APPLICATION OF BIOTECHNOLOGICAL TOOLS TO MODEL PLANT, *Brachypodium distachyon*

by

BAHAR SOĞUTMAZ ÖZDEMİR

Submitted to the Graduate School of Engineering and Natural Sciences
in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

Sabancı University

August 2009

APPLICATION OF BIOTECHNOLOGICAL TOOLS TO MODEL PLANT, *Brachypodium distachyon*

APPROVED BY:

Assoc. Prof. Dr. Hikmet Budak (Thesis Supervisor)	.....
Assoc. Prof. Dr. Sertaç Önde	.....
Prof. Dr. Zehra Sayers	.....
Prof. Dr. İsmail Çakmak	.....
Assoc. Prof. Dr. Levent Öztürk	.....

DATE OF APPROVAL: 04.08.2009

ABSTRACT

APPLICATION OF BIOTECHNOLOGICAL TOOLS TO MODEL PLANT, *Brachypodium distachyon*

Bahar Soğutmaz Özdemir
Biological Sciences and Bioengineering
PhD Thesis, 2009

Assoc. Prof. Dr. Hikmet Budak (Thesis Supervisor)

Keywords: *Brachypodium distachyon*, model plant, microprojectile bombardment, *Agrobacterium*-mediated gene transfer, plant tissue culture

Brachypodium distachyon has recently emerged as a model plant species for the grass family (Poaceae) which includes cereal crops and forage grasses. In the present study, it was aimed to create inbred lines of *Brachypodium distachyon* that were collected from different geographic regions of Turkey and characterize this diverse collection morphologically and cytologically. Besides, an efficient tissue culture and a plant transformation system using microprojectile bombardment and *Agrobacterium*-mediated gene transfer methods for three selected genotypes were established.

Phenotypic characterization of the lines and macro-/microelement analysis in *Brachypodium* seeds showed extensive variation and significant differences. Callus formation percentages (50% to 100%) of the mature embryos and regeneration capacities of these calli (11.7% to 52.3%) were found to be genotype dependent. Each genotype displayed different responses to tissue culture conditions. Bombardment of the calli with the GUS (*β-glucorinidase*) gene revealed an average transient transformation efficiency of 28.7% within all three genotypes. *Agrobacterium tumefaciens* strains (EHA105, LBA4404 and AGL1), first two conferring resistance to kanamycin (*nptII* gene) and the later to glufosinate ammonium based herbicides (*bar* gene), were used for the transformation of calli that were wounded by microprojectile bombardment with two different sizes of gold particles or cutting with a blade. The GUS expression ranged from 0.0 to 10.4 (GUS foci/callus). All parameters were found to have significant effect on transient gene expression efficiency.

Our well-characterized germplasm collection (146 inbred lines and 116 of them diploid) is freely available for scientific community and the lines that were used for the first time in this research for plant tissue culture and transformation studies could aid for further genetic and genomics studies in *Brachypodium* and other grass species including energy grasses.

ÖZET

MODEL BİTKİDE, *Brachypodium distachyon* BİYOTEKNOLOJİK YÖNTEMLERİN UYGULANMASI

Bahar Soğutmaz Özdemir
Biyolojik Bilimler ve Biyomühendislik
Doktora Tezi, 2009

Doç. Dr. Hikmet Budak (Tez Danışmanı)

Anahtar Kelimeler: *Brachypodium distachyon*, model bitki, partikül bombardımanı, *Agrobacterium* aracılığıyla gen aktarımı, bitki doku kültürü

Brachypodium distachyon, tahılları ve yem bitkilerini içeren çim familyasını (Poaceae) temsil etmesi sebebi ile yakın zamanda model bir bitki olarak sunulmuştur. Bu çalışmada, Türkiye'nin farklı coğrafi bölgelerinden toplanan *Brachypodium distachyon* ile saf hatlar oluşturmak ve bu çeşitlilik içeren kolleksiyonu morfolojik ve sitolojik olarak karakterize etmek amaçlanmıştır. Bunun yanısıra, seçilen üç genotip için etkin bir doku kültürü sistemi ve partikül bombardımanı ve *Agrobacterium* aracılığı ile gen aktarım yöntemlerini kullanarak bir transformasyon sistemi kurulmuştur.

Hatların fenotipik karakterizasyonu ve *Brachypodium* tohumlarından yapılan makro-/mikroelement analizleri geniş bir çeşitlilik ve belirgin farklılıklar göstermiştir. Olgun embriyoların kallus oluşturma oranlarının (%50 - %100) ve bu kallusların rejenerasyon kapasitelerinin (%11.7 - %52.3) genotipe bağlı olduğu bulunmuştur. Her genotip farklı doku kültürü şartlari ortaya koymuştur. Kallusların GUS (*β-glucorinidaz*) geni ile partikül bombardımanı sonucunda, tüm üç genotip içinde %28.7 ortalama geçici gen aktarım etkinliği görülmüştür. Kalluslara gen aktarımında; *Agrobacterium tumefaciens* suşları (EHA105, LBA4404 ve AGL1), ilk ikisi kanamisin antibiyotiğine direnç (*nptII* geni) ve sonuncusu glufosinate amonyum bazlı herbisitlere dayanıklılık göstererek (*bar* geni), iki farklı büyüklükte altın partkülleri yada jilet ile yaralanmış kalluslara gen aktarımında kullanılmıştır. GUS geni ifadesi 0.0 ile 10.4 (GUS odakları/kallus) arasında değişmektedir. Tüm parametrelerin geçici gen ifadesi verimi üzerinde anlamlı bir etkisi olduğu bulunmuştur.

Bilim camiasi için mevcut olan karakterize ettiğimiz bu germplasm koleksiyonu (146 saf hattın 116 tanesi diploid) ve bu araştırmada doku kültürü ve gen aktarımı çalışmaları için ilk kez kullanılan hatlar, *Brachypodium* ve enerji çimlerini de içeren diğer çim türlerinde ileride yapılacak olan genetik ve genomik çalişmaları destekleyebileceklerdir.

With all my heart,

To my family

&

In loving memory of my grandmother

ACKNOWLEDGEMENTS

First of all, I would like thank my supervisor and mentor, Assoc. Prof. Hikmet Budak, for his guidance and support throughout this study. His expertise, advices, comments and contribution to this study and my graduate experience is so valuable.

I would like to give my special thanks to my lovely and faithful friends Gözde Korkmaz, Özge Cebeci, Dilek Telci, İrem Karamollaoğlu, Neslihan Ergen and Filiz Yeşilırmak who were with me whenever I needed. Thank you for your friendship, patience, assistance and support...

I would like to express my sincere appreciation to my previous supervisor and one of the members of my thesis committee, Assoc. Prof. Sertaç Önde for his endless support and guidance for 13 years. I would also like to thank each member of my committee, Prof. Dr. Zehra Sayers, Prof. Dr. İsmail Çakmak, Assoc. Prof. Levent Öztürk and Assoc. Prof. Alpay Taralp for their helpful criticism, advices and support. I am grateful to Prof. Dr. Hüveyda Başağa and Prof. Dr. Selim Çetiner for their academic support.

I wish to express my gratitude to my lab mates for their friendship and assistance. I am also thankful to all members of the physiology lab, Atilla Yazıcı, Yusuf Tutuş, Uğur Atalay, Veli Bayır, Özay Özgür Gökmen and Hugo Ferney Gomez Becerra for their friendship and technical support. I would like to thank all graduate students at the Biological Sciences and Bioengineering Program in Sabancı University and to my friends at Biological Sciences Department in METU for sharing this great experience.

Finally, I would like to thank all the members of my large family. But, I would like to give my special thanks to my parents Engin and Sezai Soğutmaz, my sisters Hülya Soğutmaz and Mine Sidem, my brother-in-law Bernard Sidem, my marvelous nephew and niece John Sidem and Olivia Sidem; for their endless support and love that they have given me at every stage of my life. And big thanks goes to my lovely husband, Mustafa Özdemir, for his patience, understanding, care, love, support and also for his help at my sleepless nights. Thank you for always being next to me. Thank you all for your infinite patience and enduring faith in me.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

BAP	6-benzylamino-purine
CaMV	Cauliflower mosaic virus
cm	Centimeter
2, 4-D	2, 4-Dichlorophenoxyacetic acid
EDTA	Ethylenediaminetetraaceticacid
g	Gram
GUS	*β*-glucuronidase
hr	Hour
hpt	Hygromycin resistance gene
IAA	Indole-3-acetic acid
kg	Kilogram
L	Liter
m	Meter
μm	Micrometer
μM	Micromollar
mg	Milligram
min	Minute
mmol	Millimole
MS	Murashige-Skoog basalt salt medium
NAA	Naphthalene acetic acid
nos	Nopaline synthase gene
nptII	Neomycin phosphotransferase gene
psi	Per square inch
PPT	Phosphinothricin

CHAPTER 1

1 INTRODUCTION

The improvements in the studies of molecular biology and genetics depend highly on the availability of a suitable model organism. *Brachypodium distachyon* has recently emerged as a model plant species for the grass family (Poaceae) which includes cereal crops and forage grasses. Temperate crops such as wheat, barley and forage grasses are the basis for the food and feed supply. They have immense agricultural, economical and industrial importance. Grasses as herbaceous energy crops are also becoming target sources for the use of renewable energy and their biological attributes are needed to be evaluated extensively to improve their bioenergy traits. However, the size and the complexity of their genomes limit the studies in molecular and genomics research and model plants can serve as templates for the improvements of their agricultural traits. Until recently, rice (*Oryza sativa*) and *Arabidopsis thaliana* has served as model species for these temperate grasses. However, *Brachypodium*, with all its biological and genetic characteristics and phylogenetic position, being closely related to grass family has become a more suitable model plant than the former ones by overcoming all their limitations.

The natural diversity of *Brachypodium* is centered around the Mediterranean region extending from north into Europe and to south into Indian subcontinent. Meanwhile, Turkey has diverse geographic regions and the human settlements in this area from ancient times make it a valuable area especially for plant genetic resources constituting also the wild relatives of cereal crops. So, by sharing the same ancestors with the grass family, it is natural that Turkey is expected to be a rich source of *Brachypodium* diversity. For this study, different ecotypes of *Brachypodium distachyon* representing diverse geographic regions of Turkey were collected. The diverse

collection of inbred lines were assembled and characterized morphologically and cytologically. It was shown that phenotypic characterization of the lines showed extensive variation in flowering time, seed yield and size, germination rate, leaf color and hairiness, plant stature, average plant height and dry weight (biomass). According to their mean nuclear DNA content, most of them were found to be diploid whereas few of them were polyploid. In order to increase the gene pool available to plant biologists using *Brachypodium* as a model system, it is necessary to establish extensive well-characterized germplasm collections. As a result, we have created 146 lines representing different geographic regions of Turkey and 116 of them are diploid inbred lines that are freely available to be used by scientific community. Micro and macro element analysis (N, K, P, S, Mg, Ca, Fe, Cu, Mn and Zn) in seeds of these *Brachypodium* genotypes also exhibited significant differences in content and concentration for most of the elements evaluated. These differences could be investigated furtherly in physiological and genetic aspect to better understand the nutrient availability for improvement of both grain quality and yield.

The increasing trend in world population and climate changes lead scientists to find out new varieties that are resistant to disease and insects, and adapted to marginal lands or extreme soil and environmental conditions. Increasing the nutritional value and quality of the food or the crop yield are other essential improvements to be assessed in agricultural studies. Therefore, better understanding of agronomic traits is necessary. Plenty of agronomic traits are highly conserved and can be transferred between model and crop species. With the use of extending availability of genome sequences and gene transfer technology, crop improvement studies have accelerated seriously. Plant genomics studies together with transgenic technology offers a wide range of advances in functional gene analysis. So, both for the study of new genes and in the production of new transgenic plant species, plant transformation technology is crucial for improvements in agricultural studies.

One of the important traits of a model species is its capacity to be transformed and ease of growing both in tissue culture and in greenhouse conditions so that we have first established an efficient tissue culture system and then a transformation system for *Brachypodium* using two different gene transfer techniques, microprojectile bombardment method and *Agrobacterium*-mediated transformation. From our

collections, three different *Brachypodium* genotypes were selected and different plant growth regulators and carbohydrate sources were tested with different explant sources for tissue culture studies. Selecting the plant growth regulator and the explant type, three factorial completely randomized design with three levels of first factor (genotype), three levels of second factor (2,4-D; 2,4-Dichlorophenoxyacetic acid; 1.0 mg/L, 3.0 mg/L and 5.0 mg/L), and two levels of third factor (BAP; Benzylaminopurine; 0.0 mg/L and 0.5 mg/L) was set and carbohydrate source effect was also evaluated to obtain regenerated plants through callus formation using mature embryos. The callus formation percentages varied from 50% to 100% and the regeneration percentages of the calli derived were between 11.7% and 52.3%. Results indicated that callus formation and regeneration capacities were genotype dependent. Each genotype displayed different responses to tissue culture conditions, and also different efficiencies of callus formations were detected.

Microprojectile bombardment method is proven to be a suitable and effective system for monocotyledonous plants. Six different bombardment pressure and sample plate distance combinations using gold particles of two different sizes were experimented with mature embryo derived callus tissues using our well-established tissue culture system for three different genotypes. They were transformed with a construct containing screenable GUS (coding for *beta-glucorinidase*) and selectable *hpt* (coding for hygromycin resistance) genes. The efficiency of gene delivery in *Brachypodium distachyon* species was evaluated by assessing the transient GUS expression on bombarded tissues. 1100 psi bombardment pressure and 6 or 9 cm sample plate distances, using gold particles of 1.0 or 1.6 μm diameter, caused an increase in GUS expression. Average transient transformation efficiency of selected and regenerated plants was 28.7% within all three genotypes. T_0 and T_1 plants were obtained. On the other hand, three different supervirulent *Agrobacterium tumefaciens* strains were used to transform the same *Brachypodium* genotypes. *A. tumefaciens* EHA105 and LBA4404 strains combined the pGUSINT binary vector conferring resistance to kanamycin antibiotic for plant selection. AGL1 strain on the other hand contained the GUS gene as the reporter and "*bar"* gene that provides resistance to glufosinate ammonium based herbicides. The injury of the explants is important in *Agrobacterium*-mediated transformation for the increase of transformation efficiency so that the effect of injury type either by applying microprojectile bombardment using the

previously set conditions or by using conventional methods was examined. Transient GUS expressions of the infected tissues were analyzed and selection was applied for regeneration of the transformed tissues. Best genotype, injury type and bacterial strain combinations were evaluated. The GUS expression was found to be ranging from 0.0 to 10.4 (average number of GUS positive foci per callus number) and 0% to 97% (percentage of GUS gene expressing calli among total number of calli used in the experiment). All parameters were found to be significant for the efficiency of transformation. Furthermore, most commonly used *Brachypodium* line (Bd21) worldwide in genomics and molecular researches was used as control in our tissue culture and transformation studies to compare the efficiency of our lines.

Our well-characterized germplasm collection that is freely available for scientific community and these genotypes that were used for the first time in this research for plant tissue culture and transformation studies could aid for further genetic and genomics studies in *Brachypodium* and other grass species including herbaceous energy crops.

CHAPTER 2

2 OVERVIEW

2.1 *Brachypodium distachyon*

Brachypodium P. Beauv (from the Greek *brachys* 'short' and *podion* 'a little foot', in reference to its subsessile spikelets, (Watson and Dallwitz, 2005) is a temperate wild grass species. In particular, *Brachypodium distachyon* (*Brachypodium*), purple false brome, has recently emerged as a new model plant for the diverse and economically important group of temperate grasses and herbaceous energy crops (Draper et al., 2001). Temperate grass species such as wheat, barley and forage grasses are the basis of the food and feed supply. However, the size and complexity of their genomes are major barriers to genomics research and molecular breeding. Similarly, although the herbaceous energy crops (especially grasses) are becoming novel target sources of renewable energy, very little is known about the biological basis underlying their bioenergy traits. Therefore, there is a growing need for a temperate grass model to address questions directly relevant both for improving grain crops and forage grasses that are indispensable to our food production systems, and for developing grasses into superior energy crops.

2.1.1 *Brachypodium* as the model species

2.1.1.1 Characteristic features and plant distribution

Brachypodium distachyon (L.) Beauv has many qualities that make it a suitable model system for both cereals and biofuel crops such as Switchgrass (Draper et al., 2001). *Brachypodium* genome is present at different ploidy levels, including diploid, tetraploid and hexaploid accessions. *Brachypodium* 2n chromosome numbers of 10, 20 and 30 have been reported (Draper et al. 2001; Hasterok et al., 2004; 2006). However, it does not appear to be a simple polyploid series as initially thought. Rather, the 2n=10 and 2n=20 cytotypes both appear to be diploids and the 30 chromosome number cytotype seems to be an allotetraploid with the ancestral genomes similar to the 2n=10 and 2n=20 cytotypes (Hasterok et al., 2004; 2006). Due to its small genome (~355 Mbp) and availability of a polyploid series with basic chromosome number of x=5, (2n = 2x = 10), the diploid race of *B. distachyon* can be used as an accurate template for the much larger polyploid genomes of crops such as bread wheat (16979 Mbp, 2n = 6x = 42), durum wheat (12030 Mbp, 2n = 4x = 28) and barley (5439 Mbp, 2n = 2x =14) (All C-values from Bennett, 2004). Besides its small genome size, other desirable attributes include a small physical stature (approximately 20 cm), self-fertility, lack of seed shattering, a short lifecycle that is normally completed within 11-18 weeks depending on the vernalization requirement (Draper et al., 2001; Garvin et al., 2008) (might be as fast as 8 weeks under optimized conditions, (Vogel et al., 2006a), and simple growth requirements with large planting density and easy genetic transformation (Vogel et al., 2006a; Christiansen et al., 2005; Özdemir et al., 2008). This combination of desirable attributes, together with the biological similarities with its crop targets, underlies the recent research interest in this species.

Brachypodium species range from annuals to strongly rhizomatous perennials that exhibit breeding systems ranging from strictly inbreeding to highly self-incompatible (Khan and Stace, 1999). The hairy terminal ovary appendage, the single starch grains, the outermost thick layer of the nucellus, the long narrow caryopsis, spicate or racemose inflorescences and hairy nodules are some of the characteristic features of the genus *Brachypodium* (Catalan et al., 1995).

Several species of this genus were studied using combined sequences of chloroplast *ndh*F gene and nuclear ITS to reconstruct the phylogeny among these selected species within the genus (Catalan and Olmstead, 2000). Similarly, RFLPs and RAPDs were used for nuclear genome analysis to establish the evolutionary position of the genus. Genus constitutes morphologically more or less closely resembling species that are native to different ecological regions such that *B. distachyon* is the Mediterranean annual, non-rhizomatous *B. mexicanum* is from the New World, *B. pinnatum* and *B. sylvaticum* are Eurasian, and *B. rupestre* is a European taxon (Catalan et al., 1995).

