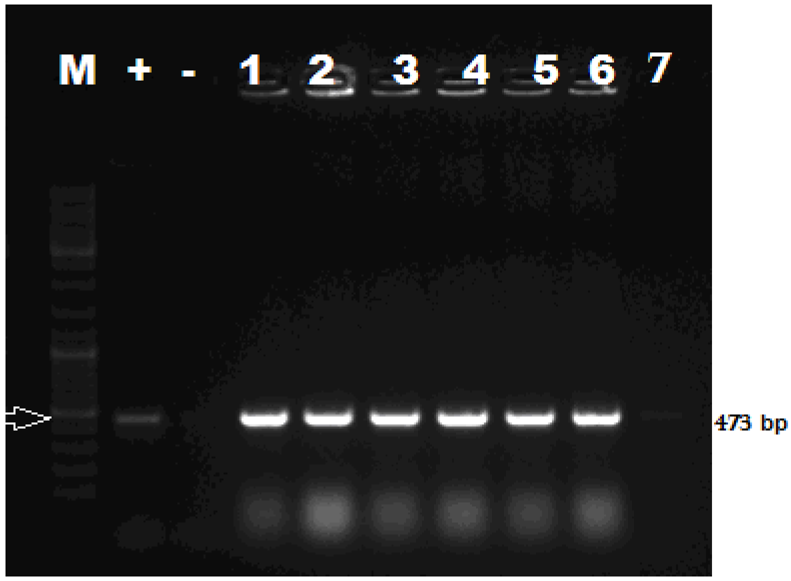

Figure 4.15. PCR assay for the confirmation of *35S:BBM* (*Brassica napus*) Agrobacterium transformation, Lane M: GeneRuler Mix DNA Ladder (Thermo Scientific), Lane +: *35S:BBM E.coli* extracted plasmid, Lane -: negative control, Lane 1-7: DNA results with *npt*II primers

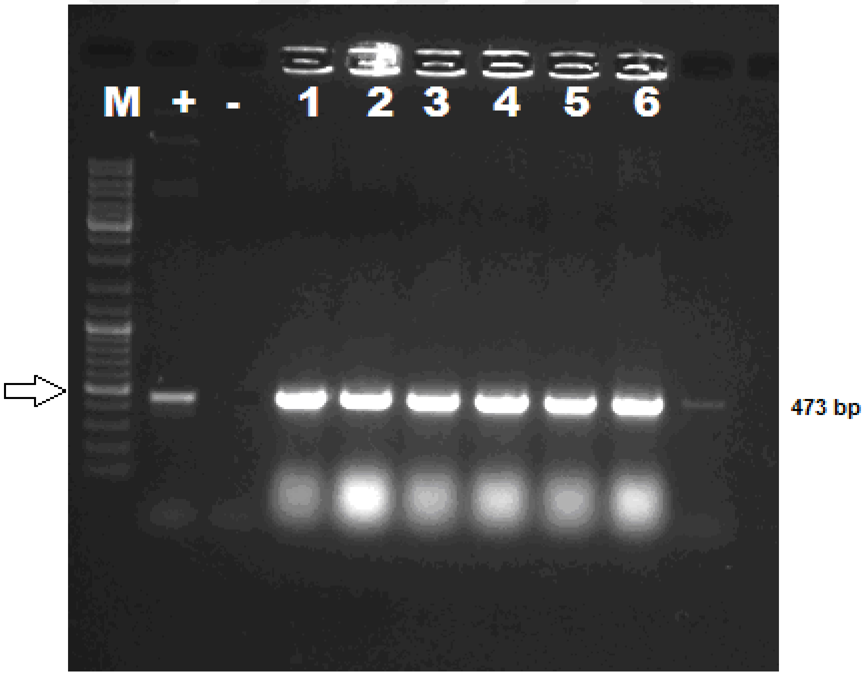

Figure 4.16. PCR assay for the confirmation of *UBI:BBM* (*Agrobacterium* transformation, Lane M: GeneRuler Mix DNA Ladder (Thermo Scientific), Lane +: *UBI:BBM E.coli* extracted plasmid, Lane -: negative control, Lane1-6: DNA results with *npt*II primers

4.3 Transformation of pEarleyGate100-*BBM1*, pBINPLUS-*BBM1*, pBINPLUS-*UBI:BBM* to Coker 312 by *Agrobacterium* Method

Positives clones from *Agrobacterium* transformation for *35S:GmBBM* (1,2 lanes Figure 4.13), empty vector control (1,4 lanes Figure 4.14), *35S:BnBBM* (1,2 lanes Figure 4.15), *UBI:BBM* (1,2 lanes Figure 4.65) were selected.

Bacterial infection harboring 4 constructs – *35S:BBM1*, *UBI:BBM, 35S:BBM* and empty vector were transformed into cotton plants by *Agrobacterium* Method. Numerous studies have shown that leaves and hypocotyl are best explant types for cotton regeneration (Ozyigit et al., 2007; Ikram-Ul-Haq, 2004).

Therefore, leaves and hypocotyl regions of cotton cultivar Coker 312 were used. Callus started emerging in 14 days that was detected as earliest time point of callus formation in this research. However, lethal browning and high amount of selection antibiotics were severely affecting explants. To face this problem, different amino acids and sugar source were tried. Moreover, different CIM were tested.

4.3.1 Optimization of CIM for the transformation of cotton plants

In order to understand optimum ranges of hormones different plant mediums were tested. Six different groups named CIM0, CIM1, CIM2. CIM3, CIM4, CIM5, CIM6 (table 4.1) with different glufosinate of 0 mg/l, 20 mg/l, 15 mg/l, 10 mg/l, 6 mg/l, 3 mg/l amounts and kanamycin 0 mg/l, 5 mg/l, 10 mg/l, 20 mg/l, 25 mg/l, 50 mgl/l amounts (Figure 4.15) were tested. CIM0 composition was selected as perfect plant medium for growth.

Table 4.1. Callus induction medium composition

Medium Name	Medium composition	
	Hormones	**Amino acids**
CIM0	2,4 D 0.1 mg/l; KT 0.5 mg/l	Ascorbic acid 1 ml/l, silver nitrate 500 µl/l, myo-inositol 400 µl/l
CIM1	IBA 1 mg/l; KT 0.5 mg/l	Ascorbic acid 2 ml/l, silver nitrate 100 µl/l, 100 µl/l L-glutamine, 50 µl/l proline
CIM2	2,4 D 0.1 mg/l; KT 0.2 mg/l; BAP 0.2 mg/l	Citric acid 1 ml/l, myo-inositol 400 µl/l
CIM3	BAP 1 mg/l; KT 1 mg/l	Charcoal 0.4 g, ascorbic acid, myo-inositol 600 µl/l, L glutamine 200 µl/l,
CIM4	Zeatin 1 mg/l; IBA 2mg/l	Charcoal 0.6 g, proline 100 µl/l, citric acid 500 µl/l
CIM5	2,4 D 1 mg/l; KT 1 mg/l	Silver nitrate 2 mg/l, myo-inositol 200 µl/l
CIM6	2,4 D 2 mg/l; KT 2 mg/l	Silver nitrate 1 mg/l, myo-inositol 400 µl/l

In 10 days, effect of culture mediums started becoming clear and antibiotics range affected growth of explants. Figure 4.17 shows development and growth of plants under effect of kanamycin antibiotic. Selection antibiotic was used for transformation selection of *UBI:BBM* and *35S:BBM* constructs. It was shown that 50 mg/l and 25 mg/l caused lethal browning. Whereas range of 0 mg/l and 5 mg/l did not effect on growth of plants. Antibiotic range in 10 mg/l effected on development in 2 months.

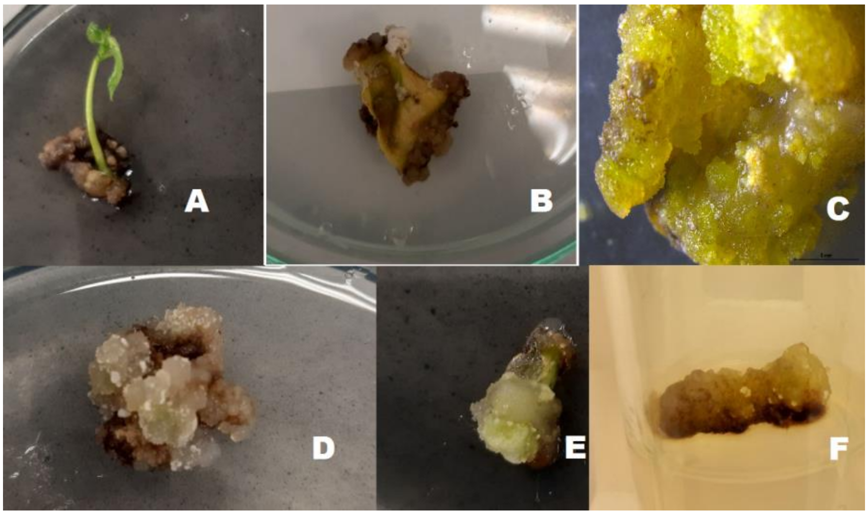

Figure 4.17. Effect of different kanamycin amount on plant regeneration and growth, 25 mg/L (a), 50 mg/L (b), 5 mg/L (c), 25 mg/L (d), 20 mg/L (e) and 10 mg/L (f)

Glufosinate antibiotic was used for selection of *35S:BBM* (*Glycine max*) construct in the range of 0 mg/l, 20 mg/l, 15 mg/l, 10 mg/l, 6 mg/l, 3 mg/l. It was shown that 0 mg/l and 1 mg/l did not effect development of most of the plants, whereas 2 mg/l killed most of the plants (Figure 4.18).