The natural diversity of *Brachypodium* is centered around the Mediterranean region extending north into Europe and south into the Indian subcontinent (Figure 2.1). However, the distribution of the 2n=10 diploid may be somewhat restricted to the center of this larger distribution but more sampling is necessary to verify this (Garvin et al., 2008). Within this region, Turkey is expected to be a rich source of *Brachypodium* diversity since the three major phytogeographical regions of Turkey (Euro-Siberian, Irano-Turanian and Mediterranean) covering hot interior regions, cooler coastal areas and colder mountainous regions represent most of the environmental diversity found within the larger geographic range (Özdemir et al., 2008). *Brachypodium* distribution area has broad range both in latitude and altitude. Besides Turkey, populations of these species are also found at different altitudes in Iran, Iraq and Afghanistan. Mediterranean like climate with hot, dry summers and mild, wet winters favors *Brachypodium* distribution. It is also found at equatorial environment, cold winters of Central Anatolia and mild temperate climate of England (Garvin et al., 2008). Since it is adapted to different abiotic and biotic conditions, unlocking and exploiting the genetic diversity found in *Brachypodium* will facilitate cereal genome studies and improve breeding programs.

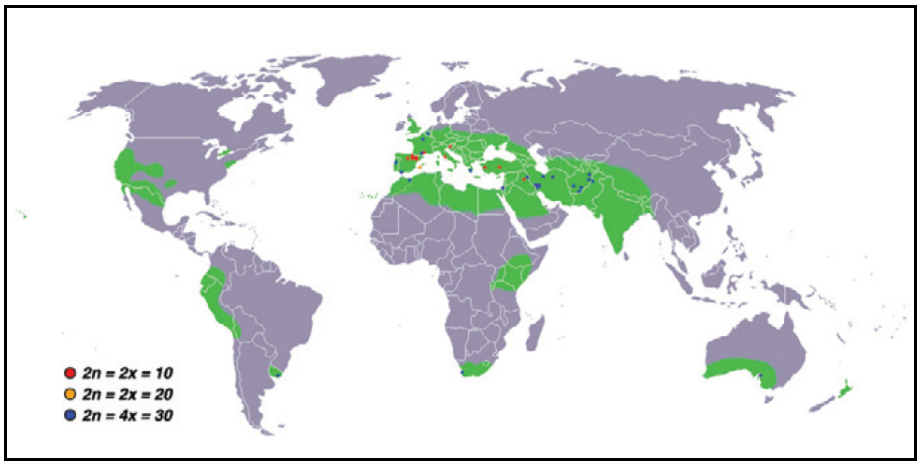


Figure 2.1 Geographic distribution of *Brachypodium* shown with green shaded areas. The 2n = 2x = 10, 2n = 2x = 20, and 2n = 4x = 30, accessions are plotted as red, yellow and blue dots, respectively (Garvin et al., 2008).

2.1.1.2 Advantages of *Brachypodium* over *Arabidopsis* and *Oryza*

The available genome sequences of the model plants *Arabidopsis* (The Arabidopsis Initiative, 2000) and rice (International Rice Genome Sequencing Project, 2005) are the major resources used in genetic and genomic studies. Nevertheless, these model species are not suitable for the genetics and genomics studies of temperate grasses. *Arabidopsis* has all the desirable attributes for a model plant: it is small in size, grows easily and quickly (reaching maturity in 6 weeks), has a small diploid genome, it is self-compatible and easily transformable. Its utility as a model system has been proven by the wealth of genomic discoveries, useful for a broad range of crops (including cereals) it has generated. However, as a dicot species, it does not share with grass crops most of the biological features related to agricultural traits and in this sense; rice would provide a better alternative. The rice plant, however, does not fulfill the requirements of short size, rapid life cycle, inbreeding reproductive strategy, undemanding growth requirements or easy transformation imposing practical limitations. As a tropical species, it does not display all the agronomic traits that are relevant to temperate grasses, especially to forage grasses, as resistance such as to specific pathogens, freezing tolerance, vernalization, perenniality, injury tolerance, meristem dormancy mechanisms, mycorrhizae, sward ecology or postharvest biochemistry of silage (Draper et al., 2001). Moreover, rice is phylogenetically distant from the *Pooidae* subfamily that

includes wheat, barley and temperate grasses (Kellogg, 2001) whereas molecular phylogenetic analysis have demonstrated that the genus *Brachypodium* diverged from the ancestral *Pooidae* clade (Figure 2.2) immediately prior to the radiation of the modern 'core pooids' (*Triticeae*, *Bromeae* and *Avenae*) which includes the majority of important temperate cereals and forage grasses (Catalan and Olmstead, 2000). Using conserved genomic and EST (expression sequence tag) sequences, divergence between *Brachypodium* and wheat was estimated to be 35-40 million years, which was significantly more recent than the divergence of rice and wheat, estimated approximately to be 50 million years (Paterson et al., 2004). Based upon the cytological, anatomical and physiological studies *Brachypodium* was placed into its own tribe *Brachypodieae* of the Poaceae family (Hasterok et al., 2004). In fact, the perennial outbreeding *B. sylvaticum* (2n = 18) was considered suitable for study of archetypal grass centromere sequences, which allowed detection of repetitive DNA sequences that are conserved among wheat, maize, rice and *Brachypodium* (Aragon-Alcaide et al., 1996). In this context, *Brachypodium distachyon* has been proposed as an alternative model for temperate grasses (Draper et al., 2001).

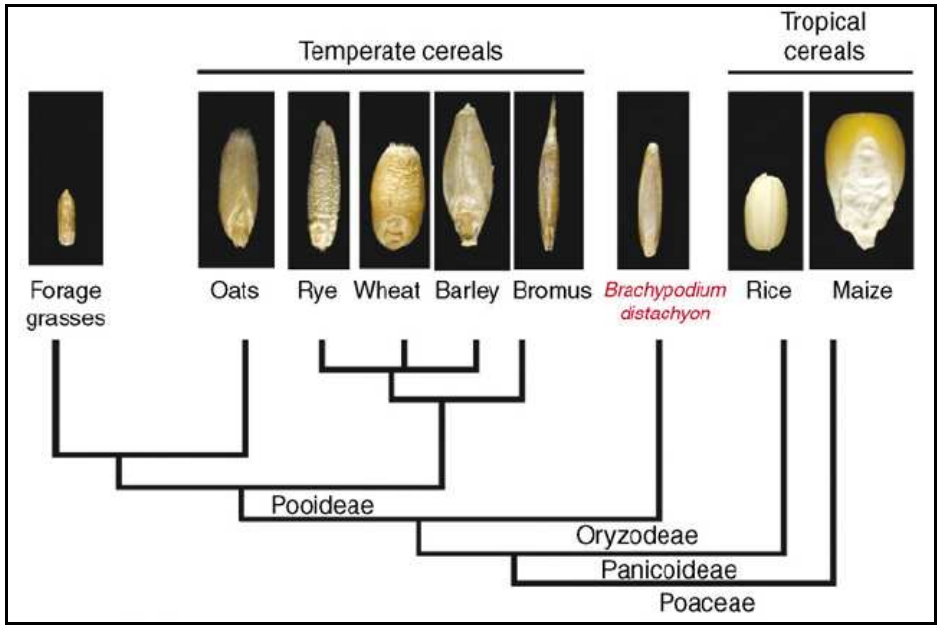


Figure 2.2 Phylogenetic position of *Brachypodium* among small cereal grains (Opanowicz et al., 2008).

2.1.1.3 The importance of model *Brachypodium* for the tribe *Triticeae*

The *Triticeae* Dumort tribe belongs to the grass family, Poaceae, and constitutes one of the economically most important plant groups. It includes three major cereal crops, wheat, barley and rye (belonging to the genera *Triticum*, *Hordeum* and *Secale*, respectively), which are traditionally cultivated in the temperate zone. The tribe has basic chromosome number of seven, and contains from diploids (2n = 2x = 14) to duodecaploids (2n = 12x = 84), including all intermediate ploidy levels (Dewey, 1984). Polyploidy (the most important cytogenetic process in higher plants, (Stebbins, 1971) and more specifically, allopolyploidy, has played and still plays a main role in the tribe's evolution. With around 350 species, crossability barriers are poorly understood, and it is remarkable how its species, even species in different genera, can be made to hybridize even if they do not occur so naturally. Therefore, the tribe also includes man made crops such as ×*Triticosecale* (triticale), ×*Tritordeum* (an amphiploid of *Triticum aestivum* × *Hordeum chilense* (Martin and Chapman, 1977; Martin and Sanchez-Monge-Laguna, 1980) and *Triticum aestivum* × *Leymus arenarius* (Anamthawat-Jonsson et al., 1997). It has also been possible to apply interspecific and intergeneric hybridization to increase the genetic variability of the tribe's crops (mainly wheat, (Cauderon, 1978; Sharma and Gill, 1983).

For these reasons, the whole tribe *Triticeae* is an enormous gene pool for crop improvement, deserving efforts not only for its morphological, physiological, genetic and genomic characterization, but also for the establishment of phylogenetic relationships among the species of the tribe. The large and complex genomes of some members of the tribe are main constraint for genetic and genomic researches within this tribe and the availability of a suitable model species like *Brachypodium distachyon* would facilitate the researches in this area.

2.1.1.4 The *Brachypodium* genome

Brachypodium distachyon accessions have chromosome numbers ranging from 10 to 30 (Robertson, 1981). The haploid genome size in diploid *Brachypodium* (2n = 2x = 10) varies from 172 Mbp to 355 Mbp (Draper et al., 2001; Bennett and Leitch, 2005),

although the former value may be an underestimate (Bennett and Leitch, 2005); the genome size is thus assumed to be approximately 355 Mbp. It is therefore one of the smallest genomes within Poaceae, which is intermediate between the genomes of *Arabidopsis thaliana* with 157 Mbp and rice with 490 Mbp (All C-values from Bennett, 2004). These data are consistent with previous reports that species of *Brachypodium* have the smallest 5S rDNA spacer of the grasses, and contain less than 15% highly repetitive DNA (Catalan et al., 1995). Additionally, GISH analysis of somatic chromosomes has shown the preponderance of repetitive DNA in the pericentromeric regions, reflecting the compactness and economy of this genome (Hasterok et al., 2004). This study also revealed the structural uniformity of the diploid accessions ABR1 and ABR5, confirming them as model genotypes, from which two BAC (bacterial artificial chromosome) libraries have recently been prepared for functional genomics analysis (Hasterok et al., 2006). Recent analysis of BAC end sequences (BES) also corroborates this unusually compact genome (Gu et al., 2007; Luo et al., 2007). The other accessions having chromosome numbers of multiples of 10 suggested that this species has evolved as a polyploidy series based upon 2n = 2x = 10, and that ecotypes that deviate from multiples of 10 evolved due to aneuploid or dysploid changes in chromosome number (Robertson, 1981). A cytotaxonomic analysis of the members of the polyploidy series has revealed a hybrid origin of several of the polyploid genotypes, thus suggesting a complex evolution of this species that is not entirely based on chromosome doubling (Hasterok et al., 2004). For example, allotetraploid artificial hybrids between *B. distachyon* and *B. sylvaticum* showed irregular meiosis and infertility (Khan and Stace, 1999), although allotetraploids were fertile, one of them (ABR100) showed normal meiosis (Hasterok et al., 2004). This indicates that either the progenitors of this allotetraploid are more compatible in hybrids, or the hybrids themselves have evolved pairing control mechanisms similar to those of wheat and other allopolyploids. The efficient separation of the genomes in the allotetraploid, together with the lack of recombination between homoeologous chromosomes has also been shown using genomic *in situ* hybridization GISH. These features make the *Brachypodium* natural polyploid hybrids a potential target for the isolation and characterization of diplodizing genes. The cytotaxonomic study of members of the polyploid series of *Brachypodium* species using (GISH) and FISH with rDNA markers states the complex evolution of this species (Hasterok et al., 2004).

2.1.2 Recent and future advances in *Brachypodium* research

Inbred lines make an important resource for genetic and genomics studies. In this view, diploid inbred lines have been developed in *B. distachyon* by selfing (Vogel et al., 2006a) as well as through selection from segragating populations derived from crosses among diploid ecotypes (Garvin, 2006). There are two sources of *Brachypodium* accessions, one collection is at Brachyomics in Aberystwyth, Wales and the source of other collection is the USDA National Plant Germplasm System (NPGS). Majority of these accessions that are diploid with haploid chromosome number of five are native to France, Slovenia, Italy, Iraq and Turkey (Garvin et al., 2008). In order to develop mutagenic lines, sodium azide as mutagenic agent was applied in *B. distachyon* and the response to this mutagen differed among accessions. The results were comparable with those earlier obtained in barley and rice under higher concentrations of mutagens (Engvild, 2005).

On the other hand, a BAC-based physical map of *Brachypodium distachyon* is being developed at the John Innes Centre (UK), as a part of the international effort to make BAC-based physical maps of Chinese Spring bread wheat (Bevan, 2006). Since establishing a physical map of bread wheat, one of the most important crops worldwide, is a major challenge due to the enormous size of the genome and its hexaploid constitution, it is expected that the availability of a *Brachypodium* physical map will greatly facilitate this task. The close phylogenetic relationship of *Brachypodium* to wheat leads to high similarity in gene sequences. An outline physical map of *Brachypodium distachyon* Bd3-1 using BAC End Sequence (BES) and fingerprinting is being established and will be used to start assembling contigs in wheat chromosome groups (Bevan, 2006). Another *B. distachyon* physical map is being developed at the University of California and USDA (Luo et al., 2007) by using two bacterial artificial chromosome (BAC) libraries constructed from *B. distachyon* Bd21 which are being fingerprinted using snapshot-based fingerprinting procedure and will serve as a resource for sequence assembly, comparative genome analysis, gene isolation and functional genomics analysis.

The genome of *B. distachyon* is also being examined for the presence, diversity, and distribution of the major classes of plant transposable elements, particularly the retrotransposons (Kalendar and Schulman, 2006), since retrotransposons comprise most of the existing DNA between genes in the large cereal genomes. These include the copia, gypsy, TRIM, and LARD groups of elements that will enable the development of retrotransposon-based molecular markers like IRAP, REMAP, SSAP and RBIP markers for the applications such as diversity and mapping tools.

A genetic linkage map of *Brachypodium distachyon* Bd21 is being developed by the International *Brachypodium* Initiative (Bevan et al., 2007). Genetic maps will provide anchor points linking the genome of *Brachypodium* with those of rice, wheat and some biofuel crops, and will establish chromosome-scale physical maps of BACs for whole genome sequencing. In order to develop genetic maps, mapping populations are being developed. The mapping populations were derived from a cross of Bd21 and Bd3-1 inbred lines. Several approaches have been taken to identify polymorphisms between parents of the mapping population. First, conserved orthologous (COS) markers derived from wheat and millet were used to identify a set of 80 confirmed polymorphisms between these two parental lines (Bd21 and Bd3-1). Another strategy was the use of sequences of Bd21 ESTs to identify additional polymorphisms and 20440 ESTs were generated (Vogel et al., 2006b).

Other resources that are currently being developed include: sequenced-indexed T-DNA populations, microarrays, and, most importantly, the complete genome sequence. The *Brachypodium* nuclear genome is currently being sequenced within a project funded in early 2006 by the US Department of Energy (DOE). The 4x draft assembly of the *Brachypodium* genome is currently available (www.brachypodium.org) whereas the final 8x version will be available in 2009. This project is generating a whole-genome shotgun sequence of the *Brachypodium* Bd21 genome, and is coupled with another to sequence nearly 250.000 ESTs. Data from both projects will be made publicly available through an online database (BrachyBase at <http://www.brachybase.org>) and a community dedicated portal (<http://www.brachypodium.org>). BrachyBase will enable efficient exploitation of genome and transcriptome sequences to identify genes underlying traits and will facilitate comparisons with other grass genomes (Mockler et

al., 2007). Other than these resources, there are other publicly available sites for *Brachypodium* research as outlined in Opanowicz et al. (2008).

BAC libraries of two diploid ecotypes of *B. distachyon*, ABR1 and ABR5 were constructed and used to determine synteny among rice, *Brachypodium* and species of Poaceae family. For this purpose, BACs were marker-selected (BAC landing) using primers designed according to previously mapped rice and Poaceae sequences. Most BACs hybridized as single loci in known *Brachypodium* chromosomes, whereas contiguous BACs co-localized on individual chromosomes, thus confirming conservation of genome syntheny (Hasterok et al., 2006). On the other hand, all plant miRNA sequences were compared with *Brachypodium distachyon* ESTs and GSS (genomic survey sequences) to identify its miRNAs and target sequences and 26 new *B. distachyon* miRNAs were detected. Their target mRNAs were found to be involved in signal transduction, metabolic processes and stress response (Unver and Budak, 2009).

Generation and analysis of over 60,000 BAC end sequences from large-insert BAC clones provided the first view of *Brachypodium* genome composition, structure, and organization (Gu et al., 2007). A 371-kb region in *B. sylvaticum* was sequenced and compared with orthologous regions from rice and wheat (Bossolini et al., 2007). *B. sylvaticum* and wheat were found to be highly identical when their sequences were compared (Griffiths et al., 2006). Chosen target loci from *Brachypodium* genome were also being sequenced and compared with genomic sequences from different plant species including wheat species (*Triticum* and *Aegilops*) with different ploidy levels, *Oryza sativa* and *Brachypodium sylvaticum*, for which a BAC library is available (Charles et al., 2006; Foote et al., 2004). This comparison revealed that there was a better conservation of microcolinearity between wheat and *Brachypodium* orthologous regions than between wheat and rice (Bossolini et al., 2007; Faris et al., 2008) that supported the results obtained from the comparison of BESs with EST databases. The BAC libraries constructed from *Brachypodium distachyon* (Bd21 diploid inbred line) would enable specific markers for wheat chromosome regions to be developed since *Brachypodium* genes with 77% were found to be matching the *Triticeae* EST and higher matching was identified with wheat EST than rice (Huo et al., 2006; 2008; 2009).

Brachypodium has also been explored as a model for the study of cereals-pathogen interactions. Varying degrees of susceptibility and resistance to *Magnaporthe grisea* (economically destructive pathogen and casual agent of Rice Blast disease that can also infect temperate cereals and forage grasses) was found in several *Brachypodium* accessions. Aetiology of fungal development and disease progression in *Brachypodium* closely resembled those of rice infections and overexpression of barley related gene probes was found (Routledge et al., 2004). Recent advances also include metabolic profiling using Fourier-transform infrared spectroscopy (FT-IR) for high-throughput metabolic fingerprinting and electrospray ionization mass spectrometry (ESI-MS). These metabolomic approaches showed considerable and differential phospholipids processing of membrane lipids during *M. grisea – B. distachyon* accessions ABR1 (susceptible) and ABR5 (resistant) interactions (Allwood et al., 2006). *Brachypodium distachyon,* being a host for *M. grisea* and other disease-causing pathogens of Pooid cereals (Allwood et al., 2006), is a suitable model for functional genomic investigations of *M. grisea* pathology and plant responses (Routledge et al., 2004).