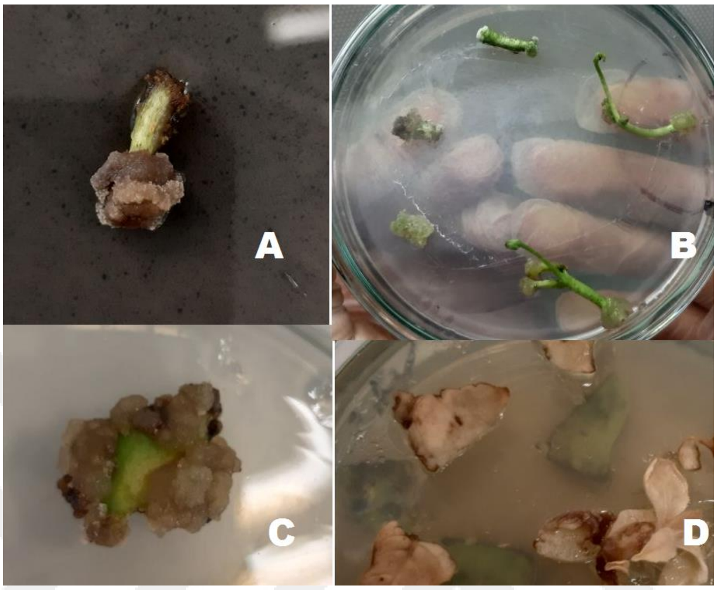

Figure 4.18. Effect of different glufosinate amount on plant regeneration and growth, 15 mg/L (a), 10 mg/L (b), 6 mg/L (c), 20 mg/L (d)

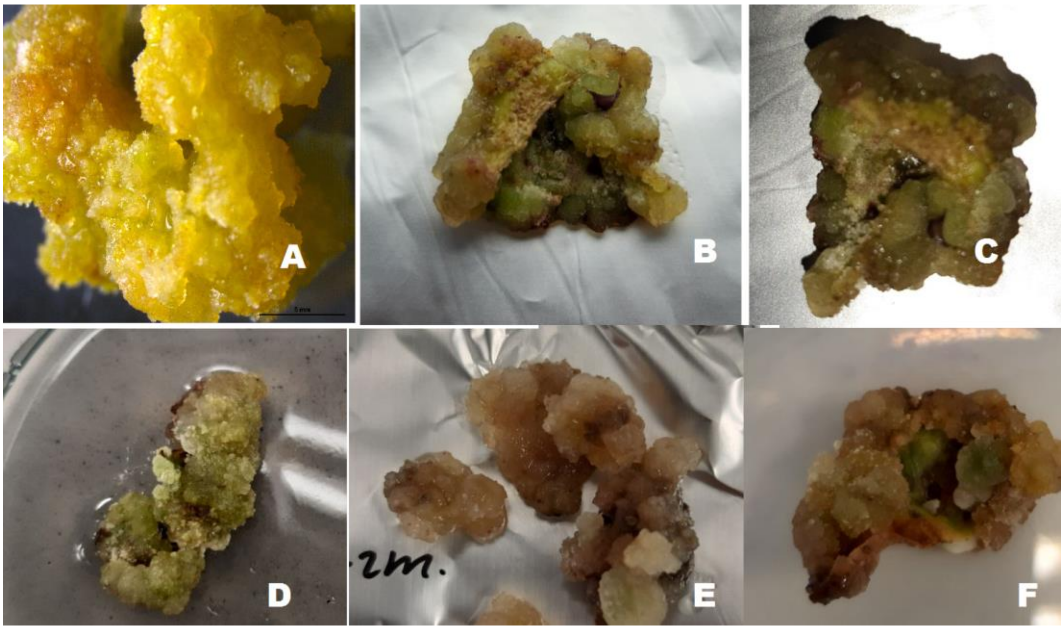

Figure 4.19. Effect of medium composition on plant regeneration and growth,CIM1 (a), CIM0 (b), CIM2 (c), CIM3 (d), CIM6 (e) and CIM0 (f)

Cotton is one of the plants producing a lot of phenolics that effect on tissue culture conditions. Therefore, different antioxidants were used for reducing phenolics activity. On Figure 4.20, different medium composition effected on levels of phenolics production and browning.

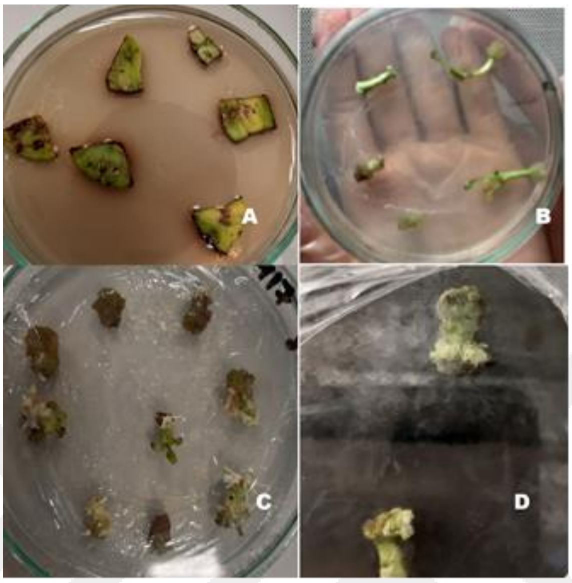

Figure 4.20. Effect of medium composition on lethal browning, CIM6 (a), CIM1 (b), CIM0 (c) and CIM4 (d)

Numerous studies showed that leaf and hypocotyl explants are the best explants having higher regeneration capacity compared to other organs. Age of the explants play crucial role in successful regeneration; therefore, 7 days old cotton plants were used. Due to excess fungal contamination of seeds, each seed was further grown in individual culture tubes (Figure 4.21).

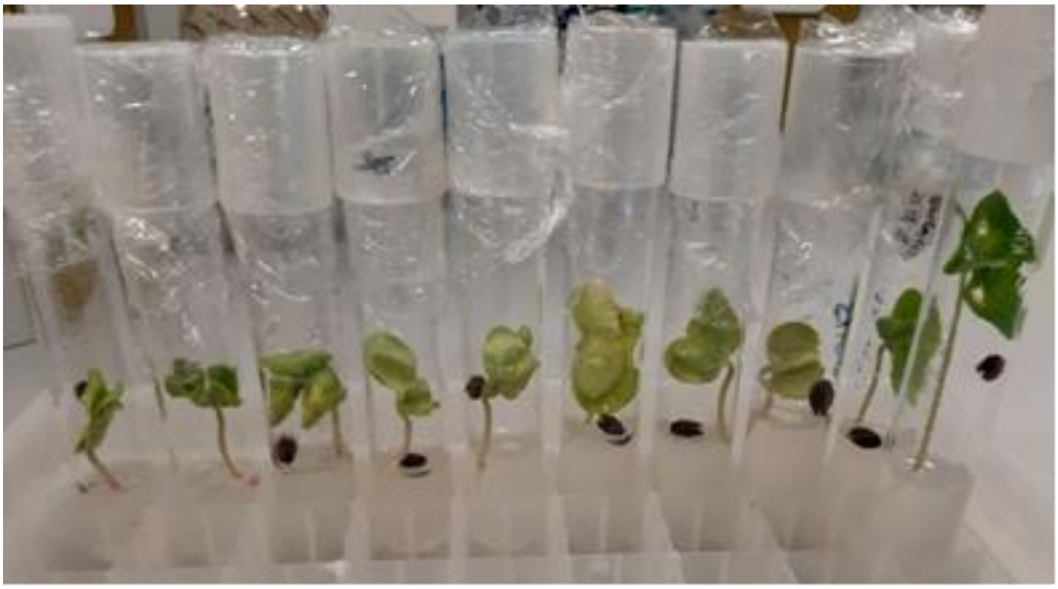

Figure 4.21. Coker 312 seeds germinated in individual tubes used for transformation. Different germination rate was observed

Comparison of explant types by appearance in the first months of culture was done.

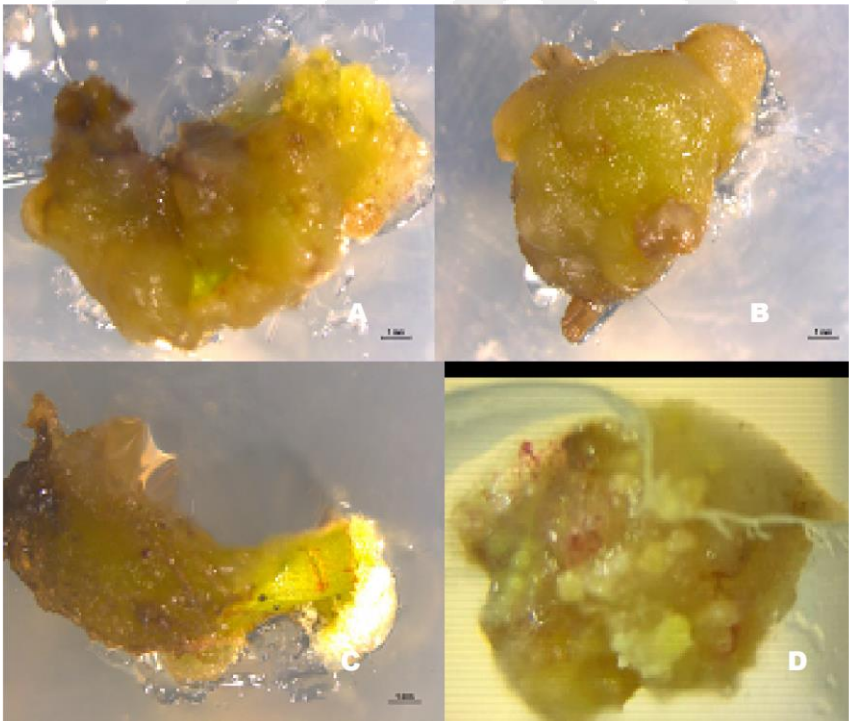

Figure 4.22. Callus formation from hypocotyl explants, 40 days old callus on CIM0 (a), 40 days old callus on CIM1 (b), formation of friable callus (c) and anthocyanin formation on 2 months old callus (d)