To test the potential of *Brachypodium* as a model for the functional analysis of ryegrass flowering genes, two *Terminal Flower* 1 orthologs, *LpTFL*1 and *TFL*1 (from perennial ryegrass and *Arabidopsis*, respectively) were expressed in two different *B. distachyon* accessions. Both floral repressors significantly delayed heading date. The short life cycle of *Brachypodium* and the fast transformation system allowed heading date scoring of T_1s within the first year upon transformation, thus demonstrating the potential of *Brachypodium* also as a ryegrass model plant (Olsen et al., 2006).

2.2 Model species and transgenic technology

The increasing trend in world population and climate changes lead scientists to find out new varieties that are resistant to disease and insects, and adapted to marginal lands or extreme soil and environmental conditions. Increasing the nutritional value and quality of the food or the crop yield are other crucial improvements to be assessed in agricultural studies. Therefore, better understanding of agronomic traits is necessary. At this juncture, the model species *Brachypodium* plays a key role for gaining the knowledge to be used in temperate grasses for studies concerning both their agricultural importance since they serve as a source for the two-thirds of the food supply (Borlaug, 1998) and use as a renewable energy source (Özdemir et al, 2008).

One of the important traits of a model species is its capacity to be transformed and ease of growing both in tissue culture and in greenhouse conditions. Plant genomics studies together with transgenic technology offers a wide range of advances in functional gene analysis such as using insertional mutagenesis by *Agrobacterium*-mediated T-DNA insertions or gene silencing technology (Pereira, 2000). Plenty of agronomic traits are highly conserved and can be transferred between model and crop species So, both for the study of new genes and in the production of new transgenic plant species, a suitable model plant and the plant transformation technology are crucial for improvements in agricultural studies. Establishment of an efficient plant regeneration system is prerequisite for the success of plant transformation applications.

2.2.1 Plant tissue culture

Plant tissue culture is an important tool for the plant development studies in accordance with its use in molecular biology and genetic engineering. It is based on the idea that plants can be manipulated *in vitro* by separating them into their organs, tissues or cells which later can differentiate into whole plants (Trigiano and Dennis, 2005). Each cell, containing a normal complement of chromosomes, has a potential to differentiate into a

complete plant, so called totipotency and this gives the capacity of the plant tissue and cell culture techniques to make rapid progress in both basic and applied studies.

One of the greatest impacts of plant tissue culture is in the area of clonal propagation. Control of development is achieved by plant growth regulators. There are five main classes of plant growth regulators: auxins, cytokinins, gibberellins, abscisic acid and ethylene. Auxins promote cell division, stimulate growth by cell elongation, maintain apical dominance, form adventitious and side roots, inhibit leaf abscission and has role in somatic embryogenesis. Cytokinins are used to stimulate cell division, shoot initiation and growth, and retardation of senescence. With respect to auxins and cytokinins, gibberellins are used less commonly. They have roles in regulating cell elongation, breaking dormancy, stimulating elongation of internodes and determining plant height and fruit-set. Enhancements of fruit and leaf abscission, inhibition of seed germination, stomatal closure due to water stress are achieved by abscisic acid. Ethylene enhances fruit and leaf abscission, and fruit ripening (Johri and Mitra, 2001; Trigiano and Dennis, 2005; Slater et al., 2003; Westhoff et al., 1998). Depending on the ratio of plant hormones, especially the ratio of auxin and cytokinin that act in synergy to regulate the cell division process, the organogenesis of roots and shoots is achieved. Macronutrients (nitrogen, phosphorus, potassium, magnesium, calcium and sulfur), micronutrients (manganese, iodine, copper, cobalt, boron, molybdenum, iron and zinc – in some formulations nickel and aluminum is also found), organic supplements (vitamins and amino acids), carbohydrate source (such as glucose, sucrose, galactose or maltose) and gelling agents are the requirements for the culture media besides the inclusion of plant growth regulators. Explants (parts or pieces of a plant) are the starting materials for the cultures. Explant source or type, species type, age of the explant, media composition and physical parameters (such as light, temperature, gaseous environment, pH, osmotic pressure and humidity) are all important factors for the cultures and the efficiency of the plant propagation. Regeneration of the plants is achieved either by somatic embryogenesis or organogenesis, directly or indirectly. In indirect organogenesis (or indirect somatic embryogenesis), different from direct organogenesis (or direct somatic embryogenesis), firstly callus is produced from the explant before differentiation since callus is somehow a form of dedifferentiation consisting of unorganized, growing and dividing mass of cells that has the potential to differentiate into a whole or any part of a plant. There are several culture types used in

plant tissue culture and transformation studies; callus, cell suspension cultures, protoplasts, root cultures, shoot tip and meristem culture, embryo culture and microspore culture (Slater et al., 2003).

2.2.2 Gene transfer systems in plants

In transgenic plant technology, only defined traits can be introduced eliminating the risk of transferring undesirable genes and in a shorter time with respect to use of conventional breeding methodologies alone. With the use of extending availability of genome sequences, the improvements in plant regeneration from cultured cells, availability of selectable marker genes and suitable vector constructs and various gene transfer methods, plant improvement studies have accelerated seriously. This technology includes various types of gene transfer methods such as chemical (PEG) treatment of protoplasts, electroporation of protoplasts or organized tissues, microinjection, pollen tube pathway, microlaser, silicon carbide whiskers or sonication. However, microprojectile bombardment and *Agrobacterium*-mediated transformation are more widely used since these two methodologies have greater advantages over the others, especially exhibiting the feature of being reproducible.

Microprojectile bombardment has the advantages of being not tissue or species specific, fast, efficient, practical, reproducible, availability of gene transfer using simple vector systems and possibility of co-bombardment of plasmids. Also, organelle transformation is possible only by using the biolistics system (Veluthamdi et al., 2003). On the other hand, multiple copies of transgene insertions, rearrangement of the transformed genes in the genome and insertion of undesired vector backbone are the main disadvantages of this technique (Bhalla et al., 2006). However, it was shown that with the usage of minimal cassette during bombardment, all these drawbacks could be eliminated (Fu et al., 2000).

Though *Agrobacterium*-mediated gene transformation does not seem to have these drawbacks, vector backbone co-transfer with *Agrobacteriu*m was reported (Mc-Cormac et al. 2001; Popelka and Altpeter 2003). Until recent years, *Agrobacterium*-mediated transformation could not have been used in major cereal crops since the system

naturally has the ability of infecting only dicot species. However, with the modifications in this system, it is now possible to use this system also in monocots. Integration of helper plasmids and the use of supervirulent strains for additional virulence genes, addition of phenolic compounds like acetosyringone to induce the virulence, use of vacuum infiltration, elimination of bacteria after co-cultivation and carrying out the co-culture conditions at lower temperatures are major parameters integrated into the system to make *Agrobacterium*-mediated transformation possible in monocots and increase its frequency of transformation efficiency (Veluthamdi et al., 2003; Bhalla et al., 2006; Shrawat and Lörz, 2006). However, not all drawbacks of *Agrobacterium*-mediated transformation of monocots are solved though there are many successful studies done on the transformation of several monocots using microprojectile bombardment method or *Agrobacterium*-mediated transformation (reviewed by Repellin et al., 2001; Cheng et al., 2004).

2.2.2.1 Microprojectile bombardment method

The biolistic process is primarily a mechanism for breaching cell walls and cell membranes, which are the principal barriers for DNA delivery. This process is also named as; The Microprojectile Bombardment Method, The Gene Gun Method, The Particle Gun Method, etc. The term "biolistics" comes from biological ballistics since DNA is being "shot" into cells (Sanford, 1990). It is one of the direct gene transfer methods that DNA coated microcarriers (microprojectiles) such as tungsten or gold particles are accelerated into target cells at high velocities.

Microprojectile bombardment method was first described by Sanford et al. (1987). It was then used in transformation of onion epidermal cells to show the integration of foreign DNA into plant cells with this method (Klein et al., 1987). After that, this technique was used for the transformation of yeast and filamentous fungi (Armaleo et al., 1990), algae (Mayfield and Kindle, 1990), crops (McCabe et al., 1988; Fromm et al., 1990; Gordon-Kamm et al., 1990), chloroplasts (Boynton et al., 1988), mitochondria (Johnston et al. 1988), edible mushrooms (Sunagawa and Magae 2002) and human cell lines (Zhang et al. 2002). From plants; embryos, meristems, suspension culture cells, callus tissue, nodes, pollen grains, young leaf discs, microspores and various plant parts

could be the target tissues of this method (Altpeter et al., 2005). Many different species has been transformed with this technique which was even used to introduce plant viruses into plant species (Hoffman et al., 2001) and to enhance virus induced gene silencing (VIGS) in important crops (Fofana et al., 2004).

There are different types of biolistic devices and one of them is the helium-driven biolistic device, PDS-1000/He. It works on the forces of vacuum and pressure. Compressed helium gas is used as the propellant and the vacuum supplied in the chamber maintains the acceleration of microcarriers by eliminating the air friction. The DNA coated microcarriers are loaded onto macrocarriers which are then placed into the macrocarrier launch assembly inside the metal discs. The target cells or tissues in the petriplates are placed to a desired distance below the macrocarrier launch assembly. For every bombardment pressure, proper rupture dics is used which is ruptured at the desired pressure and afterwards a shock wave is exerted onto the macrocarriers. A stopping screen (wire mesh) holds the macrocarrier from flying but enables microcarriers to spread onto the target cells (Figure 2.3). The target distance determines the spread of the particles pattern over the target (Yang and Christou, 1994).

The gene transfer efficiency by microprojectile bombardment method is related not only to the optimization of the chemical and physical parameters like vacuum, bombardment pressure and distance, microprojectile choice, but also to biological parameters such as plant type, choice of explant, cell age and physiology, osmotic pressure and regeneration capacity of that tissue which are main components of all gene delivery systems (Altpeter et al., 1996; Racso-Gaunt et al., 1999; Taylor and Fauquet, 2002). Also, the design of the vector is important. Structure of the vector is not a limited factor but the choice of reporter gene and the promoter is necessary. Therefore, higher transformation efficiencies are accomplished by optimizing each component.

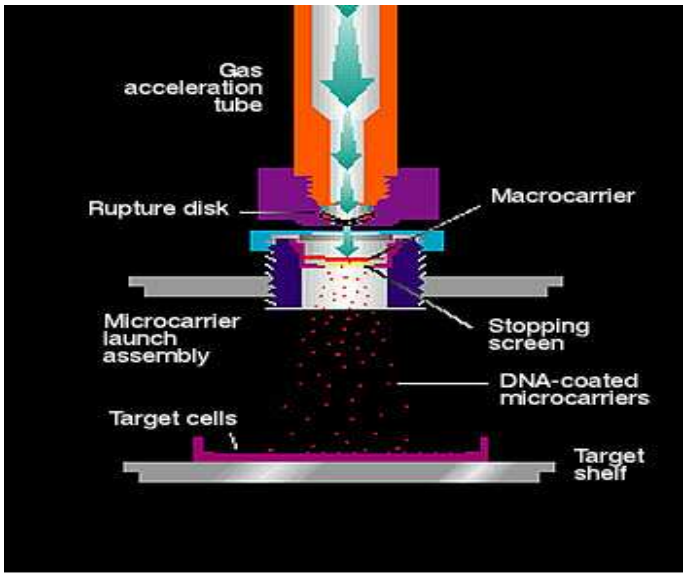


Figure 2.3 The microprojectile acceleration assembly (Bio-Rad®). (www.biorad.com)

2.2.2.2 *Agrobacterium*-mediated transformation

The initial studies on *Agrobacterium* and wheat were aided by the phenomenon of agroinfection, which for the first time demonstrated that the soil bacterium could interact in a limited manner with the cells of a monocot plant (Woolston et al., 1988; Dale et al., 1989). First transgenic monocot plants were obtained from rice immature embryos infected with *Agrobacterium* (Chan et al., 1993). Agroinfection is the introduction of viral DNA residing between the T-DNA border sequences of *Agrobacterium* into plant cells and provides a suitable system to monitor DNA delivery into target explants by detection of disease symptoms.

The genus *Agrobacterium* has been classified into a number of species. *A. radiobacter* is an 'avirulent' species, *A.rhizogenes* causes hairy root disease, *A. vitis* causes galls on grape and a few other plant species and *A. tumefaciens* causes crown gall disease. The most widely used species in plant transformation is *A. tumefaciens* since many species can be its host like many dicot and monocot plants and fungi. It is a natural soil pathogenic bacterium and causes grown gall disease by infection of the plant at wounded sites.

Transfer of the T-DNA (transfer DNA), from a large tumor-inducing (Ti) plasmid of the bacterium to the plant cell causes the crown gall disease. This T-DNA integrates itself into the plant genome where it is expressed (Fig. 2.4). The Ti plasmid contains two elements which are required for T-DNA transfer to plants. The first element is the T-DNA border sequences that consist of 25 bp imperfect repeats (right border-RB and left border-LB) defining the boundaries of T-DNA. The second group of elements is the virulence (*vir*) genes outside the T-DNA region. The *vir* genes encode a set of proteins responsible for the excision, transfer and integration of the T-DNA into the plant genome (Stanton, 2003; Zupan et al., 2000; Sheng and Citovsky, 1993). This naturally occurring transfer mechanism have been used to establish a plant transformation system and DNA vectors were designed from the Ti plasmid DNA to transfer desired genes into the plant genome. None of the T-DNA sequences is required for transfer and integration besides the border repeats meaning that the T-DNA genes can be replaced by any desired gene with varying lengths (from few to 150 kb) and can be transferred into the plant genome (Hamilton et al., 1995). Besides, T-DNA and *vir* genes do not have to be in the same plasmid for transfer of T-DNA. This information leads to the construction of a binary vector system for which two plasmids are used (Hoekema et al, 1983). One of them is the Ti plasmid containing the *vir* genes with oncogenes elimination. Naturally the T-DNA includes genes for tumor formation and opine (octopine and nopaline) and plant hormone (auxin and cytokinin) biosynthesis. If it loses its oncogenic property, it is named as "disarmed" plasmid or "*vir* helper" plasmid. The other one is the genetically engineered T-DNA plasmid where desired genes are inserted. The plasmids in T-DNA binary vectors are smaller than plasmids in *Agrobacterium* so that they can be easily manipulated in both *E. coli* and *Agrobacterium* (Klee, 2000).

At approximately 35 *vir* genes together create the 30 kb *vir* region harboring at least six operons involved in T-DNA transfer (Klee, 2000; Zupan and Zambrysky, 1995). With the induction of plant phenolic compounds due to wounding, *vir*A and *vir*G are expressed and induce the expression of other *vir* genes. Expression of *vir* genes induces the formation of single-stranded T-DNA copy (T-strand) which is then transported into the host cell. The *vir*D and *vir*E, alone with T-strand form the T-complex, is transferred to plant cells by *vir*B and other genes (Zupan et al., 2000). In monocot systems, *Agrobacteriu*m-mediated plant transformation was made possible with the use of super

virulent strains. These super-virulent vector systems were constructed by modification of the Ti plasmid. Addition of extra copies of *vir* B, *vir* C and *vir* G genes increased the virulence of the *Agrobacterium* strains. Supplementation of growth media with phenolic compounds and other modifications in the transformation protocols enabled *Agrobacteriu*m-mediated transformation possible in monocot plant systems (Repellin et al, 2001).

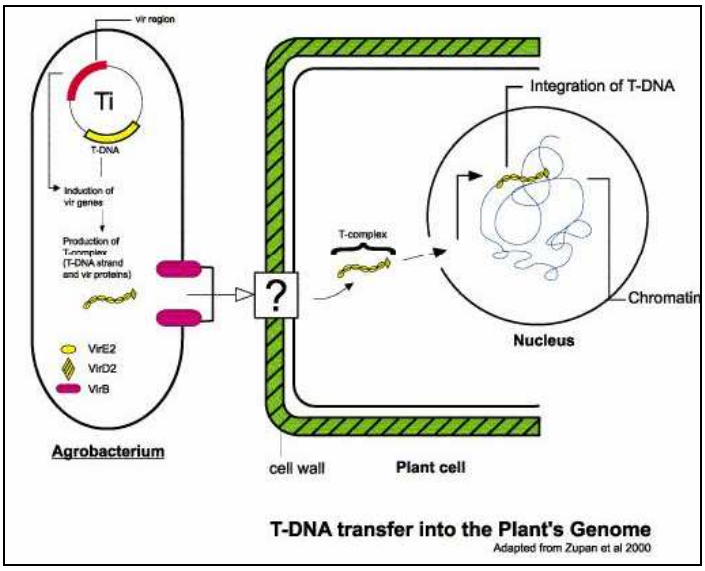


Figure 2.4 T-DNA transfer from *Agrobacterium* to the plant genome. (www.cambiaip.org)

In *Agrobacterium*-mediated transformation, many wounding types were applied in different plant species. Cutting with a scalpel blade (Mooney et al., 1991) or wounding with a needle (Xu et al., 2006), crushing explants prior to inoculation (Norelli et al., 1996), shaking with glass beads (Grayburn and Vick, 1995), application of sonication (Trick and Finer, 1997), pectolytic enzyme treatment (Alibert et al., 1999), carborundum (Çürük et al., 2005) or microprojectile bombardment mediated (Bindley et al., 1992) wounding were used to increase the transformation efficiency of the target plants. Due to the plant type and wounding application, different transformation efficiencies were achieved and results showed that not all wounding types were suitable for every species or explant type.

2.2.3 Gene transfer to *Brachypodium distachyon*: *Agrobacterium*- vs. biolistics

The efficiency of transformation using either system, *Agrobacterium*-mediated transformation or the microprojectile bombardment method, is mainly affected by the genotype, explant type and age, culture conditions, choice of promoter type and the type of selectable markers used. So, the tissue culture conditions before and after transformation and optimization of the parameters of the transformation system are crucial for the success of transformation.

Optimization and a better understanding of gene delivery and tissue culture system in this model grass species are needed for genetics and genomics studies. For several cereal crops, the success of tissue culture response mainly depends on genotype (Powell and Caligari, 1987; Zamani et al., 2003; Hoque and Mansfield, 2004; Zale et al., 2004), medium composition (Powell and Caligari, 1987; Saharan et al., 2004), growth conditions and other environmental factors (Lazar et al., 1984; Caswell et al., 2000; Delporte et al., 2001).