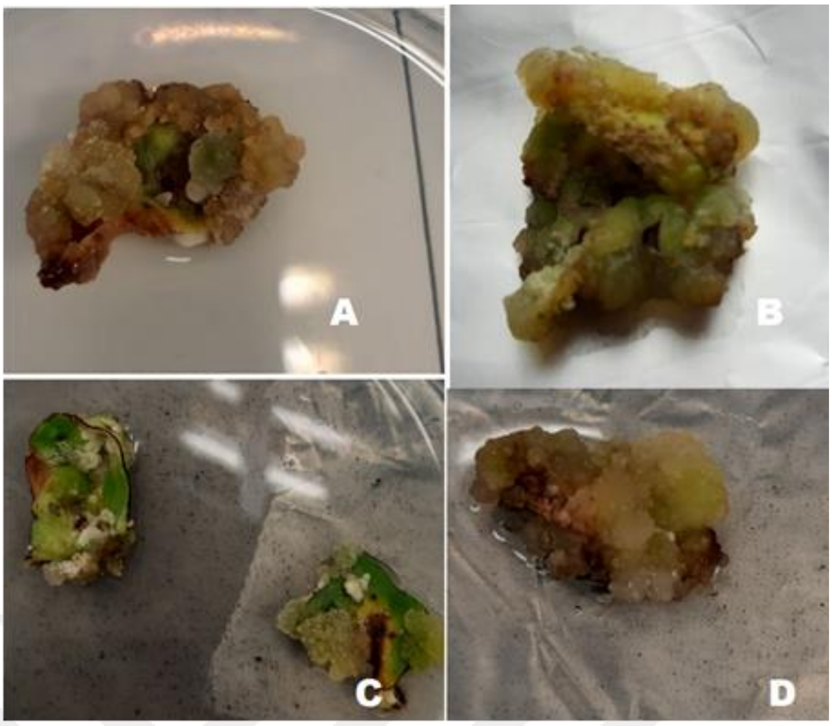

Figure 4.23. Effect of constructs on callus formation from leaf explants. Formation of compact callus on different types of media, *35S:BnBBM* (a), *35S:GmBBM* (b), control empty vector (c) and *UBI:BBM* (d)

4.4 Analysis of Regeneration Efficiency

Analysis of variance was performed for the type of constructs. According to the statistical analysis of variance by ANOVA there is no significant difference among constructs ($P > 0.05$). Figure 4.24 shows that *UBI:BBM* construct has the highest regeneration efficiency of leaf explants compared to other constructs. However, highest hypocotyl regeneration efficiency was observed in *35S:BnBBM* construct.

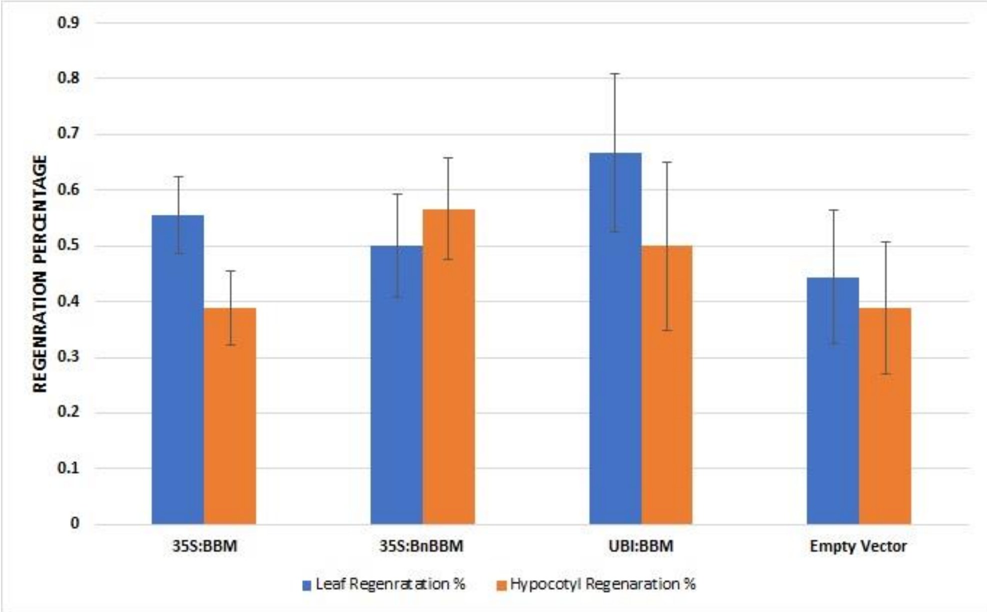

Figure 4.24. Regeneration efficiency of explant types among different constructs. Blue graphs represent regeneration percentage from leaf explants, whereas orange graph illustrates regeneration percentage of hypocotyl explants

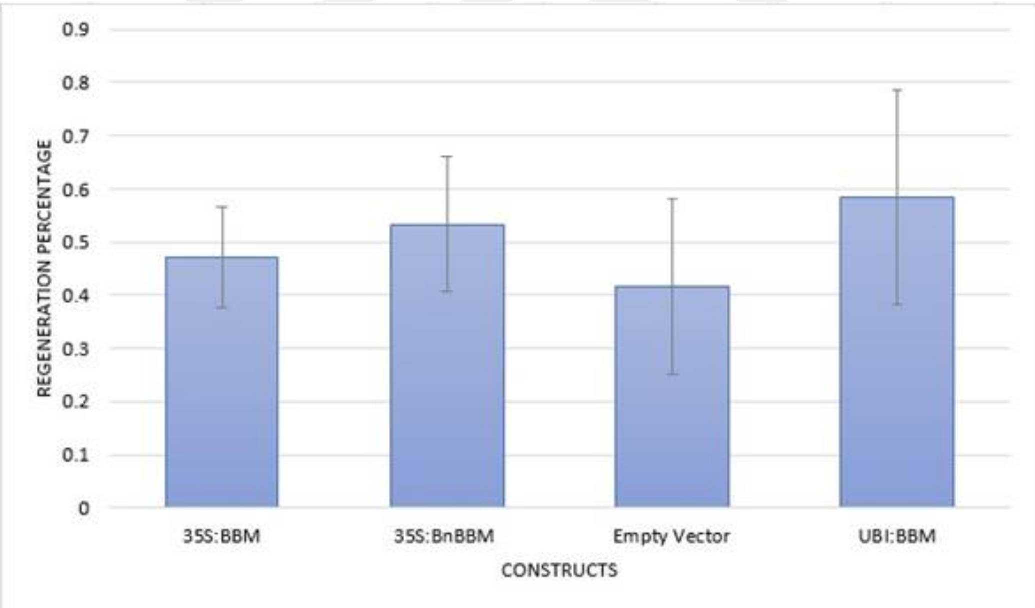

Figure 4.25. Regeneration percentage efficiency of constructs. Efficiency of constructs is illustrated regardless explant type used in transformation

Figure 4.25 illustrates difference of constructs efficiency regardless of explant source used in transformation. Statistical analysis was performed to detect regeneration efficiency of explants regardless of construct type in total. It was illustrated that leaves have a higher rate of regeneration (figure 4.26).

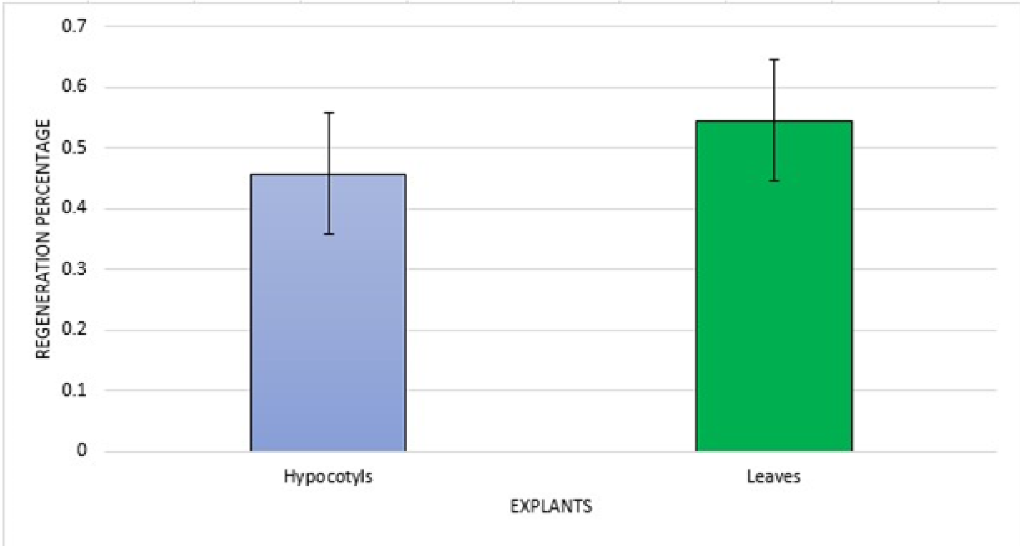

Figure 4.26. Regeneration percentage efficiency of explants. Blue bar represents efficiency of hypocotyl explants compared to green bar illustrating efficiency of leaf explants

4.5 Confirmation of Transgenic Plants by Molecular Analyses

4.5.1 PCR assays

Samples of 14 cDNA were checked for the detection of transgenic calli. (Figure 4.27 and 4.28). The PCR test was performed with *npt*II primers for the *35S:BnBBM* and *UBI:BBM* constructs (Figure 4.27). Two cotton *UBI:BBM* constructs were tested positive after the PCR test. One of the *35S:BnBBM* constructs showed positive detection, whereas another sample showed non-specific band. *Bar* primers were used for the test of *35S:GmBBM* construct, where 3 samples were checked positive. Moreover, 1 empty pEarleyGate100 sample was tested positive using *bar* primers (Figure 4.28).