2.2.3.1 Source of explant type for *Brachypodium* transformation

Embryogenic callus, due to its higher regeneration capacity, has a great influence on the transformation efficiency. Potential production of embryogenic callus differs according to the species, cultivars of that species (Vogel et al., 2006a) and the source of explant and media composition used for callus initiation. For *Brachypodium* species, both mature (Bablak et al., 1995; Vogel et al., 2006a) and immature embryos (Bablak et al., 1995; Draper et al., 2001; Christiansen et al., 2005; Vogel et al., 2006a; Pacurar et al., 2007; Vogel and Hill, 2007; Vain et al., 2008) were used to obtain embryogenic callus formation according to the conditions described in Bablak et al. (1995). Tetraploid accessions produced callus at higher percentages compared to diploid ones. Meanwhile, immature embryos were regenerated more efficiently with respect to mature ones in overall scheme (Vogel et al., 2006a). Though immature embryos have good regeneration capacities, mature embryos are available explant sources throughout the year that it speeds up the procedures in genetic transformation studies (Özgen et al., 1998).

2.2.3.2 Transformation of *Brachypodium via* microprojectile bombardment method

Brachypodium genetic transformation was firstly achieved with the study of Draper et al. (2001). Calli were formed using immature embryos of different sizes with two different media composition that were described in the study of Bablak et al. (1995). The immature embryos (ABR1) in the size of 0.3-0.7 produced embryogenic callus at higher percentages (up to 54%). ABR100 accession was bombarded and GUS activity was detected. Hygromycin-resistant plants had the average transformation frequency of 5 plants per gram tissue.

Two diploid and two tetraploid accessions were selected and embryogenic callus formation up to 46% in diploid (BDR001 & BDR018) and 91% in tetraploid (BDR017 & BDR030) accessions was observed from immature embryos (Christiansen et al., 2005). These tissues were bombarded and transformation efficiencies of 9-14% for single bombardments were recorded with higher values achieved from tetraploids. However, for all the lines tested, the numbers of BASTA-resistant plants were almost at the same range. When they compared the number of transient GUS-positive results, the number of blue spots more than 1000 per bombardment exhibited significantly higher number of independent transgenic lines. The average transformation efficiency was 5.3%. In one year time, they had tested T_0 and T_1 generations and produced T_2 seeds due to short life cycle of *Brachypodium* species, implying the importance of *Brachypodium* as a model species.

2.2.3.3 *Agrobacterium*-mediated transformation of *Brachypodium*

Immature embryos of BDR018 were used to optimize a transformation protocol and to produce BASTA-resistant transgenic plants by using the *Agrobacterium tumefaciens* strain AGL1 with the vector pDM805 (Pacurar et al., 2007). They showed the effect of pre-culture period on stable transformation and frequencies up to 80% and 30% insertion of single gene copy were recorded. Transient expression of the reporter GUS gene and stable transformation frequency displayed no correlation in between. On the basis of generated protocol, *Brachypodium* lines were transformed with the vector pWBV-Ds-Ubi-bar-Ds (Zhao et al., 2006) including the selection genes *bar* and *hpt* to

analyze the protocol for full-length integration rate and the influence of selection which was 97% of the transformants when only bialaphos was used in selection procedure. They proposed that T-DNA tagging of diploid *Brachypodium* was possible since the transformation frequency in their study was high enough to achieve in large quantities of transformed lines.

Inbred lines of *Brachypodium distachyon* from 27 accessions were developed by applying single seed descent for three or more generations and five of them were found to be diploid that presented different morphological characteristics such as in vernalization requirement (Vogel et al., 2006a). Bd21 was found to have the fastest generation time when compared to others and became a model inbred line. The ability to manipulate flowering of some lines was found out to serve us to obtain either biomass or seed production which is a good argument for the use of this model species also for the study of forage and turf grasses. The embryogenic callus formation varied greatly among the genotypes and also the regeneration percentages were in varying rate, being affected both by the genotype and the explant type used for callus induction. No correlation was observed between callus initiation and regeneration percentages. Besides, albino shoot formation was detected. Albino shoot formation frequencies increased with the use of mature embryos as explant source. The 10 of the 19 lines were transformed with 0.4-15% range of efficiencies using both mature and immature embryos *via Agrobacterium*-mediated transformation method. Bd17-2, hexaploid accession, had the highest average transformation efficiency of 13.6% and callus initiation was high both using immature and mature embryos. Super-virulent strain AGL1 was used with three DNA constructs conferring hygromycin resistance (Lazo et al., 1991). Most of the T_1 generations contained single gene insertions.

Agrobacterium-mediated transformation of the inbred line Bd21-3 was performed (Vogel and Hill, 2007) using AGL1 with seven DNA constructs (Lazo et al., 1991) and hygromycin selection since Bd21 exhibited poor development in tissue culture with low quality callus and mainly albino shoot formation. Bd21-3 embryos which were smaller than 0.7 mm were highly efficient in callus induction. Immature embryos were transformed with transformation efficiency ranging from 10-41%. One of the modifications was to perform the co-cultivation under desiccating conditions. This step and proper initiation of the embryogenic callus highly improved transformation

efficiency with ~15 fold. Since promoter choice and vector design is an important factor in transformation studies, some of the parameters were tested in this study and found out that rice actin promoter with 5' intron was less efficient than 35S promoter with an *hpt* gene containing an intron, and 35S promoter without an intron or dicot-specific 35S promoters could be used safely in *Brachypodium* species.

The inbred line Bd21 was transformed using *Agrobacterium*-mediated gene transfer system and immature embryos as the source of compact embryogenic callus formation by applying hygromycin selection for T-DNA insertional mutagenesis (Vain et al., 2008). The transformation efficiency was reported to be 17% in this study. GFP expression was observed in both T_0 and T_1 progenies. Optimization of duration of selection process, use of $CuSO_4$ in culture media of the plants and desiccation of callus after inoculation were tested for the improvements of the transformation system. They have performed flanking sequence tags (FSTs) of T-DNA inserts and are available publicly. T-DNA insertions and FSTs produced in this study well define the arguments about the use of model species and transgenic technology together with the functional genomics studies.

CHAPTER 3

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals, growth media, plant growth regulators, antibiotics and enzymes

The chemicals, growth media, plant growth regulators, antibiotics and enzymes used in this research are listed in Appendix A.

3.1.2 Buffers and solutions

All buffers and solutions used in this research were prepared according to the protocols as outlined in Sambrook et al., 2001.

3.1.3 Molecular biology kits

All molecular biology kits and biolistic particle delivery system kits used in this research are listed in Appendix B.

3.1.4 Equipments

Equipments used in this research are listed in Appendix C.

3.2 Methods

3.2.1 Plant materials and inbred line production

A total of 146 inbred lines (116 freely available) were created from 1101 *Brachypodium* individuals representing diverse geographic regions of Turkey (Appendix D). Each line was put through five generations of single seed descent and plants were covered during anthesis to prevent cross pollination in case it persisted. In addition to the inbred lines we created, we used previously developed *Brachypodium* inbred lines Bd21 (Vogel et al. 2006a).

3.2.2 Growth conditions of *Brachypodium* plants

Seeds were stratified at 4 ºC for 7-10 days in dark between moist filter papers in petri plates. After cold treatment, they were kept under light at room temperature for 5-7 days. Following germination, they were transferred to peat-soil mixture in the viols. After the seedlings were established, they were transplanted into plastic pots (no. 6) containing 1.7 kg soil (from Sultanönü-Eskişehir) and grown under controlled environment (16/8 h light/dark photoperiod at 25/22 °C, relative humidity 60-70%, and a photosynthetic photon flux of 320 μmol m^{-2} s^{-1} at canopy height provided by fluorescent lamps) in the greenhouse (Özdemir and Budak, 2008; Filiz et al., 2009). For basal fertilization, the growth media was treated with 200 mg kg^{-1} N ($Ca(NO_3)_2$), 100 mg kg^{-1} P (KH_2PO_4), 20 mg kg^{-1} S (K_2SO_4), 5 mg kg^{-1} Fe (Fe-EDTA) and 2.5 mg kg^{-1} Zn ($ZnSO_4$).

3.2.3 Morphologic and cytological characterization

3.2.3.1 Phenotypic evaluation

Phenotypic characterization was conducted in the greenhouse during spring-summer seasons from 2006-2008. We determined anthesis date, seed size and yield, germination percentage, plant height, leaf characteristics, plant stature, dry stem weight and vernalization requirement. The inbred lines were under 13 phenotypic groups. For the biomass calculation, the dry weight of each plant was recorded following drying of the green parts in incubator at 70 ºC for 2-3 days.

3.2.3.2 Flow cytometry analysis

Flow cytometry analysis was performed as described by Arumuganathan and Earle (1991) to identify the ploidy levels of the accessions used in this study. Briefly, mean DNA content was based on analysis of 1,000 nuclei. Each genotype was analyzed by four separate extractions and flow cytometric runs (Budak et al. 2004; Filiz et al. 2009). Base pair composition calculations were done as outlined by Godelle et al. (1993).

3.2.3.3 Cytological analysis

Seeds were germinated between two moistened filter papers. The seeds were kept in 4 ºC for the first 10 days. Then, they were incubated at 23 ºC in the dark until germination. Root tips were collected when roots were 1-1.5 cm in length and then pre-treated with cold water. The water was discarded after 16 hours and replaced by farmer solution which is 3:1 (v/v) ethanol: acetic acid. Root tips were then stained in %2 acetocarmine dye for 3 hours. Preparations were made by squashing root tips onto microslides.

3.2.4 Macro- and microelement analysis

Macro- and micronutrient element concentration (K, P, S, N, Mg, Ca, Fe, Cu, Mn, Al and Zn) in mature *Brachypodium* seeds were measured for 11 different genotypes.

Three inbred lines were selected for each genotype and experiments were performed with two repeats per line. Palea and lemma of 7000-8000 seeds were removed manually for the analysis.

For macro- and microelement analysis, approximately 0.2 g seeds were weighed for each repeat of *Brachypodium* line. The concentration of macro- and microelements in seeds was measured after digesting them in a microwave (CEM-MARS Xpress system) in 5 ml 65% (w/v) HNO_3 and 2 ml 30% (w/v) H_2O_2 for 50 min. After complete digestion and dilution with distilled water, the concentration of nutrients was measured by inductively coupled plasma optical emission spectrometer (ICP-OES) (Eker et al., 2006). Wheat flour was used as the standard. For analysis of nitrogen (N) concentration, approximately 0.3 g seeds were weighed for each repeat of *Brachypodium* line and then they were covered with tin foils. The nitrogen concentration was measured using LECO CN analyzer. Protein content was calculated using the nitrogen (N) concentration.

3.2.5 Tissue culture conditions of *Brachypodium* plants

3.2.5.1 Seed surface sterilization

After manual removal of the palea and lemma, mature seeds of *B. distachyon* were surface sterilized with 70% (v/v) ethanol for 5 min, washed 3 times with sterile distilled water, treated with commercial bleach (53% NaOCl) for 20 min and rinsed 3-5 times with sterile distilled water. Seeds of Bd21 line were surface sterilized in 15% bleach plus 0.1% triton X-100 for 30 min according to Vogel et al. 2006a.

3.2.5.2 Explant preparation

As the explant source; stem (mesocotyl tissue), root tip, leaf segment and mature embryos of *B. distachyon* were used for the preliminary optimization of callus initiation. For explant preparation from stem, leaf and root parts, sterile seeds were kept in sterile distilled water for 1 hr at room temperature. Then the seeds were directly placed in sterile magenta boxes (5 seeds/box) containing 4.43 g/L MS basal salt medium (Murashige and Skoog, 1962) including vitamins, 20 g/L sucrose and 8 g/L plant agar at

pH 6.0 and they were incubated under a 16/8-h (light/dark) photoperiod at 25 °C ± 1 until plantlets reached the height of 5-6 cm. Following the seedling formation, essential root, stem and leaf parts were removed under microscope. On the other hand, mature embryos were aseptically excised after imbibition of surface sterilized seeds in sterile distilled water for 1-2 hrs at 33 °C in water bath. All explants were put on callus induction media. Seeds were also directly put onto the callus induction media to observe if efficient callus formation might have existed so that the embryo excision step could be removed. When the calli could be handled, they were removed from the seeds and transferred to fresh callus induction media.

3.2.5.3 Callus induction and regeneration

For callus induction of all explants two different carbohydrate sources (maltose or sucrose) at an amount of 30 g/L, three levels of auxin (2,4-D; 1 mg/L, 3 mg/L and 5 mg/L), and two levels of cytokinin (BAP; 0.0 mg/L and 0.5 mg/L) or different auxin hormones (2.5 mg/L 2.4-D or IAA or NAA) were applied, all including 8 g/L plant agar and 4.43 g/L MS basalt salt medium with vitamins at pH 6.0. The experiments were designed as in Table 3.1. The cultures were kept in the dark at 25 °C ± 1 for callus initiation and subcultured to fresh media every 3-4 weeks.

Table 3.1 Types and concentrations of plant growth regulators and carbohydrate sources used in callus initiation of *Brachypodium distachyon*.

Growth regulator type	Concentration (mg/L)	Carbohydrate source (30 g/L)
2,4-D	1.0	sucrose
2,4-D / BAP	1.0 / 0.5	sucrose
2,4-D	3.0	sucrose
2,4-D / BAP	3.0 / 0.5	sucrose
2,4-D	5.0	sucrose
2,4-D / BAP	5.0 / 0.5	sucrose
IAA	2.5	sucrose
NAA	2.5	sucrose
2,4-D	2.5	sucrose
2,4-D	2.5	maltose

Since preliminary experiments showed that efficient callus formation was mostly obtained from mature embryos, we used mature embryos in subsequent experiments to compare three different *Brachypodium distachyon* genotypes, BdTR4, BdTR6 and BdTR13. Callus induction media with different carbohydrate source and 2,4-D concentration were used to compare these three genotypes. The composition of the media was as follows: Two different carbohydrate sources (maltose or sucrose) at an amount of 30 g/L, three levels of (2,4-D; 1 mg/L, 3 mg/L and 5 mg/L) all including 8 g/L plant agar and 4.43 g/L MS basalt salt medium with vitamins at pH 6.0. Each experiment was set with four replicates (20 embryos/replicate). The petri plates were kept in the dark at 25 °C ± 1 for callus initiation and subcultured to fresh media every 3-4 weeks. Embryogenic calli were selected and broken into pieces before transferring to new media. After 2 months, the calli were put onto regeneration media in sterile magenta boxes containing 4.43 g/L MS basal salt medium including vitamins, 30 g/L sucrose and 8 g/L plant agar at pH 6.0 and they were incubated under a 16/8-h (light/dark) photoperiod at 25 °C ± 1. In order to make comparison with our results, previously optimized Bd21 line was used in all our experiments. Callus formation of Bd21 lines from excised mature embryos and regeneration were carried out according to the procedure used by Vogel et al. 2006a. Callus induction medium contained LS salts (Linsmaier and Skoog, 1965) plus 3% sucrose, 11.25 µM 2,4-D and 0.2% phytagel whereas regeneration medium included LS salts plus 3% maltose, 0.93 µM kinetin and 0.2% phytagel. Also, we used MS medium with 3mg/L 2,4-D for callus initiation and MS medium without kinetin for regeneration.

3.2.6 Genetic transformation of *Brachypodium* with Biolistic® PDS-1000/He device

3.2.6.1 Plant materials

Brachypodium distachyon genotypes, BdTR4, BdTR6, BdTR13 and Bd21, were used for the bombardment experiments. Six weeks-old embryogenic calli were arranged in a circle of 2.5 cm-diameter at the center of the petri plates containing callus induction medium (4.43 g/L MS basal salt medium including vitamins, 30 g/L sucrose, 8 g/L plant agar, with optimized 2,4-D concentration according to the genotype used- the concentrations range from1 to 5 mg/L, pH 6.0) prior to bombardment and incubated

under same conditions for 1 day until the bombardment process started (Figure 3.1 a, b). Common bean (*Phaseolus vulgaris* L.*)* cotyledons were used as control explants for the system control. Bean seeds were surface sterilized with 70% (v/v) ethanol for 3 minutes, washed 2 times with sterile distilled water, treated with commercial bleach (53% NaOCl) for 15 minutes and rinsed 3 times with sterile distilled water, and then, placed in regeneration media under light at 25 °C ± 1. The cotyledons could be excised after ten days when the seed coats were removed and placed abaxial side up within a 2.5 cm circle in the center of the petri plates.

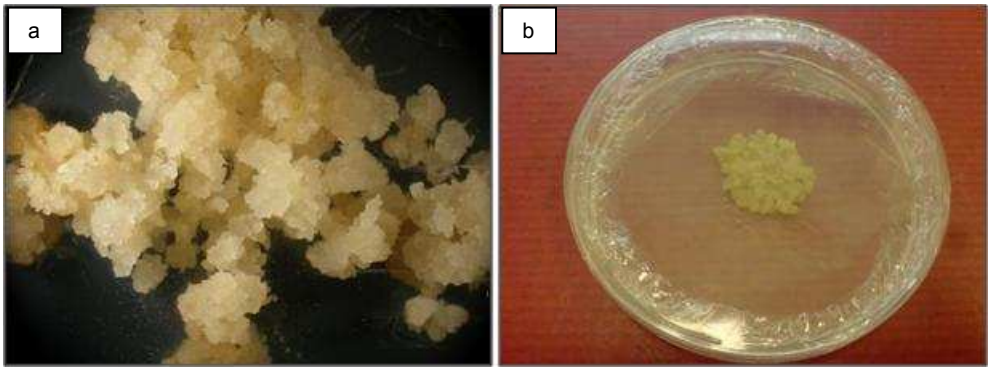


Figure 3.1 (a) Callus formation from mature embryos of *Brachypodium distachyon*, and **(b)** arrangement of these calli in the middle of petri plates prior to bombardment.

3.2.6.2 DNA material

The plasmid pCAMBIA1301 (CAMBIA, Canberra, Australia) containing the *β*–glucuronidase uidA (GUS) reporter gene and the selectable marker hygromycin phosphotransferase (*hpt*) gene that gives resistance to the antibiotic hygromycin, was used for this study (Appendix E-1). Both GUS and *hpt* genes were driven by a Cauliflower mosaic Virus '35S' (CaMV35S) promoter.

3.2.6.3 Plasmid preparation

3.2.6.3.1 Chemically competent cell preparation

DH5α strain of *E. coli* was used for transformation. First of all, chemically competent cells were prepared. Cells were plated on LB agar and grown overnight in a shaking incubator at 37 °C with 250 rpm. Single colony was inoculated in 5 ml LB and again grown overnight. 1 ml of this culture was inoculated into 50 ml LB and grown until OD_{600} reached 0.3-0.4. The culture was centrifuged at 6000 rpm for 15 min, resuspended in 25 ml ice-cold 0.1 M Ca Cl_2 and kept on ice for 15 min. The suspension was centrifuged again at 6000 rpm for 15 min, resuspended in 3.3 ml (1/15 vol) 0.1 M Ca Cl_2-15% glycerol and kept on ice overnight. They were freezed as aliquots in liquid nitrogen and stored at -80 °C.

3.2.6.3.2 Plasmid transformation

For transformation of the plasmid vector, 1 µl of vector and 200 µl of DH5α competent cells (thawed on ice) were mixed briefly, kept 20-30 min on ice, then put at 42 °C for 90 seconds, and lastly kept on ice for 1-2 min. Then 800 µl LB medium was added to transformed cells and put at 37 °C for 45 min. From culture, 50 µl was spread over LB agar plates supplemented with 50 mg/L Kanamycin antibiotic. The plates were kept at 37 °C overnight and single colonies were inoculated in 5 ml LB media with antibiotic. The liquid culture was grown overnight at 37 °C in a shaker and 800 µl culture was mixed with 200 µl sterile 87% glycerol. The stock cultures were freezed as aliquots in liquid nitrogen and stored at -80 °C.

3.2.6.3.3 Plasmid isolation

Plasmid was isolated from overnight grown culture using QIAprep® Spin Miniprep Kit (Qiagen) and Genopure® Plasmid Midi Kit (Roche) and the procedures were carried out according to manufacturer's instructions. Isolated plasmids were checked by enzyme digestion and the concentrations were determined using the NanoDrop Spectrophotometer. 1 µg plasmid was digested with Xho I enzyme (Fermentas) at 37 C

for 2-3 hrs and the fragments were checked by agarose (1%) gel electrophoresis (Appendix E-1).

3.2.6.4 Microprojectile bombardment

3.2.6.4.1 Coating gold particles with DNA

Two different sizes of gold particles (1.0 μm and 1.6 μm in average diameter) were used as microcarriers. For every 4-5 shots, 50 μl gold suspension was used. The required amount of gold particles was weighed and 1 ml of 100% ethanol for 60 mg of gold was added, vortexed for 1-2 minutes, centrifuged at 10000 rpm at 4°C for 1 minute and the supernatant was removed. These steps were repeated for three times. Then ethanol was replaced with 1 ml of sterile distilled water and rinsed with sterile distilled water two times by vortexing for 1-2 minutes, centrifugating at 10000 rpm at 4°C for 1 minute. The pellet was resuspended in 1 ml of 50% sterile glycerol.

For every 50 μl gold suspension, 6 μl DNA (1μg/1μl), 50 μl of 2.5 M $CaCl_2$ and 20 μl of 0.1 M spermidine were added in this order with continuous vortexing and centrifuged at 10000 rpm for 10 seconds and the supernatant was discarded. The pellet was then washed in 250 μl of 100% ethanol, centrifuged at 10000 rpm for 10 seconds, resuspended in 60 μl of 100% ethanol and kept at 4°C until bombardment.

3.2.6.4.2 The bombardment process

A Bio-Rad Biolistic® PDS-1000/He particle delivery system was used (Figure 3.2 a). Calli and cotyledons were bombarded under a partial vacuum of 27" Hg pressure with three different bombardment pressures (650, 900 and 1100 psi rupture disks) and two sample plate distances (6 and 9 cm).

Before each bombardment**,** vacuum chamber, components (stopping plate, sample plate and sample chamber) of the biolistic device, stopping screens, rupture disks, macroprojectiles and its holders were sterilized with the proper chemical (Figure 3.2 b-

e). Each macroprojectile was loaded with 8 µl of suspension and allowed to dry under vacuum (Figure 3.2 f). All steps of bombardment process were carried out as described in manufacturer's protocol.

Figure 3.2 (a) Bio-Rad Biolistic® PDS-1000/He particle delivery system and **(b)** its components **(c)** disk retaining cap, macrocarrier launch assembly **(d)** rupture disks **(e)** stopping screens, macroprojectiles and its holders **(f)** DNA-gold loaded macroprojectiles shown.

3.2.6.5 Assay of transient gene expression

Transient expression of the GUS gene was detected by "Histochemical GUS Staining" method (Jefferson, 1987). The GUS gene products (*β*–Glucuronidase enzyme) produced blue dyes (blue spots) at the site of enzyme activity when the embryos supplied with the chromogenic substrate 'X-gluc' (5,bromo-4,chloro-3,indolyl *β*–D-glucorinic acid) in the presence of atmospheric oxygen. X-gluc solution was prepared as follows: 1 ml (0.1 M) EDTA, 1 ml (5 mM) potassium ferricyanide, 1 ml (5 mM) potassium ferrocyanide, 5.0 ml sodium phosphate buffer (%61 0.1 M Na_2HPO_4, %39 0.1 M NaH_2PO_4 at pH 7.0), 100 µl (0.1 %) Triton X-100, 0.3 mg/ml X-gluc (dissolved in DMF until transparent) and sterile distilled water added up to 10 ml.

After bombardment, the plant materials were incubated for 48 hours at 25°C in dark. Then, half the number of embryos were put into X-gluc solution for the GUS assay and half of them were cultured in callus induction medium. X-gluc solution was added into the tubes until it covered the surface of the explants which were then incubated at 37 °C in darkness for at 24-48 hours. After incubation, explants were kept in absolute ethanol or in GUS fixative solution (10% formaldehyde, 20% ethanol and 5% acetic acid) and the GUS expression was recorded by counting the blue spots (putative transformed cells or cell aggregates) under microscope.

3.2.6.6 Selection and regeneration of the putative transformants

After bombardment, the calli that were not subjected to assay were kept in callus induction medium at dark for further 4-5 days. For the selection of the transformed cells, the calli were transferred to callus induction medium supplemented with 15mg/L hygromycin antibiotic under same conditions. For shoot and root initiation, calli that showed resistance to the hygromycin-B antibiotic were transferred to regeneration medium supplemented with 30mg/L hygromycin and antibiotic selection was applied for 2 months. The resistant plantlets were transferred to magenta boxes containing regeneration media without antibiotic.

The regenerated plants that exhibited well developed root and shoot formation were taken out of the media; the roots were rinsed under tap water and then transferred to peat-soil mixture in pots. However, the plants were kept at 70-90% humidity for 10 days and then they were grown in normal conditions. Plants were regenerated and grown in growth room under a 16/8-h (light/dark) photoperiod at 25 °C ± 1.T_0 and T_1 hygromycin resistant lines were recovered.

3.2.6.7 Molecular analysis of the putative transgenic plants

Genomic DNA was isolated from fresh leaves of the regenerated resistant plants and non-transformed control plants according to the protocol of Doyle and Doyle 1987 with some modifications or by Wizard® Genomic DNA purification kit (Promega). For genomic DNA extraction using CTAB isolation; after homogenization of the leaf samples with CTAB buffer 25 µl (10 mg/ml), proteinase K was added. At the end of DNA isolation, genomic DNA was treated with 5 µl (10 mg/ml) RNase at 37 °C for 30 min. Then DNA was precipitated by adding 2X volume of 100% ethanol 0.1X volume of 2M NaCl onto 1X volume of DNA After incubation of the mixture at -80 °C for 30 min, it was centrifuged at 14000 rpm for 10 min and pellet was washed with 80% ethanol and centrifuged again at 14000 rpm for 10 min. Pellet was dried and resuspended in TE or sterile distilled water. The DNA concentrations were determined using the NanoDrop Spectrophotometer and checked by gel electrophoresis.

PCR amplification was carried out to detect approximately 195 bp region of the CaMV35S promoter by using the primers CaMV35SF (5' GCTCCTACAAATGCCATCA3'), CaMV35SR (5'GATAGTGGGATTGTGCGTCA3') under the following conditions: initial denaturation at 94°C for 5 min; 35 cycles at 94°C for 1 min, 55°C for 45 sec and 72°C for 1 min; and final extension at 72°C for 10 min. PCR reaction was carried out in a final volume of 25 µl consisting of 100 ng of the genomic DNA (or 50 ng of plasmid DNA for the positive control), 0.15 mM dNTPs, 2.0 mM $MgCl_2$, 1X PCR Buffer, 0.4 µM of each primer and 0.025 units/µl of Taq DNA polymerase (Fermentas) as the final concentrations. PCR products were resolved by electrophoresis and visualized on 1% agarose gel stained with ethidium bromide.

3.2.7 *Agrobacterium*-mediated transformation of *Brachypodium*

3.2.7.1 Plant material

3.2.7.1.1 Preparation of explants

Six weeks-old embryogenic calli from mature embryos *Brachypodium distachyon* genotypes BdTR4, BdTR6, BdTR13 and Bd21 were used. Callus formation from BdTR4, BdTR6 and BdTR13 with the appropriate media (4.43 g/L MS basal salt medium including vitamins, 30 g/L sucrose, 8 g/L plant agar, with optimized 2,4-D concentration according to the genotype used- the concentrations range from 1 to 5 mg/L, pH 6.0), and from Bd21 (as outlined in section 3.2.5.3) was obtained for transformation with *Agrobacterium*.

3.2.7.1.2 Wounding of tissues

Callus tissues were wounded by two types of methods. One set of calli were wounded by classical method with a sterile blade (referred as "macro-wounding"), and microprojectile bombardment-mediated wounding (referred as "micro-wounding") was used for the other set. The tissues were wounded by bombardment with previously optimized pressure and distances at 650 psi-9 cm with gold particles of 1.0 μm in size and 1100 psi -6 cm using 1.6 μm sized gold particles. These two methods were compared to see the difference in transformation efficiency due to wounding type.

3.2.7.2 Plasmids and bacterial strains

Three different *Agrobacterium tumefaciens* strains AGL1, EHA105 (supervirulent strains) and LBA4404 were used for transformation of *Brachypodium distachyon*. These strains were chosen according to their transformation efficiency and frequency of usage in monocot systems (Nadolska-Orczyk et al., 2000 and Jones et al., 2005).

AGL1 strain carried the plasmids pAL154 and pAL156 (Appendix E-2). Plasmid pAL156, helper plasmid, contained the *GUS* gene as the reporter and the *bar* gene

coding for resistance to glufosinate ammonium based herbicides like PPT (phosphinothricin) for plant selection that were under the control of *ubi* (ubiquitin) promoter. Kanamycin and carbenicillin resistance genes were used for bacterial selection. EHA 105 and LBA4404 carried the plasmid pGUSINT (Appendix E-3) that contained the GUS gene under the control of CaMV35S promoter. For bacterial and plant selection, they had the *nptII* (neomycin phosphotransferase) gene for kanamycin resistance.

3.2.7.3 Growth of *Agrobacterium tumefaciens* strains

AGL1 was grown on MGL medium (5 g/L mannitol, 2.5 g/L yeast extract, 1 g/L glutamic acid, 0.1 g/L $MgSO_4.7H_2O$, 0.25 g/L KH_2PO_4, 0.25 g/L NaCl, 5 g/L bacto-tryptone, pH 7.0 and 1 µg/L filter sterile biotin might be added after autoclave) efficiently though could also be grown in YEB medium. When growing, the following antibiotics were needed to be used in the media: Kanamycin 100 mg/L and Carbenicillin 200 mg/L. EHA105 and LBA4404 were grown in YEB media (13.5 g/L nutrient broth, 1 g/L yeast extract, 5 g/L sucrose, 2mM $MgSO_4.7H_2O$ and pH 7.2) with supplementation of following combinations of antibiotics respectively: Kanamycin 100 mg/L and Rifampin 20 mg/L; Kanamycin 100 mg/L and Streptomycin 100 mg/L.

From glycerol stocks, the culture was streaked onto agar plates containing the proper antibiotics to get single colonies by incubating overnight. Single colony was inoculated into liquid medium with antibiotics and incubated at 28 ºC at 250 rpm. At all stages of growth of *Agrobacterium*, more than 24 hours and up to 48 hours was needed for the activation of bacterial cells.

3.2.7.4 *Agrobacterium*-mediated transformation

Inoculation and cocultivation steps were optimized according to the procedures applied in the grasses (Li et al., 2005; Luo et al, 2004; Jones et al., 2005; Pacurar et al., 2007; Vogel et al., 2006a and Vogel and Hill, 2007).

3.2.7.4.1 Induction of *vir* genes

When bacteria are in log phase, the culture was inoculated into YEB-MES medium (YEB medium containing at a final concentration of 10 mM MES [2-(N-Morpholino) ethanesulfonic acid], pH 5.6) with proper antibiotics and 20 µM of acetosyringone as final concentration (add freshly prepared and filter-sterile). However, AGL1 was inoculated into MGL medium with proper antibiotics and acetosyringone at a final concentration of 20 µM. Acetosyringone plays an important role as phenolic compound in the induction of virulence. When OD (Abs=600 nm) was between 0.6-0.8, the cells were collected by centrifugation at 3500 rpm for 15 min at 4 ºC. Then cells were resuspended in MMA medium (4.3 g/L MS basal salt medium, 20 g/L sucrose, 10 mM MES, pH 5.6) containing 200 µM acetosyringone and then incubated at 22 ºC for 1 hour or up to 2 hours in dark.

3.2.7.4.2 Inoculation and co-cultivation

After induction, bacteria were ready to infect plant cells. Plant cells were wounded with needle or by bombardment of the tissues with gold particles without coating of DNA on it under previously optimized conditions and suspended in bacterial suspension for 15-20 min at 22 ºC. The explants with the bacterial cells were placed into vacuum infiltrator for 45-60 minutes under 50 mm Hg, which might have increased the transformation efficiency. Then, the calli were placed on sterile blotting papers to remove the excess bacteria on the plants and co-cultivated in MMD medium (4.4 g/L MS basal salt medium, 10 mM MES, 30 g/L sucrose, 1 to 5 mg/L 2,4-D - according to the optimized need of the ecotype used, 8 g/L plant agar and at pH 5.6; filter-sterile, 100 mg/L ascorbic acid and 200 µM acetosyringone added after autoclave) for 2-3 days at dark.

3.2.7.4.3 Elimination of *Agrobacterium*

After co-cultivation period, calli were washed with MMA medium containing 500 mg/L Cefotaxime or 250 mg/L Augmentin for 40 minutes up to 2 hours. Then, the calli were placed on sterile blotting papers. If needed, the calli were rinsed few times with sterile

water before antibiotic washing step. After blotting of the explants on the filter paper, the explants should not be so dry or so wet. At least 3-4 days, they were kept at their normal callus induction medium to allow the expression of transformed genes. Since these supervirulent strains had high capacity to infect, *Agrobacterium* contamination could be observed and washing steps with antibiotic was repeated at these times before transfer of these calli to new media.

3.2.7.5 Assay of transient gene expression

Transient GUS expression was carried out as outlined in Section 3.2.6.5.

3.2.7.6 Selection and regeneration of the transformed calli

The other half of the explants that are not subjected to the assay were transferred to callus induction medium containing 3 mg/L PPT (phosphinothricin) for AGL1 or 50 mg/L Kanamycin for EHA105 and LBA4404 transformation and 250 mg/L Cefotaxime under same conditions for 2-3 weeks. Selection was carried out in regeneration medium (as described in Section 3.2.5.3) containing 4 mg/L PPT (phosphinothricin) for AGL1 or 100 mg/L Kanamycin for EHA105 and LBA4404 and 250 mg/L Cefotaxime under 16/8 light/dark at 25±1 °C. Lastly, the resistant plantlets will be put in regeneration media without antibiotic. The regenerated plants should then be transferred to soil but first 70-90% humidity for 10 days is necessary to be applied.

3.2.8 Statistical analysis

Analysis of the data collected from morphologic characterization, callus formation, plant regeneration and transient GUS expression were performed with two-way ANOVA using the GenStat® statistical analysis software and Student's t-test analysis. Macro- and microelement analysis were also statistically analyzed by GenStat® statistical analysis software.

CHAPTER 4

4 RESULTS

4.1 Turkish *Brachypodium dist*achyon collection

Brachypodium distachyon is a small grass with biological and genomic attributes necessary to serve as a model system for all grasses including small grains and grasses being developed as energy crops. In order to add natural variation to the toolkit available to plant biologists using *Brachypodium* as a model system, it is imperative to establish extensive well-characterized germplasm collections. For this reason, we created inbred lines using the diverse *Brachypodium* accessions sampled from Turkey, characterized them both morphologically and cytologically and then evaluated the variation within this collection.

4.1.1 Growth conditions and inbred line production

One thousand one hundred and one *Brachypodium* individuals sampled from 45 locations in Turkey (Figure 4.1a, b) were used for our analysis. From these accessions we created 146 inbred lines and named as “BdTR”. The inbred lines were placed into 13 phenotypic groups (BdTR1 to BdTR13) based on their morphological characteristics (Table 4.1) and each inbred line was represented by a unique letter followed by the group number such as “BdTR1a”.

These lines were grown under greenhouse conditions and morphological interpretation was performed (Figure 4.2a-f). The seeds were stratified at cold to achieve synchronous germination and then germinated under light. The seedlings were first planted into viols

for their roots to be fully developed. After that the plantlets were transplanted to soil pots. We observed that if the seedlings were planted directly to soil in pots, the viability decreased and plant development completed in a longer time than the normal period. Before anthesis, the seed heads were covered to prevent cross-pollination in case it persisted. Though *Brachypodium distachyon* is an obligate self-pollinator, it was discussed that occurrence of cross-pollination could have been possible (Christiansen *et al*., 2005).

4.1.2 Morphological characterization

There was considerable phenotypic variation within this collection (Appendix D and Figure 4.3). Plant height ranged from 25 to 52 cm with an average of 35 cm. Leaf color was rated as 1 (light green), 2 (green) and 3 (dark green) (Figure 4.4a). The degree of leaf hairiness was classified from 1 to 5 with 1 being almost hairless and 5 being very hairy (Figure 4.4b). Plant stature varied from fairly erect to branchy (Figure 4.5). The time to from planting to first seed production ranged from 7 weeks to 22 weeks with an average of 12 weeks. Like all other characteristics, the number of seeds produced per plant varied greatly. Among the genotypes, BdTR4 had the highest seed yield (average of 793 seeds/plant) and BdTR12 exhibited the lowest seed yield (average of 4 seeds/plant). Since there was a great deal of variation in seed production we vernalized all the lines for 6 weeks at 4 ºC to determine if vernalization affected seed production. There was no significant correlation between vernalization and seed production under these conditions. Viability of the harvested seeds (as measured by germination) ranged from 60% to 100% with an average of 90%. Like all other morphological features examined, above-ground biomass showed considerable variation and ranged from 0.40 to 15.77 grams/plant of average dry weight. There was also a great deal of variation in spikelet and grain morphology (Figure 4.6).