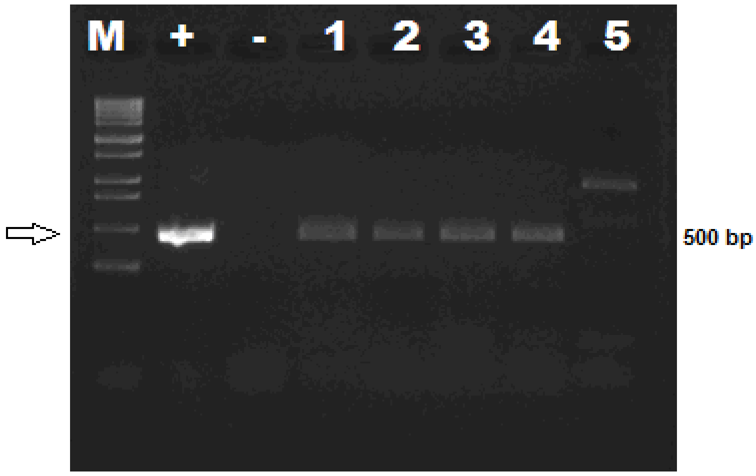

Figure 4.27. PCR assay for the confirmation of putative transgenic plants by *npt*II primers, Lane M: 1 kb plus DNA Ladder (Transbiotech), Lane -: Non-transgenic Coker 312 cultivar, Lane +: 35S:BnBBM plasmid, Lane 1: control *UBI:BBM*; Lane 2,4: Coker *UBI:BBM*; Lane 3,5: *35S:BnBBM*

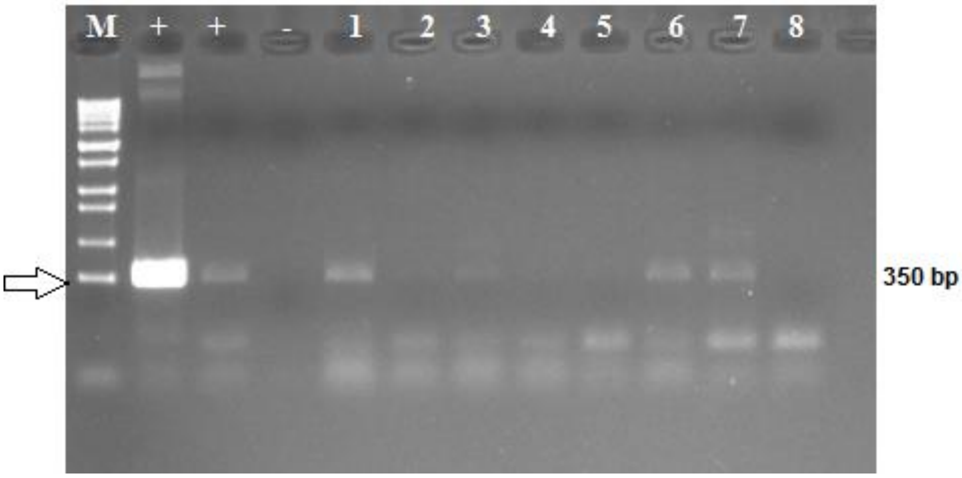

Figure 4.28. PCR assay for the confirmation of putative transgenic plants by *bar* primers, Lane M: 1 kb plus DNA Ladder (Transbiotech), Lane +: empty plasmid; Lane +: *35S:GmBBM* plasmid; Lane -: Non-transgenic Coker 312 cultivar, Lane 1,2,3 : control *35S:GmBBM*; Lane 4,5,6: Coker *35S:GmBBM*; Lane 7: empty pEarleyGate100; Lane 8: Coker *35S:GmBBM*

4.6 Gene Expression Analysis by qRT-PCR

Total RNA was isolated from all construct groups and treated by DNase in order to remove contamination. Agarose gel representing raw RNA is on Figure 4.29. Several raw RNA samples were showing two sharp clear bands (Figure 4.29, lanes

20,21,23,26,27,28). However, most of them were showing detection of DNA and degraded RNA (Figure 4.29, 1-17 lanes).

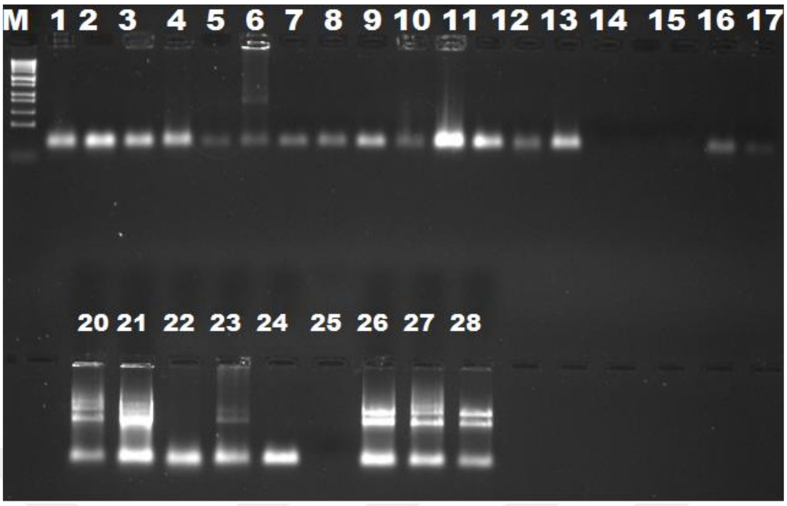

Figure 4.29. Raw RNA samples run on gel electrophoresis. Lane M: 1 kb plus marker; Lane 1-28: RNA samples

Several raw RNA samples were chosen for the DNase treatment according to the gel image (Figure 4.29) of raw RNA samples. Samples (1-12, 20-28) were used for DNase treatment (Figure 4.30).

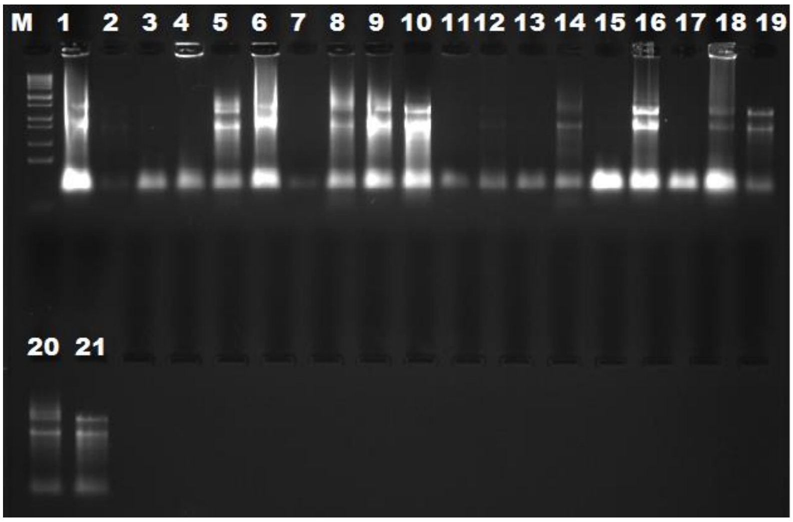

Figure 4.30. Agarose gel analysis of isolated DNase treated RNAs, Lane M: 1 kb plus marker; Lane 1-21: RNA samples

Nanodrop device was used to investigate amount and purity of RNA extracted. Results are shown on table 4.2.

Table 4.2. Total RNA concentration in candidate transgenic cotton lines by nanodrop device before DNase treatment

	Sample Name	**Nucleic Acid Conc (ng/ µl)**	**OD 260/280**	**OD 260/230**	**OD260**	**OD280**
1	empty	527.440	1.911	0.251	13.186	12.248
2	empty	622.680	1.951	0.302	15.567	7.977
3	empty	1048.080	1.991	0.438	26.202	13.157
4	empty	136.44	2.068	0.344	3.411	1.641
5	35S:GmBBM(18)	839.160	1.907	0.343	20.979	11.004
6	35SS(19)	1289.120	2.093	0.467	32.228	15.397
7	Empty (24)	905.480	2.014	0.381	22637	11.240
8	UBI:BBM(36)	332.440	2.089	0.582	8.311	3.978
9	35S:BnBBM (41)	595.360	1.957	0.324	14.884	7.600
10	UBI(45)	469.400	2.004	0.332	11.432	10.086
11	35S:BnBBM (47)	1372.200	2.093	0.576	34.305	16.435
13	35S:GmBBM	1435.840	2.106	0.573	35.986	17.047
14	35S:GmBBM	1665.560	2.180	0.604	41.439	19.006
15	Empty	834.880	2.028	0.334	20.872	10.293

As a purpose of provided research and this thesis to investigate BBM gene levels under different constructs, qRT-PCR was done. For this reason, one non transgenic, one empty vector, two *35S:GmBBM*, two *35S:BnBBM*, two *UBI:BBM* samples were checked. In this performed qRT-PCR, 18S rRNA was used as a reference gene for normalization purpose. Change of proportional expression of genes was calculated based to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Relative expression levels of *BBM, PKL, FUS, LEC2* genes were measured in several independent transgenic lines. Quantitative real-time RT-PCR analysis was performed to examine relative expression levels of *BBM*, *PKL, FUS*, *LEC2* genes in transgenic lines. Expression of genes were compared to non-transgenic control expression. Numbers given to the transgenic cotton lines are based on the numeration of total calli samples obtained from transformation work.