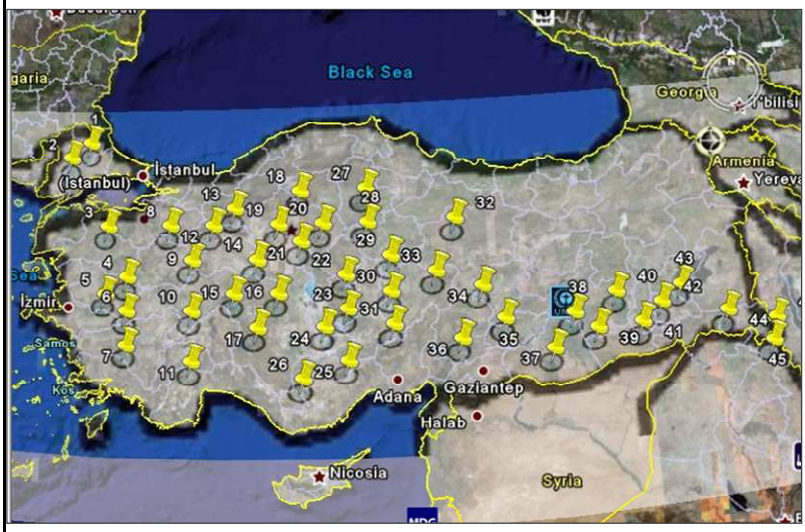
4.1.3 Cytological characterization

The mean nuclear DNA content ranged from 0.70 pg/2C (diploid) to 1.35 pg/2C (polyploid) (Table 4.1 and Appendix D). Based on these data, *Brachypodium* lines were

classified as either diploid (116 lines) or polyploid (30 lines). Thus, the most prevalent ploidy level of Turkish *Brachypodium* was diploid.

Karyotype was produced for plants of two groups (BdTR1 and BdTR13) to confirm that it was similar to previously described karyotypes for the 2n=10 diploid form (Draper et al. 2001; Hasterok et al. 2004, 2006; Jenkins et al. 2003, 2005). Similar to previous reports, we observed 10 chromosomes with four pairs of BdTR1 appearing submetacentric to metacentric and one pair appearing acrocentric (Figure 4.7).

a



b

Location ID	Latitude	Longitude
1	41°25'17.86"N	27°28'36.81"E
2	41° 5'7.15"N	26°55'53.29"E
3	39°44'17.39"N	28° 2'24.71"E
4	38°45'50.79"N	28°35'6.42"E
5	38°25'0.42"N	28° 1'52.75"E
6	38° 5'35.03"N	28°34'59.02"E
7	37°25'38.24"N	28°35'6.75"E
8	39°45'34.18"N	29°40'40.96"E
9	39° 5'12.20"N	30°15'8.93"E
10	38° 5'59.53"N	30°14'44.11"E
11	37° 7'38.06"N	30°13'59.22"E
12	39°45'10.62"N	30°47'19.07"E
13	40° 4'55.55"N	31°19'52.01"E
14	39° 5'8.89"N	31°53'29.40"E
15	38°25'46.78"N	31°18'33.71"E
16	38°25'40.97"N	32°24'16.47"E
17	37°46'41.64"N	31°53'5.68"E
18	40°23'37.13"N	32°59'7.32"E
19	39°45'23.35"N	32°25'56.46"E
20	39°45'16.02"N	33°32'16.37"E
21	39°24'46.28"N	32°59'17.24"E
22	38°45'28.75"N	34° 4'18.34"E
23	38° 5'52.78"N	34° 5'40.38"E
24	37°46'4.28"N	33°31'10.58"E
25	37° 6'31.87"N	34° 4'17.06"E
26	36°46'58.92"N	32°57'46.71"E
27	40°24'19.91"N	34°38'10.10"E
28	39°44'53.45"N	34°39'1.15"E
29	39° 5'31.64"N	35°11'18.62"E
30	38°24'16.86"N	35° 9'34.32"E
31	37°46'43.23"N	35°12'9.55"E
32	39°44'51.33"N	36°48'56.85"E
33	38°45'25.33"N	36°16'56.90"E
34	38°25'12.10"N	37°23'26.99"E
35	37°45'58.01"N	37°53'23.56"E
36	37°26'4.39"N	36°49'9.55"E
37	37° 7'2.52"N	39° 1'49.56"E
38	37°45'41.57"N	39°34'56.72"E
39	37°26'13.88"N	40° 7'50.51"E
40	38° 5'43.50"N	40°39'30.84"E
41	37°27'31.68"N	41°14'39.03"E
42	37°47'51.69"N	41°46'25.09"E
43	38° 5'48.94"N	42°19'18.79"E
44	37°28'28.23"N	43°26'45.08"E
45	36°45'59.24"N	44°32'6.90"E

Figure 4.1 (a) Map of Turkey representing the geographical regions with yellow labels from where *Brachypodium* samples collected. The regions are labeled according to **(b)** GPS locations using Google Earth.

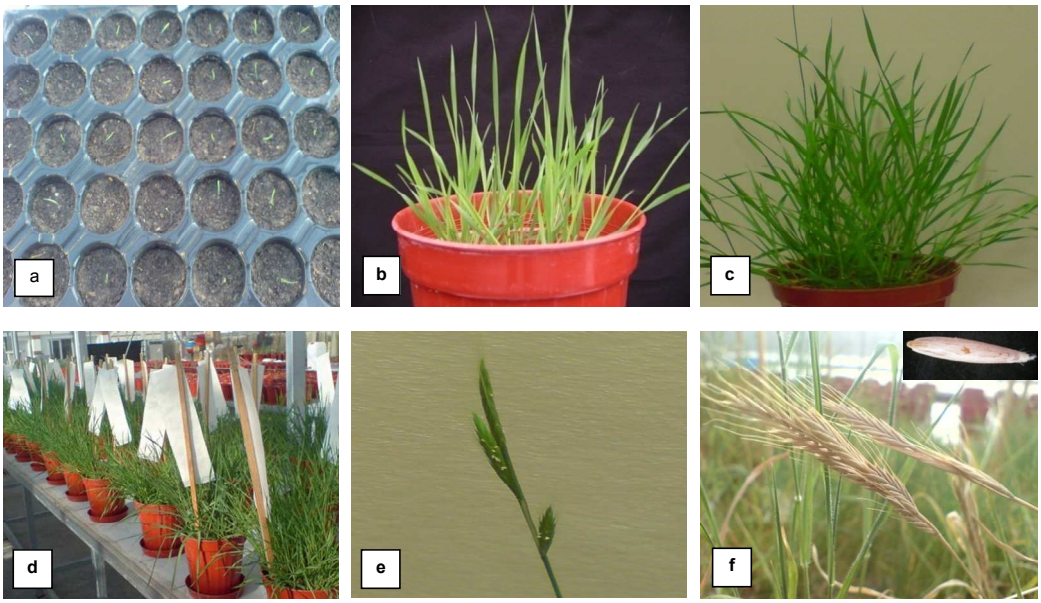


Figure 4.2 *Brachypodium distachyon* grown in the greenhouse. **(a, b, c, d, e, f)** Different developmental stages of growth.

Table 4.1 Morphological characteristics of *Brachypodium distachyon* genotypes collected from different locations of Turkey.

Genotypes	Latitude	Longitude	Elevation (m)	†Average Height (cm)	Leaf Hairiness*	Leaf Color**	Plant Stature	†Average Seed size (width/length) (cm)	***Seed Production	†Average Biomass (g)	††Seed yield (seed #/plant)	Germination%	DNA Contents (pg/2C)
Bd TR-1	**39°44'17.39"N**	**28° 2'24.71"E**	**363**	29.2 - 40.0	2	2	Fairly erect	0,121 / 0,645	8 weeks after	0,9	63	93.8 %	0,693
Bd TR-2	**37° 7'38.06"N**	**30°13'59.22"E**	**1073**	29.7 - 40.6	2	2	Fairly erect	0,115 / 0,599	10 weeks after	0,78	183	100%	0,696
Bd TR-3	**38° 5'52.78"N**	**34° 5'40.38"E**	**1406**	30.0 - 38.3	1	2	Erect	0,104 / 0,658	12 weeks after	2,06	34	100%	0,678
Bd TR-4	**38° 5'43.50"N**	**40°39'30.84"E**	**688**	38.3 - 49.3	2	2	Erect	0,127 / 0,678	7 weeks after	0,95	793	60%	1,34
Bd TR-5	**37°28'28.23"N**	**43°26'45.08"E**	**1957**	29.2 - 37.4	3	3	Fairly erect	0,108 / 0,682	14 weeks after	1,6	20	100%	0,699
Bd TR-6	**37°47'51.69"N**	**41°46'25.09"E**	**612**	35.0 - 51.6	5	3	Erect	0,141 / 0,784	10 weeks after	0,43	443	100%	1,34
Bd TR-7	**40°24'19.91"N**	**34°38'10.10"E**	**1088**	26.0 - 36.0	2	1	Erect	0,112 / 0,633	22 weeks after	15,77	49	100%	0,694
Bd TR-8	**39°45'23.35"N**	**32°25'56.46"E**	**787**	25.0 - 35.0	2	1	Erect	0,127 / 0,647	22 weeks after	8,72	67	100%	1,27
Bd TR-9	**37°46'43.23"N**	**35°12'9.55"E**	**3196**	30.0 - 45.0	3	1	Branchy	0,123 / 0,611	9 weeks after	2,71	14	85.7 %	0,69
Bd TR-10	**39° 5'8.89"N**	**31°53'29.40"E**	**864**	30.0 - 40.5	3	1	Erect	0,123 / 0,621	20 weeks after	2,22	17	92.9 %	0,7
Bd TR-11	**38°45'50.79"N**	**28°35'6.42"E**	**612**	28.6 - 38.3	3	1	Erect	0,124 / 0,617	17.5 weeks after	1,98	100	95%	0,692
Bd TR-12	**38°45'50.79"N**	**28°35'6.42"E**	**612**	25.0 - 32.3	3	1	Erect	0,112 / 0,578	20 weeks after	4,72	4	66.7 %	0,7
Bd TR-13	**39° 5'31.64"N**	**35°11'18.62"E**	**1249**	30.7 - 42.1	4	3	Branchy	0,104 / 0,663	19.5 weeks after	0,4	214	95%	0,7

* The degree of feathery leaf structure from 1 to 5 in ascending order.

** The degree of greenish leaf color from 1 to 3 in ascending order.

*** The time of first seed production observed.

† Values are the means of 15 replicates (individual plant).

†† Values are the means of 7 replicates (individual plant).

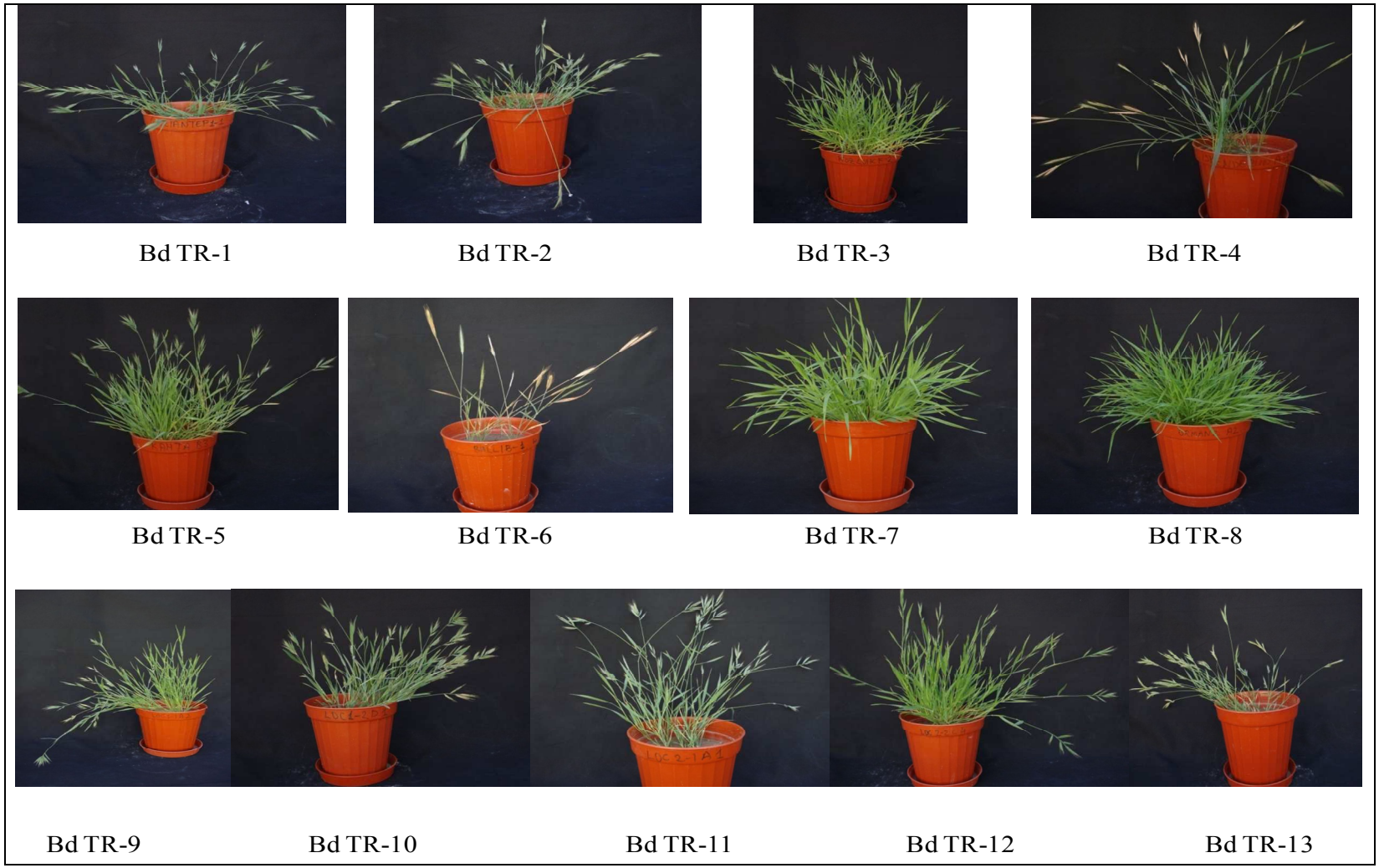


Figure 4.3 Illustrations of 13 different Turkish *Brachypodium* genotypes.

a

b

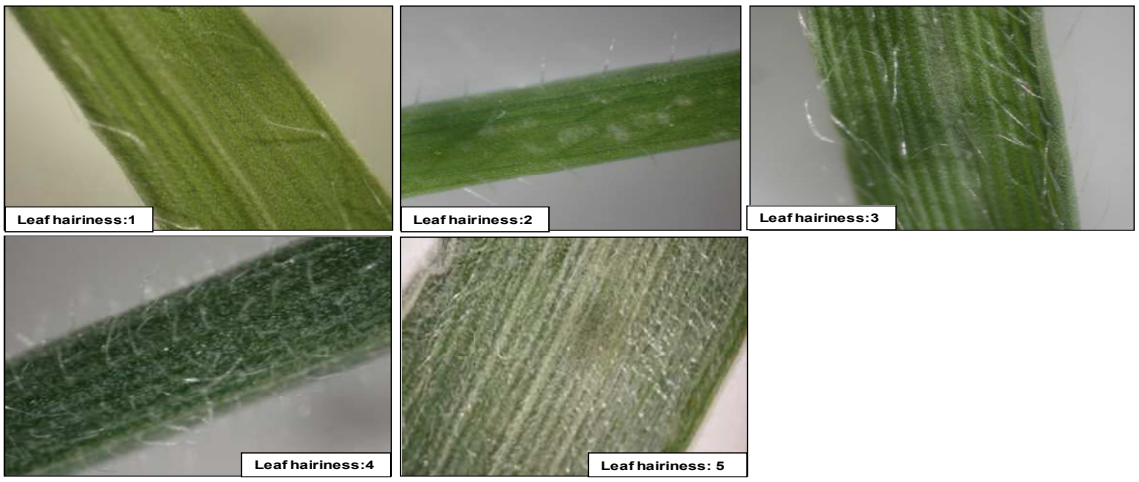


Figure 4.4 (a) The leaf color scale of *Brachypodium distachyon* exhibiting three different degrees of greenish leaf color from one on the left to three on the right. **(b)** Leaves of *Brachypodium distachyon* showing 5 different degrees of hairiness from one at the upper right to five at the lower left. The variation in trichome density is notable.

Figure 4.5 Plant statures in *Brachypodium* from **(a)** erect, and **(b)** fairly erect, to **(c)** branchy.

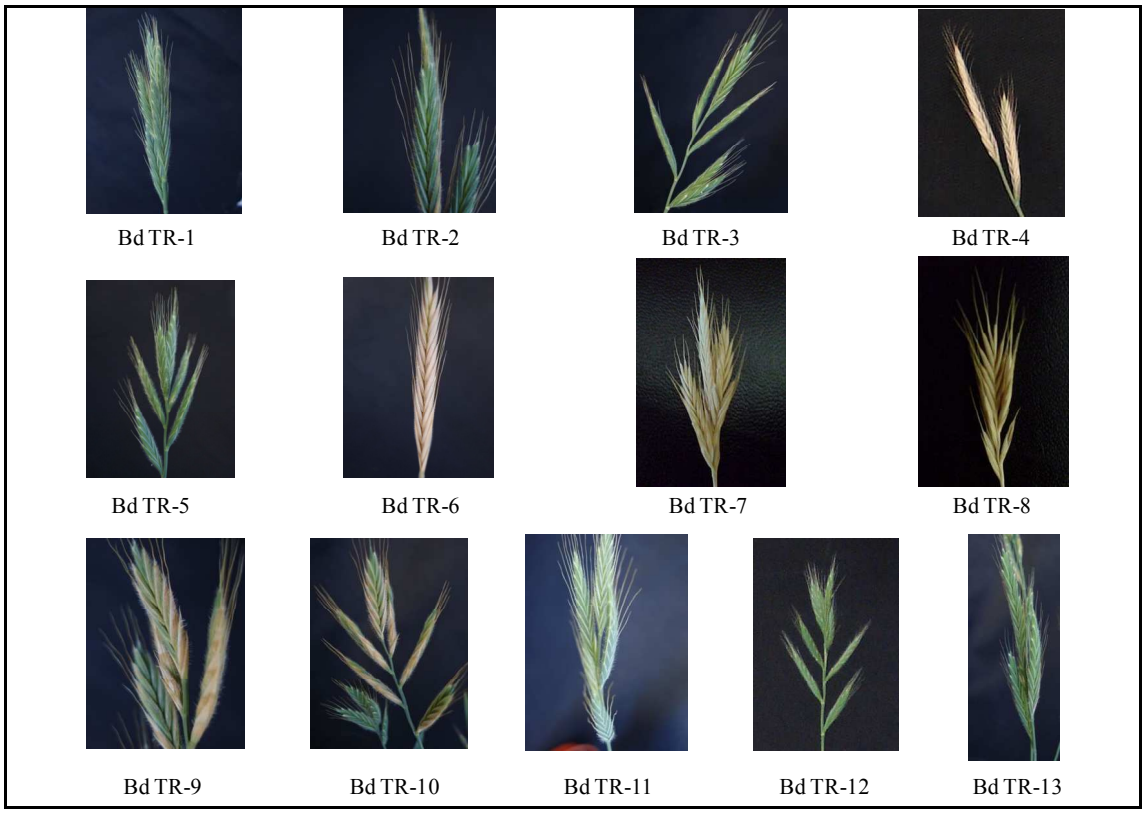


Figure 4.6 Illustrations of the *Brachypodium distachyon* seed heads.