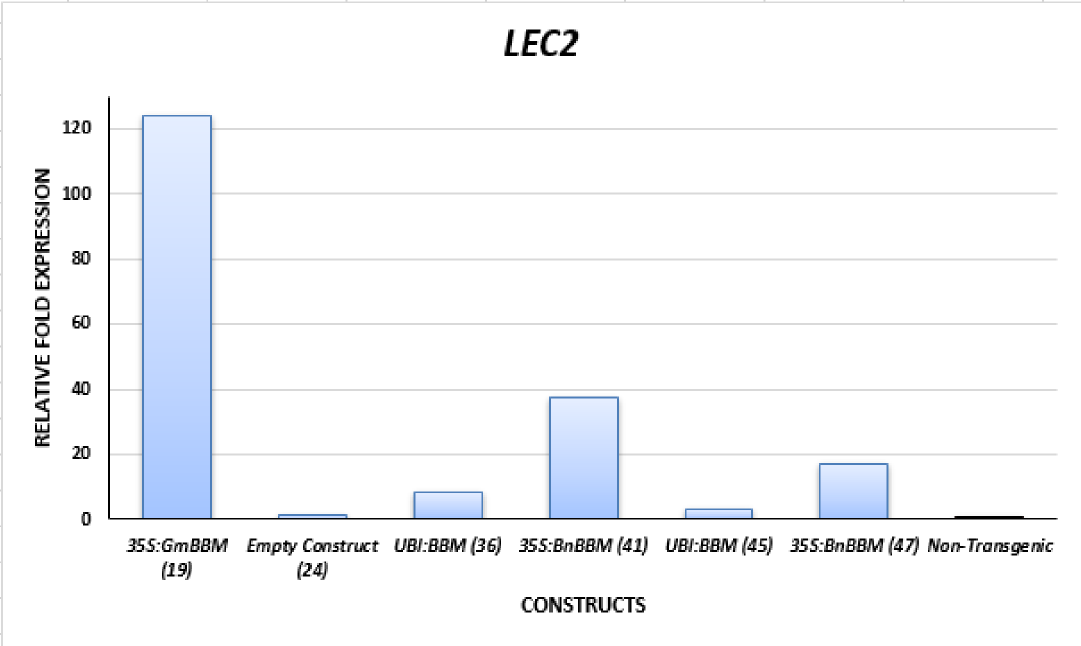

Figure 4.31. Relative expression level of *LEC2* gene in transgeniccotton lines, (19) refer to *35S:GmBBM*, (24) refer to empty vector control, (36) refer to *UBI:BBM*, (41) refer to *35S:BnBBM*, (45) refer to *UBI:BBM*, (47) refer to *35S:BnBBM*, (nt) refer to non transgenic control used as the calibrator

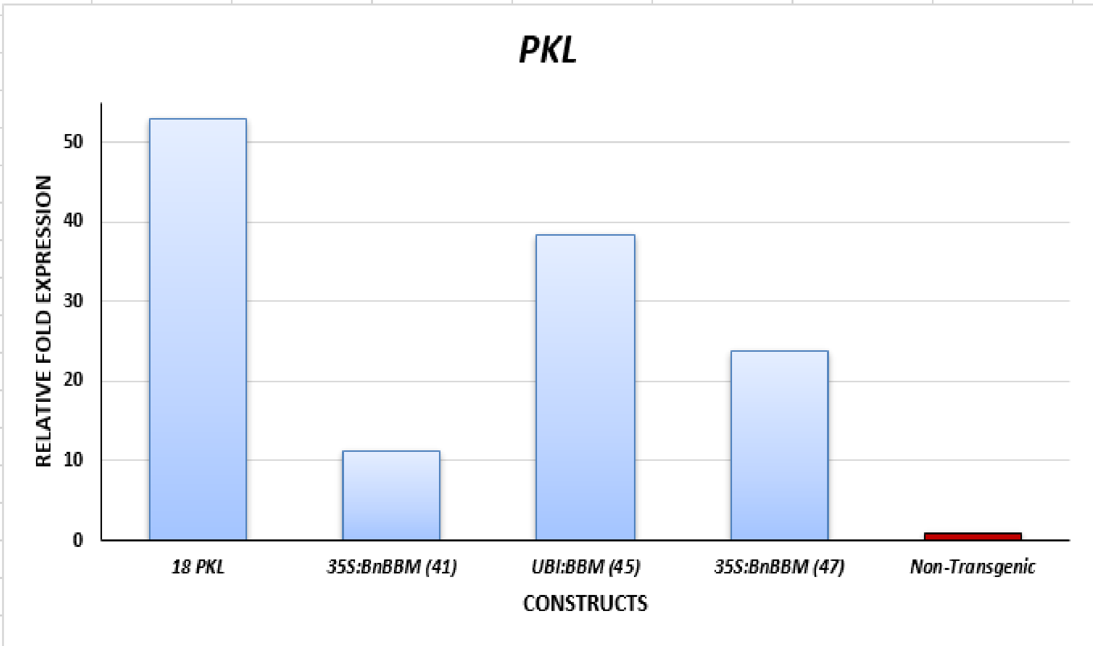

Figure 4.32. Relative expression level of *PKL* gene in transgenic cotton lines (18) refer to *35S:GmBBM*, (41) refer to *35S:BnBBM*, (45) refer to *UBI:BBM*, (47) refer to *35S:BnBBM*, (nt) refer to non transgenic control used as the calibrator

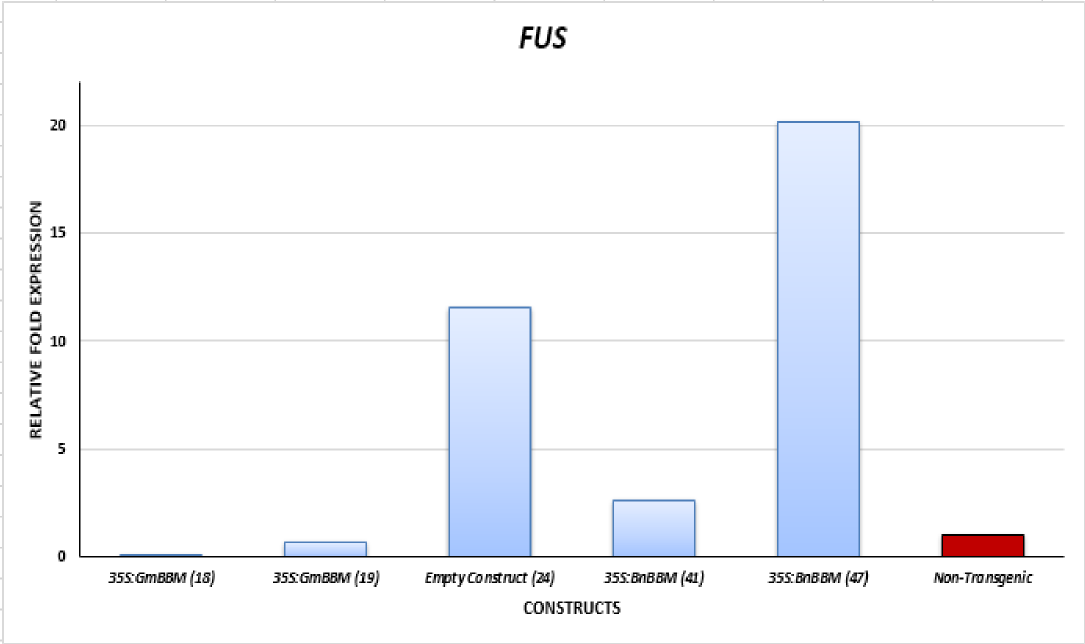

Figure 4.33. Relative expression level of *FUS3* gene in transgenic cotton lines, (18-19) refer to *35S:GmBBM*, (24) refer to empty vector control, (41) refer to *35S:BnBBM*, (47) refer to *35S:BnBBM*, (nt) refer to non transgenic control used as the calibrator

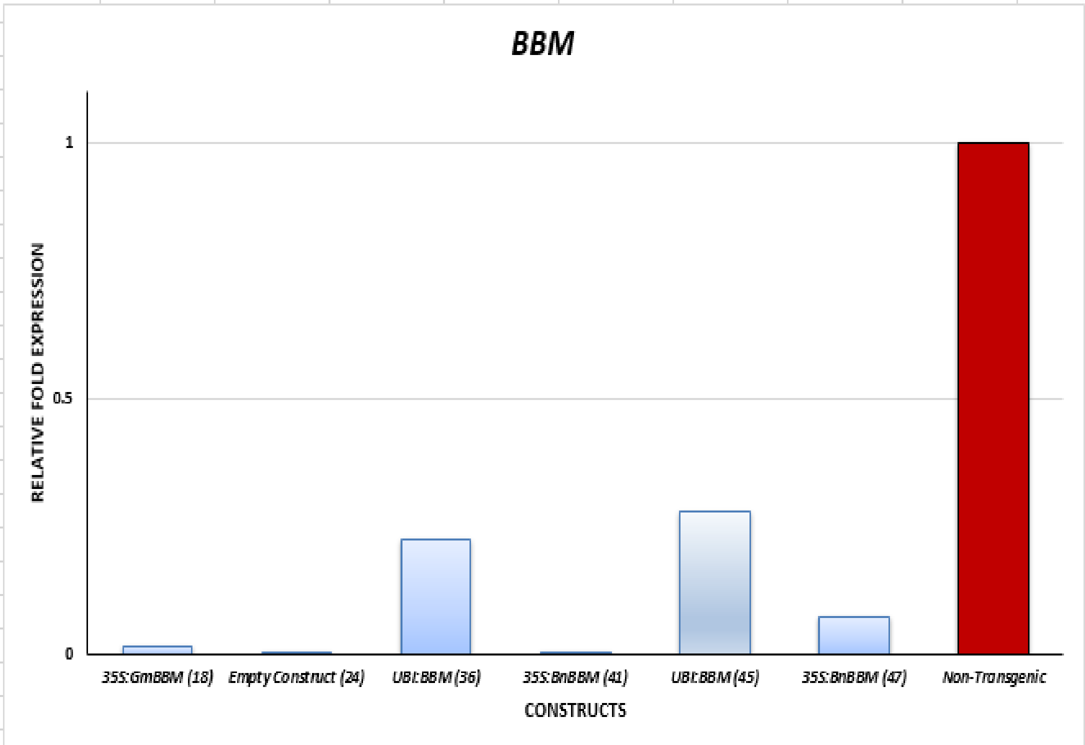

Figure 4.34. Relative expression level of *BBM* gene in transgenic cotton lines, (19) refer to *35S:GmBBM*, (24) refer to empty vector control, (41) refer to *35S:BnBBM*, (45) refer to *UBI:BBM*, (nt) refer to non transgenic control used as the calibrator