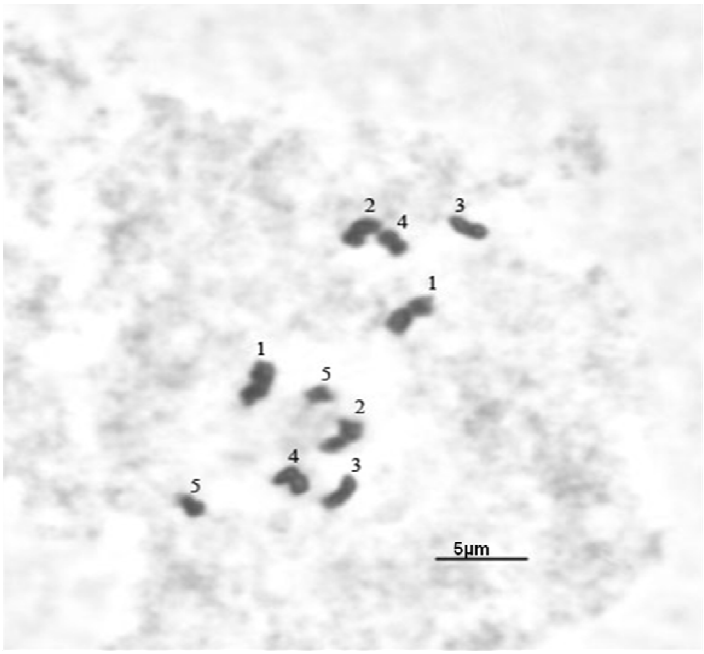


Figure 4.7 Mitotic chromosomes of diploid *Brachypodium* line BdTR1.

According to the principal component analysis performed for morphological traits obtained from *Brachypodium* genotypes showed the variation in the distribution of genotypes (Figure 4.8a-f). The qualitative descriptors (leaf color, leaf hairiness and plant stature) were grouped together. The quantitative descriptors (seed yield, biomass, seed production time and germination as one group and average seed sizes and plant height as another group) were analyzed in two groups. All the traits in each group were in different line and the genotypes were scattered according to the correlation of the variables.

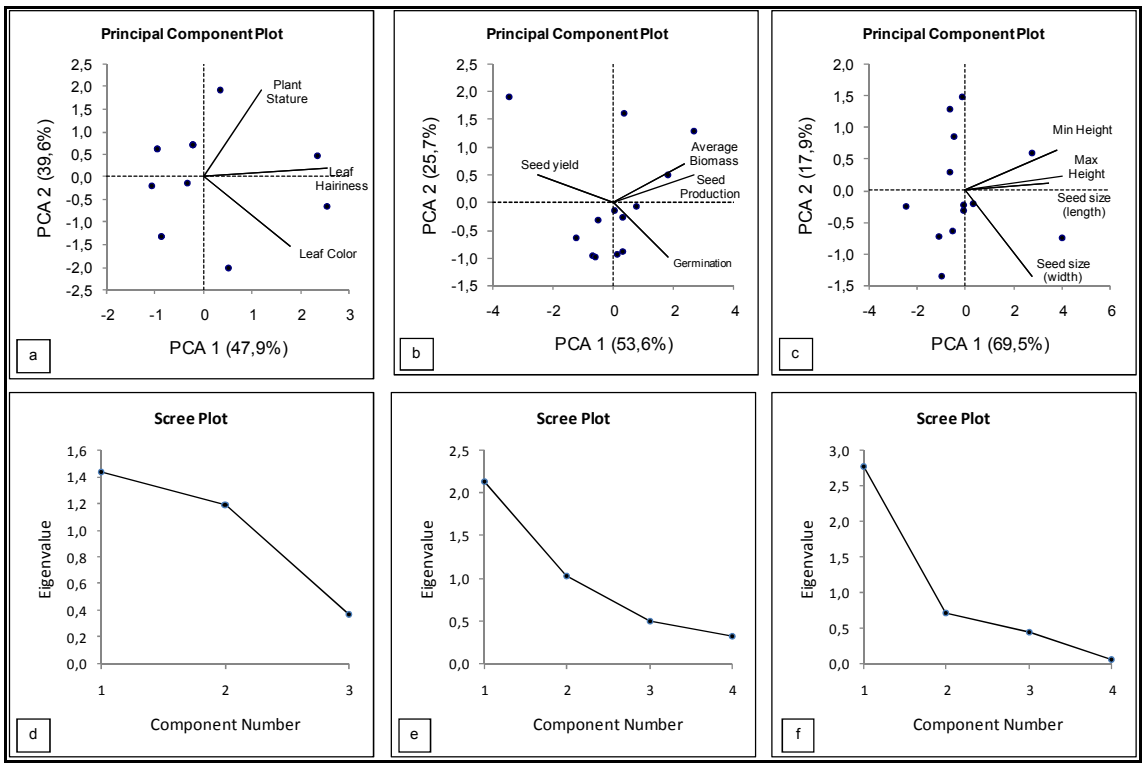


Figure 4.8 Principal component analysis diagram of morphological traits in *Brachypodium* genotypes based on correlation of variables: **(a)** plant stature, leaf hairiness and color as qualitative descriptors; **(b)** seed yield, average biomass, seed production time and germination percentage; **(c)** min and max height, seed size in terms of width and length as qualitative descriptors; and **(d, e, f)** their eigen values shown respectively.

As a consequence, we observed a high degree of variation in this collection. This phenotypic data can be used to select parents for developing mapping populations to map genes that control vernalization, yield, and other important traits. The diverse *Brachypodium* lines developed in this study will allow experimental approaches dependent upon natural variation to be applied to this new model grass. These results will also help efforts to have a better understanding of grass species with complex large genomes.

4.2 Macro- and microelement analysis

Macro- and microelement analysis of Turkish *Brachypodium* genotypes was performed using three lines for each genotype with two replicates. For identification of macronutrient composition in seeds; nitrogen (N), potassium (K), phosphorus (P), sulphur (S), magnesium (Mg) and calcium (Ca) concentrations were measured. Protein content was calculated by multiplying N% by a constant number 6.25. Micronutrient composition was identified by measuring iron (Fe), copper (Cu), manganese (Mn) and zinc (Zn) concentrations in seeds. Aluminum (Al) concentration was used as the indicator of soil contamination. Thousand kernel weights were also calculated for each genotype (Table 4.2). Wheat flour was used as the reference material.

The data for each genotype was also represented by the mean value of three inbred lines and gave us actual values for each genotype. The elements were outlined both as concentration (Table 4.3) and content (Table 4.5) since small seed size might effect concentration. Both macro and microelement concentration and content could be compared to achieve the precise result. Maximum, minimum and mean values of both concentration and content were interpreted and LSD (5%) values were calculated.

Furthermore, grain macroelement **(**K, P, S, Mg, and Ca) and microelement (Fe, Cu, Mn, and Zn) concentrations and contents in *Brachypodium* were further analyzed and plotted to evaluate the correlation between (Figure 4.9a-i). For some of the elements (K, S and Ca), the correlation was found to be very low and was highest in manganese (Mn).

The largest variation among the macroelements was observed in grain Ca concentration (2.3-fold) and the variation in grain concentration of the other macroelements was between 1.73- (for N and K) and 1.86-fold (for S). Micronutrients displayed larger variations in grain concentration with respect to macroelements. Highest variation was achieved with grain Fe concentration ranging from 18-82 mg kg^{-1} (4.56-fold) and the variation for the rest of the other microelements was as follows: Cu (2.78-fold), Mn (2.7-fold) and Zn (1.83 fold) (Table 4.2).

The statistical analysis of macro and microelement concentration in seeds (Table 4.4) revealed that four elements (N, S, Mn and Zn) were highly significant at $p \leq 0.001$ and three elements (K, Fe and Cu) were significant at $p \leq 0.05$. Genotypic effect on these nutrient concentrations was clear. Besides these significant differences of the elements among *Brachypodium* genotypes, concentrations of the three elements (P, Mg, and Ca) did not show any significance difference among genotypes.

On the other hand, analysis of variance for macro- and microelement content in seeds (Table 4.6) showed slightly different results with respect to the concentration analysis results which were outlined above. The statistical analysis performed on the macro- and microelement content of seeds showed that the six elements (K, P, S, Mg, Cu and Mn) were highly significant at $p \leq 0.001$ whereas Ca and Zn were significant at $p \leq 0.05$. Only iron (Fe) had no significant difference among genotypes. Meanwhile, protein content was also significant at $p \leq 0.001$.

Correlation coefficients of among mineral concentration were also calculated and the significance levels were indicated. The significant correlations between some of the macro- and microelements could be observed (Table 4.8). The significant correlations were between: N and S, N and Mn, N and Zn ($p \leq 0.01$); P and Mg ($p \leq 0.01$); S and Mn ($p \leq 0.05$), S and Zn ($p \leq 0.001$); Mg and Cu ($p \leq 0.05$); Cu and Mn ($p \leq 0.05$).

Some important relations between or within macro- and microelements were plotted for graphical visualization of these elements (Figure 4.10a-h). The correlations between N and Zn as well as S and Zn were found to be high with correlation coefficients (r) of 0.83 and 0.88 respectively. The correlations between these elements were strong and significant. There was moderate correlation between N and Fe, S and Fe, Zn and Fe, P and Mn with r values ranging from 0.39-0.43 and relatively low correlation between P and Zn ($r = 0.21$). The association between P and Fe was negative ($r = -0.21$).

These data might be further evaluated since genotypic variation was distinct. The genotypes that had the highest level of element composition could be analyzed to asses the nutrient use efficiency or the element accumulation at physiologic, genetic and molecular level for enhanced crop yield.

Table 4.2 Macro- and microelement analysis in seeds of *Brachypodium* genotypes using three inbred lines for each genotype. (Each data representing the mean ± SE of 2 replicates)

Macro and micro element analysis of *Brachypodium distachyon*																
			Concentration													
Sample Labels Genotype-line#	1000Kernel weight	Protein content (N% × 6.25)	N	K	P	S	Mg	Ca	Fe	Cu	Mn	Zn	Al			
	(g)	(%)	(%)								(mg kg^{-1})					
BdTR1-1	2,96	18,0	2,87 ± 0,02	0,66 ± 0,00	0,39 ± 0,00	0,22 ± 0,00	0,11 ± 0,00	0,12 ± 0,01	31 ± 0	9,3 ± 0,1	31 ± 1	34 ± 2	3,22 ± 0,50			
BdTR1-2	3,15	18,7	3,00 ± 0,05	0,71 ± 0,02	0,40 ± 0,00	0,23 ± 0,00	0,11 ± 0,00	0,12 ± 0,02	36 ± 2	10,6 ± 0,3	36 ± 2	38 ± 3	2,32 ± 0,37			
BdTR1-3	3,56	17,7	2,83 ± 0,03	0,67 ± 0,01	0,46 ± 0,01	0,23 ± 0,00	0,12 ± 0,00	0,12 ± 0,01	29 ± 0	10,1 ± 0,1	34 ± 0	36 ± 2	2,81 ± 0,20			
BdTR2-1	3,40	18,8	3,01 ± 0,07	0,69 ± 0,01	0,36 ± 0,01	0,23 ± 0,00	0,11 ± 0,00	0,11 ± 0,00	35 ± 1	10,2 ± 0,0	36 ± 1	33 ± 0	2,73 ± 0,08			
BdTR2-2	3,36	18,9	3,02 ± 0,02	0,61 ± 0,02	0,43 ± 0,02	0,24 ± 0,00	0,11 ± 0,00	0,10 ± 0,00	34 ± 0	11,1 ± 0,3	36 ± 1	40 ± 1	2,34 ± 0,33			
BdTR2-3	3,61	18,0	2,89 ± 0,01	0,62 ± 0,01	0,42 ± 0,01	0,22 ± 0,00	0,11 ± 0,00	0,11 ± 0,00	30 ± 1	8,8 ± 0,5	33 ± 0	32 ± 1	2,21 ± 0,09			
BdTR3-1	2,47	21,4	3,42 ± 0,07	1,04 ± 0,25	0,50 ± 0,01	0,26 ± 0,01	0,13 ± 0,00	0,12 ± 0,04	23 ± 0	11,0 ± 0,3	23 ± 4	44 ± 1	2,91 ± 0,00			
BdTR3-2	2,84	17,1	2,74 ± 0,02	0,72 ± 0,02	0,29 ± 0,02	0,23 ± 0,00	0,10 ± 0,00	0,09 ± 0,01	33 ± 1	8,2 ± 0,2	28 ± 0	38 ± 1	3,12 ± 0,19			
BdTR3-3	2,84	16,7	2,66 ± 0,01	0,62 ± 0,01	0,28 ± 0,01	0,21 ± 0,00	0,10 ± 0,00	0,09 ± 0,00	34 ± 1	7,8 ± 0,4	26 ± 1	35 ± 1	3,34 ± 0,33			
BdTR4-1	3,56	12,6	2,01 ± 0,03	0,62 ± 0,01	0,33 ± 0,00	0,14 ± 0,00	0,10 ± 0,00	0,09 ± 0,00	23 ± 1	10,8 ± 0,3	41 ± 1	24 ± 1	3,08 ± 0,14			
BdTR4-2	3,86	13,0	2,09 ± 0,10	0,62 ± 0,03	0,33 ± 0,00	0,16 ± 0,00	0,11 ± 0,00	0,10 ± 0,00	27 ± 0	11,2 ± 0,2	46 ± 0	25 ± 0	3,27 ± 0,74			
BdTR4-3	4,01	13,1	2,09 ± 0,04	0,61 ± 0,00	0,33 ± 0,00	0,15 ± 0,00	0,11 ± 0,00	0,09 ± 0,00	28 ± 1	11,4 ± 0,2	44 ± 1	24 ± 0	2,69 ± 0,36			
BdTR5-1	3,52	19,7	3,15 ± 0,01	0,65 ± 0,00	0,31 ± 0,00	0,21 ± 0,00	0,11 ± 0,00	0,09 ± 0,00	35 ± 0	10,7 ± 0,1	32 ± 0	37 ± 1	2,69 ± 0,16			
BdTR5-2	3,62	21,7	3,47 ± 0,01	0,63 ± 0,00	0,32 ± 0,01	0,20 ± 0,00	0,10 ± 0,00	0,11 ± 0,00	22 ± 0	8,5 ± 0,0	21 ± 0	32 ± 0	2,47 ± 0,06			
BdTR5-3	3,24	20,0	3,20 ± 0,03	0,60 ± 0,00	0,31 ± 0,02	0,22 ± 0,00	0,10 ± 0,00	0,08 ± 0,00	35 ± 1	10,1 ± 0,0	30 ± 1	36 ± 1	3,52 ± 0,43			
BdTR6-1	5,64	15,3	2,46 ± 0,10	0,70 ± 0,02	0,48 ± 0,01	0,21 ± 0,00	0,14 ± 0,00	0,10 ± 0,00	21 ± 1	14,8 ± 0,2	50 ± 2	33 ± 1	1,85 ± 0,13			
BdTR6-2	5,88	16,0	2,56 ± 0,07	0,81 ± 0,00	0,46 ± 0,00	0,20 ± 0,00	0,14 ± 0,00	0,10 ± 0,00	24 ± 1	15,3 ± 0,4	54 ± 1	33 ± 1	2,90 ± 0,65			
BdTR6-3	4,30	13,2	2,11 ± 0,00	0,70 ± 0,01	0,38 ± 0,01	0,19 ± 0,00	0,12 ± 0,00	0,11 ± 0,00	24 ± 1	13,0 ± 0,3	53 ± 2	27 ± 1	2,77 ± 0,08			
BdTR9-1	3,56	16,6	2,66 ± 0,03	0,66 ± 0,01	0,28 ± 0,01	0,21 ± 0,00	0,09 ± 0,00	0,08 ± 0,00	31 ± 1	7,5 ± 0,1	31 ± 1	31 ± 0	2,82 ± 0,45			
BdTR9-2	3,25	17,7	2,84 ± 0,09	0,62 ± 0,01	0,41 ± 0,01	0,23 ± 0,00	0,13 ± 0,00	0,10 ± 0,00	31 ± 1	10,1 ± 0,2	27 ± 1	35 ± 0	6,63 ± 1,44			
BdTR9-3	2,97	19,1	3,06 ± 0,05	0,60 ± 0,01	0,31 ± 0,00	0,24 ± 0,00	0,10 ± 0,00	0,07 ± 0,00	36 ± 1	7,0 ± 0,0	30 ± 0	32 ± 0	5,43 ± 1,11			
BdTR10-1	3,11	16,7	2,67 ± 0,11	0,84 ± 0,00	0,30 ± 0,01	0,21 ± 0,00	0,10 ± 0,00	0,07 ± 0,01	41 ± 0	7,2 ± 0,4	24 ± 1	35 ± 1	3,98 ± 0,09			
BdTR10-2	3,61	21,3	3,41 ± 0,06	0,85 ± 0,01	0,42 ± 0,02	0,25 ± 0,00	0,13 ± 0,00	0,11 ± 0,00	36 ± 0	14,4 ± 0,1	34 ± 1	36 ± 0	3,88 ± 0,19			
BdTR10-3	4,99	18,8	3,00 ± 0,03	0,72 ± 0,00	0,36 ± 0,01	0,23 ± 0,00	0,12 ± 0,00	0,13 ± 0,00	82 ± 2	5,5 ± 0,2	25 ± 0	44 ± 1	3,65 ± 0,36			
BdTR11-1	3,41	17,3	2,76 ± 0,01	0,71 ± 0,02	0,35 ± 0,00	0,22 ± 0,00	0,11 ± 0,00	0,12 ± 0,00	31 ± 0	12,1 ± 0,2	30 ± 1	34 ± 0	4,24 ± 0,79			
BdTR11-2	2,97	18,2	2,92 ± 0,15	0,82 ± 0,03	0,32 ± 0,01	0,22 ± 0,00	0,11 ± 0,00	0,10 ± 0,00	35 ± 2	10,5 ± 0,2	25 ± 0	35 ± 1	4,40 ± 1,67			
BdTR11-3	3,02	18,4	2,94 ± 0,20	0,75 ± 0,02	0,31 ± 0,01	0,21 ± 0,01	0,11 ± 0,00	0,16 ± 0,00	30 ± 1	9,3 ± 0,3	27 ± 2	31 ± 1	3,88 ± 0,13			
BdTR12-1	3,28	16,8	2,69 ± 0,03	0,72 ± 0,00	0,37 ± 0,01	0,21 ± 0,00	0,12 ± 0,00	0,11 ± 0,00	35 ± 0	9,2 ± 0,1	27 ± 1	30 ± 0	3,05 ± 0,13			
BdTR12-2	3,40	17,8	2,85 ± 0,05	0,76 ± 0,01	0,46 ± 0,01	0,22 ± 0,00	0,13 ± 0,00	0,12 ± 0,00	34 ± 1	12,9 ± 0,1	31 ± 0	37 ± 1	4,03 ± 0,02			
BdTR12-3	3,29	18,0	2,88 ± 0,00	0,81 ± 0,02	0,30 ± 0,01	0,22 ± 0,00	0,10 ± 0,00	0,09 ± 0,00	38 ± 1	7,8 ± 0,0	24 ± 1	32 ± 0	3,79 ± 0,76			
BdTR13-1	2,94	18,4	2,95 ± 0,02	0,71 ± 0,00	0,43 ± 0,01	0,23 ± 0,00	0,12 ± 0,00	0,12 ± 0,00	25 ± 0	13,3 ± 0,2	24 ± 1	41 ± 2	3,32 ± 0,28			
BdTR13-2	3,18	18,8	3,01 ± 0,03	0,69 ± 0,01	0,35 ± 0,00	0,22 ± 0,00	0,11 ± 0,00	0,10 ± 0,00	33 ± 0	10,2 ± 0,3	32 ± 1	35 ± 0	3,04 ± 0,17			
BdTR13-3	2,86	18,8	3,01 ± 0,03	0,69 ± 0,00	0,45 ± 0,00	0,23 ± 0,00	0,14 ± 0,00	0,13 ± 0,00	18 ± 0	12,0 ± 0,0	20 ± 0	40 ± 0	2,62 ± 0,13			
maximum	5,88	21,7	3,47	1,04	0,50	0,26	0,14	0,16	82	15,3	54	44	6,63			
minimum	2,47	12,6	2,01	0,60	0,28	0,14	0,09	0,07	18	5,5	20	24	1,85			
Total mean	3,50	17,7	2,82	0,70	0,37	0,22	0,11	0,10	32	10,4	32	34	3,30			