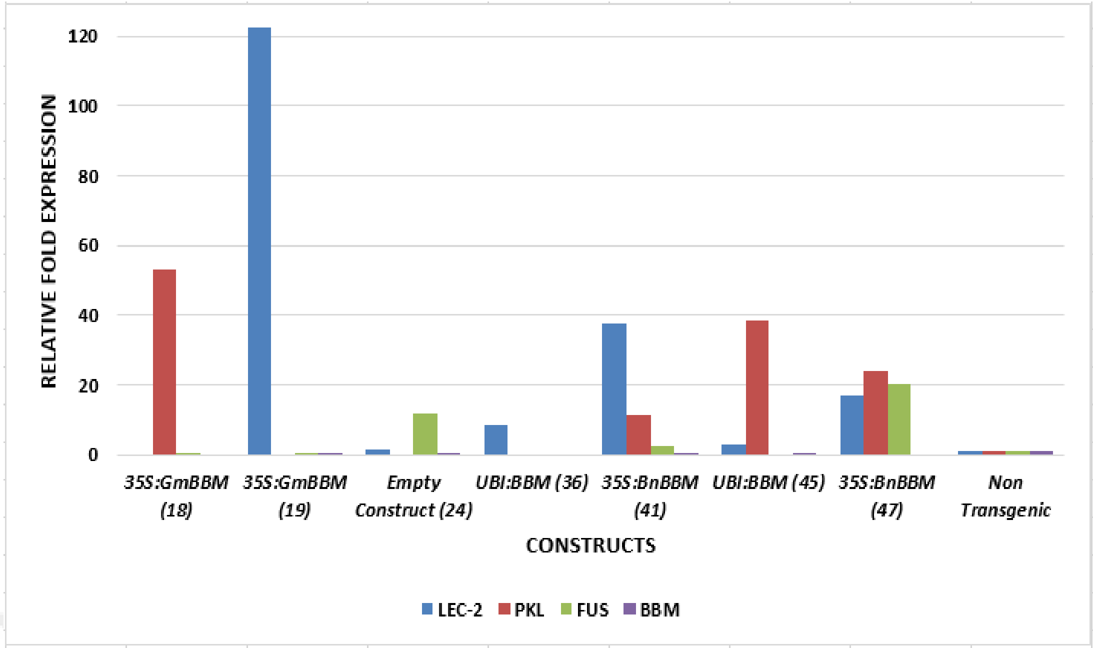

Figure 4.35. Relative expression level of *LEC2, PKL, FUS3, BBM* genes in transgenic cotton lines, whereas (18-19) refer to *35S:GmBBM*, (24) refer to empty vector control, (36) refer to *UBI:BBM*, (41) refer to *35S:BnBBM*, (45) refer to *UBI:BBM*, (47) refer to *35S:BnBBM*, (nt) refer to non-transgenic control used as the calibrator. Red color represents *PKL* gene, blue represent *LEC2* gene, green represent *FUS3* gene, purple represent *BBM* gene

Results of qRT-PCR indicate that *BBM* gene expression (Figure 4.34) is very low when *PKL* expression (Figure 4.32) is high regardless of driven constitutive expression of the target gene. Therefore, it was suggested that *BBM* gene has a strong correlation with some of the negative regulators of somatic embryogenesis. However, BBM expression could be low die to inappropriate extraction of RNA used in qRT-PCR. Moreover, it was illustrated that expression of *LEC2* and *FUS3* (Figures 4.31 and 4.33) were not affected by high expression of the negative regulator – *PKL* gene suggesting that these genes can independently regulate SE. Visual representation of the gene expression levels (Figure 4.35) indicate that *35S:GmBBM* has the highest expression in *LEC2* gene, whereas the lowest *LEC2* level is represented in non-transgenic calli. *PKL* gene has the highest expression level in the *35S:GmBBM* construct. The highest expression of *FUS3* gene was observed in *35S:BnBBM*, whereas the lowest were in *UBI:BBM* and *35S:GmBBM* constructs.

Control samples that are indicated as non transgenic (nt) are extracted from Coker 312 calli. Non-transgenic (nt) sample has the highest expression of *BBM* gene compared to other samples. Other genes in control samples (*LEC2*, *PKL*, *FUS3*) have relatively low expression compared to other transgenic samples from different constructs. Illogical data represented in Ct values from qRT-PCR were not used in calculation.

Analysis of Variance (ANOVA) was performed for all genes for estimating how much variations are there in dependent variables. All sections are estimated as non-significant according to provided p-values (Significance) shown in the Tables 4.3, 4.5, 4.7. Post hoc tests are indicating differences among treatment levels and it shows that there is no significant difference among treatments (Tables 4.4, 4.6, 4.8).

Table 4.3. Analysis of Variance (ANOVA) of *LEC2* qRT-PCR data

Tests of Between-Subjects Effects					
Dependent Variable: Gene Expression Rate					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1442.640[a]	6	240.440	.836	.579
Intercept	642.546	1	642.546	2.234	.179
Treatment	1442.640	6	240.440	.836	.579
Error	2013.755	7	287.679		
Total	4098.941	14			
Corrected Total	3456.395	13			

Table 4.4. The multiple-comparison post hoc test of qRT-PCR data of *LEC2* gene

Gene Expression Rate			
	Treatment	N	Subset
			1
Tukey HSD[a,b]	NonTrans-*LEC2*	2	.2458859
	24-*LEC2*	2	.3975038
	45- *LEC2*	2	.7337477
	36- *LEC2*	2	2.0550725
	47- *LEC2*	2	4.1988088
	41- *LEC2*	2	9.2118705
	19- *LEC2*	2	30.5798071
	Sig.		.590

a. R Squared = .417 (Adjusted R Squared = -.082)

Table 4.5. Analysis of Variance (ANOVA) of *PKL* qRT-PCR data

Tests of Between-Subjects Effects					
Dependent Variable: Gene Expression Rate					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	181.378[a]	4	45.345	.502	.738
Intercept	341.285	1	341.285	3.775	.110
Treatment	181.378	4	45.345	.502	.738
Error	452.022	5	90.404		
Total	974.685	10			
Corrected Total	633.400	9			

a. R Squared = .286 (Adjusted R Squared = -.285)

Table 4.6. The multiple-comparison post hoc test of qRT-PCR data of *PKL* gene

Gene Expression Rate			
	Treatment	N	Subset
			1
Tukey HSD[a,b]	NonTrans-*PKL*	2	.2295344
	41-*PKL*	2	2.5822790
	47-*PKL*	2	5.4543878
	45-*PKL*	2	8.8069646
	18-*PKL*	2	12.1366290
	Sig.		.727

Table 4.7. Analysis of Variance (ANOVA) of *BBM* qRT-PCR data

Tests of Between-Subjects Effects					
Dependent Variable: Gene Expression Rate					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	18.752[a]	4	4.688	.939	.511
Intercept	8.285	1	8.285	1.659	.254
Treatment	18.752	4	4.688	.939	.511
Error	24.963	5	4.993		
Total	52.000	10			
Corrected Total	43.716	9			

Table 4.8. The multiple-comparison post hoc test of qRT-PCR data of *BBM* gene

Gene Expression Rate			
	Treatment	N	Subset
			1
Tukey HSD[a,b]	24-*BBM*	2	.0031169
	19-*BBM*	2	.0056057
	41-*BBM*	2	.0146632
	45-*BBM*	2	.9854195
	NonTrans-*BBM*	2	3.5421976
	Sig.		.561

Table 4.9. Analysis of Variance (ANOVA) of *FUS* qRT-PCR data

Tests of Between-Subjects Effects					
Dependent Variable: Gene Expression Rate					
Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	1134.928[a]	4	283.732	.714	.617
Intercept	924.468	1	924.468	2.326	.188
Treatment	1134.928	4	283.732	.714	.617
Error	1986.881	5	397.376		
Total	4046.277	10			
Corrected Total	3121.809	9			

a. R Squared = .364 (Adjusted R Squared = -.146)

Table 4.10. The multiple-comparison post hoc test of qRT-PCR data of *FUS* gene

Gene Expression Rate			
	Treatment	N	Subset
			1
Tukey HSD[a,b]	18-*FUS*	2	.0604680
	19- *FUS*	2	.9306034
	41- *FUS*	2	3.5826961
	24- *FUS*	2	15.8415232
	47- *FUS*	2	27.6593323
	Sig.		.661

CHAPTER V

DISCUSSION

This performed research was dedicated to investigating potential of morphogenic gene BBM to increase regeneration capacity and transformation efficiency. During research *35S:BBM* construct was developed using conventional and GateWay cloning techniques. This gene is a part of AP2/ERF transcription factor family that has a broad range of functions in plants. This TF family is unique for plant kingdom and essential for numerous plant developmental processes. Traditional cloning was used to introduce *BBM* gene to vectors of pBIN61 and pDrive using TA cloning. On the contrary, GateWay cloning was used to clone *BBM1* gene from soybean. This gene has predicted sequence in cotton and designed primers were degenerate. Unfortunately, TA cloning was giving different size of gene in restriction analysis. Due to this problem, TA cloning was stopped. GateWay cloning showed perfect performance during cloning steps and both BP and LR reactions were successful. Digestion confirmation showed matching results and further steps were done. Invitrogen Gateway recombination cloning technology allowed to perform quick reactions and enabled accurate results.

Over the last 40 years genetic engineering applications especially over-expression studies have stimulated many significant plant biological discoveries, as well as improvement of economically important crops. In many over-expression studies, the 35S promoter has been used to drive constitutive expression of desired transgenes, promoting to elucidate functions of genes and giving insight into plant processes. Nowadays the CaMV 35S promoter is one of the most well studied and mostly used promotors. Besides being used as a constitutive driver of processes in plant cells, this promoter has been used as a backbone for developing various transcriptional regulatory systems (Amack and Antunes, 2020).

Selection of promoter used for driving gene expression is very important. There is a necessity of understanding native regulation of genes by promotors; however, designing and predicting effect of a promoter on the genome of a transformed plant is more important. One of the constitutive promotors used in transgene expression is the maize ubiquitin 1 (Ubi-1) promoter. This promoter is actively used in driving transgene expression in monocotyledonous plants. It was stated that Ubi promoter can express strong and constitutive expression in all tissues in the wheat. However, there is a little information about activity of Ubi promoter in cotton (Rooke et al., 2000).

Several reports describing overexpression of morphogenic genes were provided; however, constitutive expression causes pleiotropic phenotypes. Therefore, studies investigating ways of controlling such effects are being performed. It was stated that there are several ways of eliminating the problem. Stimulation of growth using inducible systems followed by stopping induction; excision of the overexpressed gene, when not needed; application of promoter that turns off allowing adequate growth are some of the ways of preventing pleiotropic phenotypes (Gordon-Kamm et al., 2019).

Cotton is described as highly recalcitrant plant due to regeneration that is highly genotype dependent. Moreover, cotton has low rates of embryos turning to mature plants. Long periods of culture in tissue conditions may promote somaclonal variation and excess production of phenolic compounds in cotton cultures. Cotton plants are being killed by lethal browning caused by excess production of phenolic compounds in tissue culture with prolonged time periods. Frequently, abnormal embryos are being formed from callus and elongation of shoots is a rare condition. Most of these problems are based on tissue culture systems (Kumria et al., 2003).

Plant material age and genotype; hormone selection and plant medium composition can be as main reasons of fails in culturing cotton plants (Ikram-Ul-Haq, 2004). Phenolics are believed and proved to be intermediates, phytoalexins and precursors of some metabolic pathways (Cvikrová et al., 1996; Lewis and Yamamato, 1990; Kessmann et al., 1990). Deposition of phenolics in the plant cell walls are used as a defence system against pathogens (Bolwell et al., 1985). When phenolics are released from root system of plants, inhibitory function of phenolics is used for effecting bacterial flora in the soil (Kefeli et al., 2003). Moreover, phenolics as a big group of secondary metabolites manage plant growth by controlling catabolism of IAA (Arnaldos et al., 2001). There are two believes regarding effect of phenolics on plants in the culture conditions.

It is shown that phenolics are useful in plant development regulation and promoting organogenesis and differentiation of cells. It was shown that oxidized phenolics negatively affect plant condition in *in vitro* conditions (Arnaldos et al., 2001).

One of the major problems of economically important plants are blackening and tissue lethal browning. In damaged cells vacuoles are mixed with components of cytoplasm, and therefore, phenolic compounds present in cells become oxidized. As a result, activity of enzymes are inhibited and culture medium is becoming darkened by tissues (Laukkanen et al., 1999; Compton, 1986). It was shown that liquid mediums can decrease oxidation of phenolics. Subculturing and addition of antioxidants believed to be reducing phenolics activity. Antioxidants like ascorbic and citric acid; PVP and activated charcoal are frequently used in tissue culture mediums. Pretreatment of cotton explants in ascorbic acid is believed to reduce stress, have beneficial effects against contamination by bacteria and fungi. (Toth et al., 1994). Therefore, glucose and ascorbic acid in combination with activated charcoal were used to prevent lethal browning of cultures.

Effect of antioxidants was tested in three ways. The first application method was addition of 100 mg/L ascorbic acid and 150 mg/L citric acid as a pre-treatment method while cutting the explants. Second method was addition of acrobic acid on 100 mg/L to the callus induction method. Third method combined 100 mg/L of ascorbic and citric acid in the callus induction medium. Experiments showed that pre-treatment and combination of two antioxidants significantly reduce phenolic activity and totally prevent lethal browning. Similar results were obtained when ascorbic acid in combination with citric acid was tested in the micrografting of *P. cynaroides* (Wu et al., 2007).

Ascorbic acid protects unsaturated (esterified and non-esterified) fatty acids and prevents from peroxidation. Hydroperoxides are comparatively stable when orin and copper is absent. Regeneration of membrane bounds of vitamin E is done by ascorbic acid and limitation of lipid peroxidation is occurring. Urate as one of the most important anti-oxidants is regenerated from urate radicals by the help of ascorbic acid. Mobilization of copper from serum ceruloplasmin is done by ascorbic acid without production of hydrogen peroxide. Developments of extracellular matrices are done by hydroxylation of proline that is also catalyzed with the help of ascorbic acid. Moreover, hydroxylation of proline forms molecules of collagen and elastin. Ascorbic acid as one of the co-factors of hydroxylphenylpyruvic acid oxidase participates in catabolism of phenylalanine and tyrosine (URL 1).

Total phenols extracted from 7 days old cotton plants grown in tissue culture condition were the lowest compared to other groups of 14, 21, 28 days old plants. Leaf, cotyledon, hypocotyls were tested among these age groups for phenolics content. Leaves had no phenolics amount in 7 days old group compared to 10% in hypocotyl and 50% of cotyledon groups. Moreover, regeneration of seeds were above 70 percent in 7 days old group and highest compared to others, therefore suggesting 7 days old group being best for using in genetic transformation (Ozyigit et al., 2007).

It was stated that the *BBM* gene from AP2/ERF TF family can induce embryogenesis in *Arabidopsis* and *B. napus*. Expression of the BBM gene is found in early stages of embryogenesis. Overexpression of this gene is believed to be sufficient for induction of SE. Different observations of *BBM* induced SE suggest developmental role of the gene in the processes of cell proliferation. It was also stated that expression of *BBM* gene in older tissues can cause pleiotropic alterations (Boutilier et al. 2002).

However, spontaneous induction of SE was not observed in transgenic lines. The same constructs (*35S:BnBBM* and *UBI:BBM*) that have been reported several times in induction of spontaneous SE did not induce SE and pleiotropic phenotypes in this research. Similar results were taken from the *BBM* gene extracted from *Rosa canina* over-expressed in *Arabidopsis* (Yang et al., 2014) and *35S:BBM* in tobacco (Srinivasan et al., 2007). Results indicate that somatic embryogenesis pathways different among species and involve different hormonal and gene pathways.

BBM TF has considerable role and potential for genetic engineering emerging *BBM* as a significant gene marker. Some studies state that precise expression of the gene can provide evidence of the significant role of the *BBM* gene for induction of SE. Moreover, *BBM* gene utilizes distinct functions in numerous signalling pathways of development. Exact signalling pathways of *BBM*-mediated SE and signalling transmissions are still unclear; however, many observations of *BBM* genes participating in developmental processes have been stated (Boutilier et al. 2002).

AP2/ERF transcription factors play different roles in fundamental plant processes like identity of flower organs, identity of epidermal cells, respond processes to biotic stress. This TF multigene family shares distinguishing characteristic having one or two domains of AP2. For the first time, as a repeated motif AP2 subfamily was found by Jofuku et al. (1994), in *Arabidopsis*. It was found that AP2 subgroup plays role in floral, ovule and seed development. Subfamily genes of AP2 have been investigated by applying mutant analysis and it was established that they have essential parts in fundamental processes (Riechmann and Meyerowitz, 1998).

Functions of *BBM* gene are broad and proteins that are encoded by *BBM* show similarity to AP2/ERF TF family (Boutilier et al., 2002; Riechmann and Meyerowitz, 1998). Functions of these TF family proteins are explained by number of binding domains existing (Moose and Sisco, 1996). AP2 subgroup has two binding domains in the contrast of ERF subgroup that has one DNA binding domain (Jofuku et al., 1994).

Presence of two binding domains is consistent with a role of these proteins in fundamental processes. Binding domains of two subgroups in BBM proteins, are shown to be similar to AP2 domains. This family includes proteins of ANT and ZMMHCF1. Protein of maize ZMMHCF1 is found in postpollination endosperm; it is also performed to be complement to deficient mutant to *E.coli* (Daniell et al., 1996). Protein ANT was studied using mutant approach and loss of functions mutants showed decreased number of cells and smaller sizes of plant organs (Klucher et al., 1996; Elliot et al., 1996). It was discovered that *BBM*, *LEP* and *ANT* share common functions such as cell proliferation, whereas these proteins are distinct. It also suggests that activation pathways are possibly intersecting (Boutilier et al., 2002).

Improved regeneration was received from another work studying Arabidopsis and fusion protein BBM. This post-translationally controlled protein resulted in fertile plants. Fusion gene under constitutive promoter without application of DEX showed normal plants, whereas medium with DEX showed better regeneration. Other genes were also checked under inducible systems to have better regeneration (Lutz et al., 2015). Transcriptionally regulated system was used to express *WUS* gene to transition of vegetative growth to embryonic (Zuo et al., 2002). Constitutively expressed *LEC1* and *LEC2* genes promote embryo formation on vegetative organs (Lotan et al., 1998). However, overexpression of *LEC1* did not form embryo-like structures (Zuo et al., 2002). Embryogenesis was developed by induction of *BBM* gene in sweet pepper (Heidmann et al., 2011) and tobacco (Srinivasan et al., 2007).

Shoot regeneration was found to be ecotype dependent in *Arabidopsis* (Luo and Koop, 1997). Regeneration capacity is highly dependent on explant type (Ma´rton and Browse, 1991). There is a high need of developing tissue culture systems for different traits such as organellar traits (Lutz et al., 2011). Plastid mutants that are fertile are showing utility to be selection mutants. These resistant mutants are showing pollen transmission in *Arabidopsis* (Azhagiri and Maliga, 2007). Regeneration of *BBM* steroid inducible systems form fertile plants. These fertile plants are formed from leaf explants after several months of growing (Lutz et al., 2015). Protocols of plastid transformation can see advantages of inducible systems because they need 12 weeks of culture (Maliga and Bock, 2011.

Frequently observed trend within AP2/ANT family phenotypes is undifferentiated cell growth in meristems (Nole-Wilson et al., 2005). More recently, role of this family in embryogenesis is being studied and importance emerged. Most of the information gathered in understanding embryogenesis was done because of ectopic overexpression of *BBM* gene in *Brassica* and *Arabidopsis* (Boutilier et al., 2002; Passarinho et al., 2008). Numerous studies propose that embryonal cells that are formed from somatic cells are activated using genetic pathways and morphogenic genes (Passarinho et al., 2008).

Some studies have been performed to understand features of homologues gene to *BBM* gene. Those genes are proposed to divide *BBM*-like genes from AP2/ERF TFs family. It is crucial to understand functional pathway of genes among this transcription factor family in order to understand how *BBM*-like genes turn somatic cells into embryonic cells. It was established that euANT members like *AIL*-like, *BBM*-like and *PLT*-like genes share common features in phylogenic and structure context. The work of El Ouakfaoui et al. (2010), established conservation of domains as well as specificity of domains. For instance, bbm-1 motif was revealed to be common for all *BBM*-like genes. Specificity of roles in fundamental processes are emerging for this group of genes (El Ouakfaoui et al., 2010).

Another example of functioning as redundant partner in embryogenesis of Arabidopsis are mutants of *plt2* and *bbm*. The work established that several genes act as redundant partners in stem cells of roots for differentiation (Galinha et al., 2007). These genes are *PLT1,2,3, AIL6 and AtBBM* that expressed in primordium of roots. It was demonstrated that *PLT2* under addition of bbm-1 motif was able to enhance better SE. Obtained results showed close relation of these genes with each other and may suggest important key feature of having similar structure. Transgenic lines harbouring *BnBBM1* and PLT2 lines were related with each other inversely showing that root and embryogenic pathways may compete between themselves (El Ouakfaoui et al., 2010). Ectopic expression of another *BBM*-like gene *AIL5* was shown to generate bigger organs, for instance, bigger flowers (Nole-Wilson et al., 2005). However, exact function pathway is not established for *AIL5*. Phenotypes generated by *AIL5* were similar to *ANT* that also results in bigger flowers (Nole-Wilson et al., 2005; Mizukami and Fischer, 2000); similar to AP2 that forms big embryos when overexpressed (Ohto et al., 2005). *AIL5* mutants believed to be redundant in action with other similar members (Nole-Wilson et al., 2005).

Another suggestion was done by Tsuwamoto et al. (2010), when double mutants of *BBM* and *AIL5* appeared to have normal phenotype without any defects in embryo and root development. It was suggested that there may present unidentified members that act in similar genetic pathway. Overexpression of *AIL5* yielded in similar phenotypes to *BBM*-like genes such as prompting SE. Discovering this feature shows that these genes possible involved in cell proliferation. Emerged embryos were found to have uplifted expression profiles of several genes like *BBM, FUS3, LEC1, LEC2, AGL15*; all of these genes have an ability to induce SE (Tsuwamoto et al., 2010). Another example of *AIL5*-like gene present in oil palm, named *EgAP2-1* is found to be expressed in growing dividing tissues, although callus growth is promoted rather than embryo formation in Arabidopsis plants (Morcillo et al., 2007).

Srinivasan et al. (2007), showed that *BnBBM1* promotes shoot organogenesis not embryogenesis showing that *BBM*-like genes able to promote activity of shoot meristem as well as embryogenesis. This feature depends on cell and genetic environment (Srinivasan et al., 2007).

Addition of specific bbm-1 motif to all *BBM*-like genes did not promote embryogenesis in *Arabidopsis* in the study of El Ouakfaoui et al. (2010), unlike to results of *PLT2*. This difference pathway is unknown and shows complex functions of *AIL5* in plant growth. Conserved motifs were described in members of AP2/ERF genes among different plants such as rice, *Arabidopsis* and soybean (Zhang et al., 2008). Nakano et al. (2006), proposed that it is a common feature of members within subfamilies to have similar sequence motifs. It proposes that members share similar functions within genetic pathways. Compared to some well described sequence motifs in subfamily of ERF, sequence motifs that are shared among members of AP2/ANT subfamilies are barely described (Nakano et al., 2006; Zhang et al., 2008).

The work of El Ouakfaoui et al. (2010), that made characterization of *BBM1* gene from soybean made possible to arrange *BBM*-like genes from other members which are featured as generators of embryo development. Collected data compared received ten conserved motifs. Motif composition of *PLT*-like and *AIL5*-like genes was similar to *BBM*-like genes. Moreover, bbm-1 motif was found to be the most common motif to be shared among *BBM*-like genes (El Ouakfaoui et al., 2010).

It was also stated that obtained phenotypes from overexpression of *BBM* and *EMK* lines share common features. Most features recorded were callus growth and delayed root development. Similar expression profiles were found when these two genes were checked. Although, *BBM* gene was found in globular and cotyledonary stage of embryos, whereas *EMK* gene was not found.

CHAPTER VI

CONCLUSION

Cotton is considered as one of the most important income sources for millions of people around the globe. FAO states that cotton is involved in numerous aspects of day-to-day lives of people for many centuries. Cotton production and worldwide trade have a significant role in national economies. Importance of these crops is equally crucial for developing and developed countries. Cotton industry is creating millions of workplaces each year and international trade helps national economies (World Cotton Day, 2019).

However, environmental changes, lack of resources and arable land are causing different problems for the agricultural sector of cotton crop. There are high expectations from cotton production regardless of problems with modern agriculture. Cotton is expected to give high yields despite shortened land areas, numerous pests, abiotic and biotic stresses. Such high expectations cannot be fulfilled without biotechnology. Each year numerous attempts to improve performance of cotton crops on the fields have been performed despite fears of GM crops. Nevertheless, biotechnology of cotton has been developing and new traits are introduced to the cotton germplasm.

Genetic improvement and engineering of cotton crops need efficient tools and protocols of transformation and regeneration due to very low transformation efficiency and regeneration. For this reason, numerous ways of improving regeneration capacity have been attempted. Several methods and ways of improving cotton regeneration such as overexpression of morphogenic genes and members of transcription factors participating in developmental processes; deletion and mutant approach methods of negative correlators, stimulation of regeneration through hormones; investigation of hormonal crosstalk; identification of putative genes that are upstream of signaling pathways have been attempted. However, results of such investigations are below expectations for the cotton biotechnology. More recently, application of morphogenic genes in plant biotechnology started giving expected results. Overexpression of *BBM* gene that is a member of AP2/ERD TF family was overexpressed in *B. napus, Arabidopsis thaliana, Glycine max, Theobroma cacao, Musa acuminate, Zea mays, Coffea arabica, Rosa canina, Populus tomentosa* for improving regeneration capacity and inducing somatic embryogenesis. Results of studies proved the positive effect of *BBM* gene on overall performance of transformation and shortened time required for induction of SE (Yavuz et al., 2020).

This phenomenon was taken as a role model for overexpressing the *BBM* gene in cotton and investigate whether transformation efficiency can be improved. Ectopic expression of *BBM* gene in cotton has not been performed before and this study is the first to be done. This study aimed to investigate whether the *BBM* gene is capable of inducing spontaneous SE and improving transformation efficiency. For this purpose, *35S:GmBBM* and empty vector constructs were developed and transferred into cotton through the Agrobacterium method. Moreover, *UBI: BBM* and *35S: BnBBM* constructs were transferred as additional to the main developed construct.

The study was successful to get calli harbouring *35S:GmBBM*, *UBI:BBM* and *35S:BnBBM*. Regeneration efficiency of transgenic calli constituted 71% out of survived calli. Results state that all constructs were highly successful in improving transformation efficiency. However, spontaneous SE induction was not observed in obtained calli. Similar results were observed from the work of Srinivsan et al. (2007) overexpressed *BBM* gene in tobacco; spontaneous induction of somatic embryogenesis as in *Arabidopsis* and *B. napus* was not observed. Such results show that *BBM* expression should be combined with hormones to induce SE (Srinivsan et al., 2007). Srinivsan et al. (2007) stated that obtained tobacco lines were having pleiotropic phenotypes; however, in this work we could not see any pleiotropic phenotypes suggesting that signaling pathways and possible effects in different crops are different.

More and more proteins regulating cell totipotency are being identified, including *LAFL* network members. The work of Horstman et al. (2017) proposed transcriptional regulation of the *LAFL* network by *BBM* gene. Experimental *lafl* mutant approach showed reduced BBM expression. However, despite the fact of direct activation of the *LAFL* network by BBM proteins, exact transcriptional loops and pathways are not clear. Therefore, this thesis work aimed to check expression profiles of several *LAFL* gene members and one repressor of embryogenesis.

Gene expression analyses of *BBM, LEC2, FUS3 and PKL* genes were provided insight to understand correlation of *BBM* gene with members of *LAFL* network. Results suggest that *PKL* gene as a negative correlator of *BBM* gene has a very high impact on expression profile of the gene. When *PKL* is high, the *BBM* gene is showing extremely reduced expression. *LEC2* and *FUS3* genes showed moderate levels of expression despite the extremely low profile of the *BBM* gene.

The overall idea of this thesis was to have some insight into the effect of several genes participating in cell proliferation and developmental processes. *BBM* gene was the target of this thesis due to strong evidence of inducing somatic embryogenesis in several crops. *BBM* gene showed itself as an effective tool in improving transformation efficiency; however, spontaneous induction of SE did not happen in cotton. Moreover, this thesis work reveals a strong correlation of *BBM* gene with negative regulators of embryogenesis. However, results of qRT-PCR indicating suppression of *BBM* gene by the *PKL* negative regulator might be affected by other hormonal or gene regulatory pathways. It has been stated that *BBM* gene expression and some of the genes participating in somatic embryogenesis are expressed during early and late stages of SE. Therefore, one of the reasons for a low *BBM* profile might be inappropriate timing of RNA extraction. For better understanding of *BBM* gene expression, future experiments in cotton transformation should involve extraction of the *BBM* gene during different embryogenesis stages. Moreover, *in situ* hybridization along with multiple analyses should be performed. Future works should be carried towards studying loss of function mutant approach of negative regulators of embryogenesis and on more precise investigation of signaling pathways of morphogenic genes. In addition to mutant approaches, gene wide analyses should be performed. Domain swap and deletion studies can explain the necessity of several conserved motifs of the AP2/ERF transcription factor family that can reveal the necessity and importance of motifs.

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