Table 4.3 Macro- and microelement concentration in seeds of *Brachypodium* genotypes.
(The data for each genotype representing the mean value of three inbred lines)

Macro and micro element analysis of *Brachypodium distachyon*												
			Concentration									
Genotype	1000Kernel weight	Protein content (N% × 6.25)	N	K	P	S	Mg	Ca	Fe	Cu	Mn	Zn
	(g)	(%)	(%)						(mg kg^{-1})			
Bd TR-1	3,22	18,1	2,90	0,68	0,42	0,23	0,11	0,12	32	10,0	34	36
Bd TR-2	3,46	18,6	2,97	0,64	0,40	0,23	0,11	0,10	33	10,1	35	35
Bd TR-3	2,71	18,4	2,94	0,79	0,35	0,23	0,11	0,10	30	9,0	26	39
Bd TR-4	3,81	12,9	2,06	0,61	0,33	0,15	0,10	0,09	26	11,1	43	24
Bd TR-5	3,46	20,5	3,27	0,63	0,31	0,21	0,10	0,09	31	9,8	27	35
Bd TR-6	5,27	14,8	2,38	0,74	0,44	0,20	0,13	0,10	23	14,4	52	31
Bd TR-9	3,26	17,8	2,85	0,63	0,33	0,23	0,11	0,08	33	8,2	29	33
Bd TR-10	3,90	18,9	3,03	0,80	0,36	0,23	0,11	0,10	53	9,0	28	38
Bd TR-11	3,13	18,0	2,87	0,76	0,33	0,21	0,11	0,13	32	10,6	27	33
Bd TR-12	3,32	17,5	2,80	0,76	0,38	0,22	0,12	0,11	36	10,0	27	33
Bd TR-13	2,99	18,7	2,99	0,70	0,41	0,22	0,13	0,12	25	11,8	25	39
maximum	5,27	20,5	3,27	0,80	0,44	0,23	0,13	0,13	53	14,4	52	39
minimum	2,71	12,9	2,06	0,61	0,31	0,15	0,10	0,08	23	8,2	25	24
Total mean	3,50	17,7	2,82	0,70	0,37	0,22	0,11	0,10	32	10,4	32	34
LSD (5%)		2,20	0,352	0,131	0,103	0,020	0,020	0,029	14,89	3,18	6,19	5,65

Table 4.4 Analysis of variance for macro- and microelement concentration in seeds.

		N		K		P		S		Mg		Ca		Fe		Cu		Mn		Zn	
		%												mg kg^{-1}							
Source of variation	df	MS		MS		MS		MS		MS		MS		MS		MS		MS		MS	
Block	2	0,04627		0,0086		0,0017		0,0000198		0,0000375		0,0000441		65,74		9,515		17,41		6,47	
Genotype	10	0,32877	***	0,0147	*	0,0054	NS	0,0016959	***	0,0002427	NS	0,0005114	NS	185,6	*	8,444	*	218,58	***	54,94	***
Error	20	0,04281		0,0059		0,0036		0,000142		0,0001414		0,0002885		76,48		3,482		13,19		11	
LSD (5%)		0,352		0,131		0,103		0,020		0,020		0,029		14,89		3,18		6,19		5,65	

*** Significant at $p \leq 0.001$

** Significant at $p \leq 0.01$

* Significant at $p \leq 0.05$

NS Not significant

Table 4.5 Macro- and microelement content in seeds of *Brachypodium* genotypes.
(The data for each genotype representing the mean value of three inbred lines)

Macro and micro element analysis of *Brachypodium distachyon*									
	Content								
Genotype	**K**	**P**	**S**	**Mg**	**Ca**	**Fe**	**Cu**	**Mn**	**Zn**
					(µg seed^{-1})				
Bd TR-1	22	13,5	7,3	3,66	3,90	0,104	0,032	0,109	0,116
Bd TR-2	22	13,9	7,9	3,82	3,62	0,114	0,035	0,121	0,120
Bd TR-3	21	9,4	6,3	2,89	2,73	0,088	0,024	0,070	0,105
Bd TR-4	23	12,6	5,7	4,00	3,47	0,100	0,042	0,166	0,092
Bd TR-5	22	10,8	7,2	3,60	3,22	0,106	0,034	0,094	0,120
Bd TR-6	39	23,4	10,6	7,09	5,47	0,122	0,077	0,274	0,165
Bd TR-9	21	10,8	7,5	3,54	2,72	0,106	0,027	0,096	0,107
Bd TR-10	31	14,2	9,0	4,47	4,10	0,222	0,034	0,108	0,153
Bd TR-11	24	10,3	6,7	3,44	3,94	0,100	0,034	0,086	0,105
Bd TR-12	25	12,5	7,3	3,89	3,55	0,119	0,033	0,091	0,109
Bd TR-13	21	12,3	6,7	3,74	3,50	0,077	0,035	0,076	0,116
maximum	39	23,4	10,6	7,1	5,5	0,22	0,08	0,27	0,17
minimum	21	9,4	5,7	2,9	2,7	0,08	0,02	0,07	0,09
Total mean	25	13,1	7,5	4,0	3,7	0,11	0,04	0,12	0,12
LSD (5%)	6,25	4,99	1,80	1,28	1,43	0,088	0,014	0,036	0,042

Table 4.6 Analysis of variance for macro- and microelement content in seeds.

		K	P	S	Mg	Ca	Fe	Cu	Mn	Zn
						(µg seed^{-1})				
Source of variation	df	MS	MS	MS	MS	MS	MS	MS	MS	MS
Block	2	8,47	3,961	0,229	0,1985	0,22	0,001916	0,000183	0,00051	0,000141
Genotype	10	94,33 ***	42,531 ***	5,401 ***	3,5633 ***	1,6867 *	0,004359 NS	0.0005819 ***	0,010155 ***	0,001394 *
Error	20	13,44	8,596	1,119	0,5685	0,703	0,002666	0.0000652	0,000442	0,000601
LSD (5%)		6,25	4,99	1,80	1,28	1,43	0,088	0,014	0,036	0,042

*** Significant at $p \leq 0.001$

** Significant at $p \leq 0.01$

* Significant at $p \leq 0.05$

NS Not significant

Table 4.7 Analysis of variance for protein content in seeds.

		Protein content	
		%	
Source of variation	df	MS	
Block	2	1,808	
Genotype	10	12,842	***
Error	20	1,672	
LSD (5%)		2,20	

*** Significant at $p \leq 0.001$

** Significant at $p \leq 0.01$

* Significant at $p \leq 0.05$

NS Not significant

Table 4.8 Correlation coefficient among mineral concentration in seeds.
(The numbers in *italics* are the significance level)

	N	K	P	S	Mg	Ca	Fe	Cu	Mn	Zn
N	1	0,17	-0,14	0,81	-0,23	0,19	0,42	-0,53	-0,79	0,83
		0,625	*0,686*	*0,002***	*0,496*	*0,579*	*0,204*	*0,093*	*0,004***	*0,002***
K		1	0,18	0,40	0,32	0,41	0,44	0,02	-0,26	0,49
			0,587	*0,218*	*0,333*	*0,207*	*0,171*	*0,951*	*0,442*	*0,125*
P			1	0,22	0,81	0,45	-0,21	0,59	0,42	0,21
				0,520	*0,003***	*0,163*	*0,535*	*0,056*	*0,197*	*0,527*
S				1	0,08	0,26	0,43	-0,45	-0,62	0,88
					0,813	*0,439*	*0,185*	*0,165*	*0,043**	*0,000****
Mg					1	0,35	-0,26	0,77	0,42	0,09
						0,293	*0,436*	*0,005**	*0,193*	*0,795*
Ca						1	-0,06	0,32	-0,17	0,36
							0,865	*0,343*	*0,616*	*0,278*
Fe							1	-0,59	-0,43	0,39
								0,056	*0,190*	*0,236*
Cu								1	0,71	-0,33
									*0,014**	*0,315*
Mn									1	-0,67

*** Significant at $p \leq 0.001$

** Significant at $p \leq 0.01$

* Significant at $p \leq 0.05$

NS Not significant

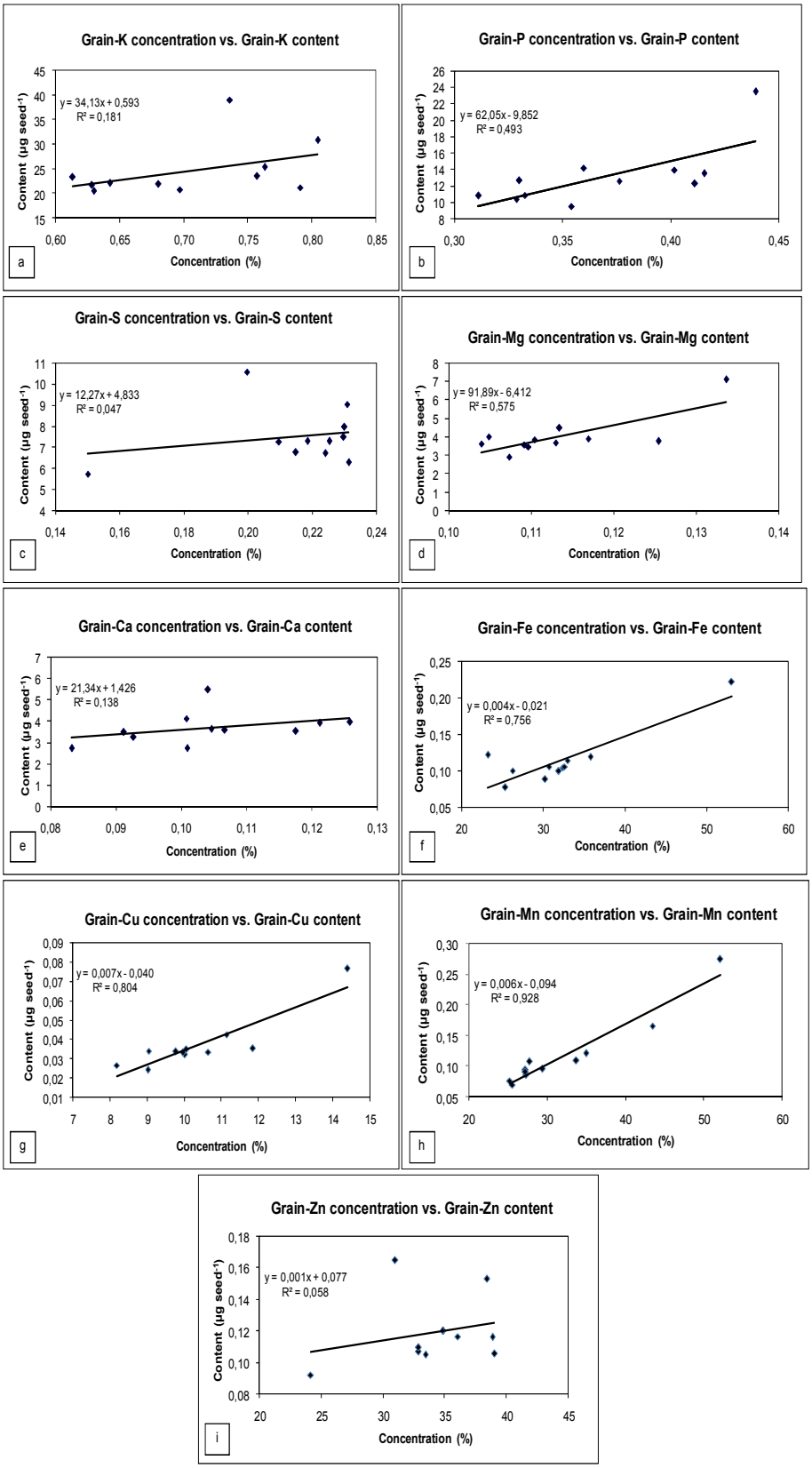


Figure 4.9 Evaluation of macroelements **(a)** K, **(b)** P, **(c)** S, **(d)** Mg, **(e)** Ca and microelements **(f)** Fe, **(g)** Cu, **(h)** Mn, **(i)** Zn in *Brachypodium* genotypes comparing the concentration and content of minerals in grain composition.

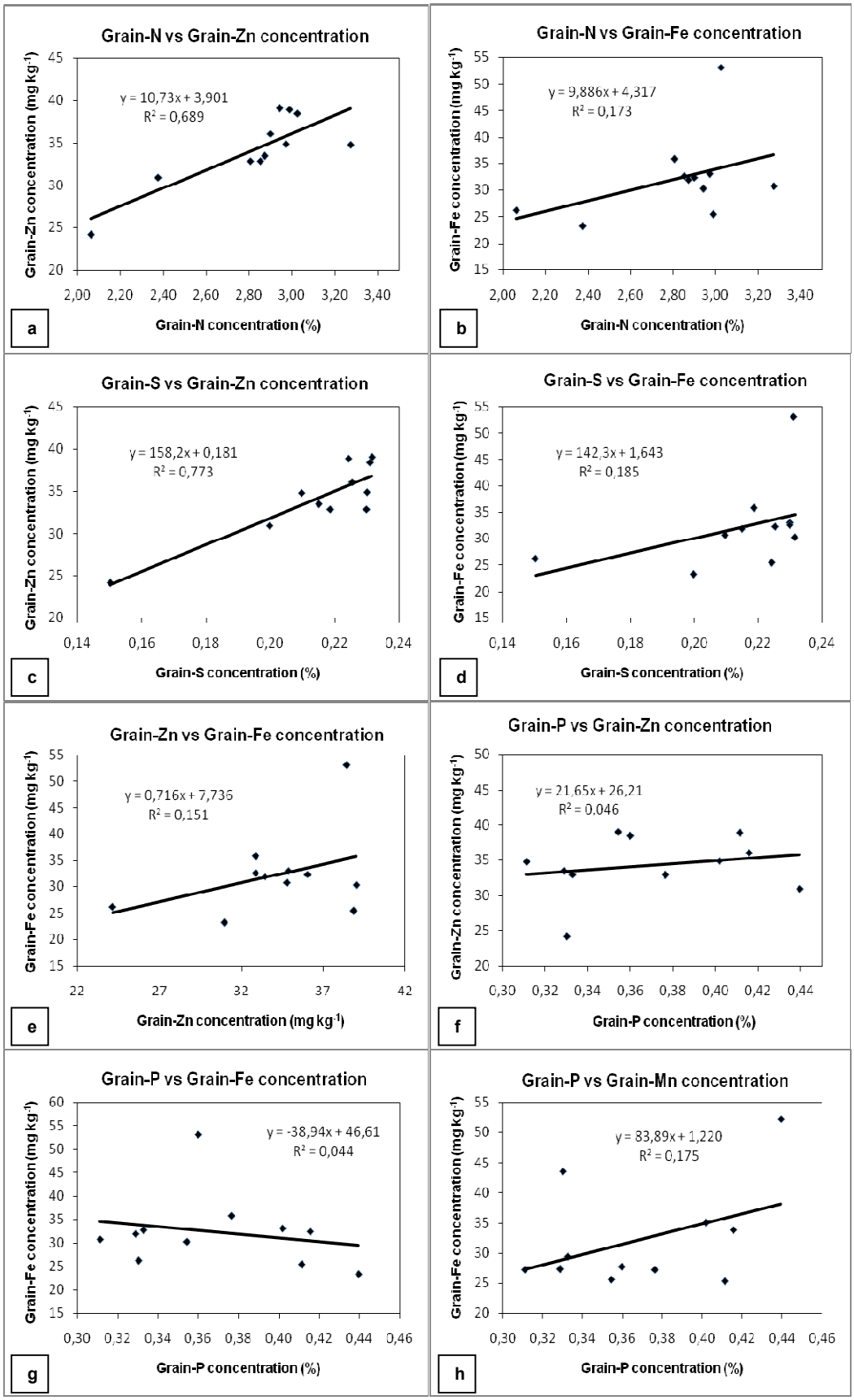


Figure 4.10 Graphical visualization of correlation between (a) N and Zn, (b) N and Fe, (c) S and Zn, (d) S and Fe, (e) Zn and Fe, (f) P and Zn, (g) P and Fe, (h) P and Mn.

4.3 Tissue culture

4.3.1 Callus formation

In order to optimize tissue culture conditions, we attempted to establish a callus induction and regeneration system. In preliminary studies, stem (mesocotyl tissue), root tip, mature embryo and leaf segment were used as the sources of explants Table 4.9). Different concentrations of auxin (1, 3 and 5 mg/L 2,4-D) and cytokinin (0.5 mg/L BAP) were applied with sucrose supplementation as the carbohydrate source. Different auxin types (IAA and NAA) rather than 2,4-D was applied at a concentration of 2.5 mg/L. At same concentrations 2,4-D was also used in media composition with differing carbohydrate source addition as maltose and sucrose. Callus initiation was achieved from all media compositions using stem mesocotyl tissues after two weeks (Figure 4.11a). Callus formation percentages were varying from 0-80% with the media used after five weeks. Most responding explant type was stem mesocotyl tissue after mature embryos. In mature embryos, only auxin type hormone addition (without cytokinin) resulted in callus formation (Figure 4.11b). Addition of cytokinin (BAP) to the medium had a negative effect on all explants and promoted necrosis. The callus formation percentages were 100% with all concentrations of 2,4-D, however NAA and IAA addition to the media resulted in 20% and 30% callus formation efficiency. Root tips exhibited callus formation (Figure 4.11c) in only three media compositions used at a range of 25-45%. Callus formation was not observed from leaf explants under any of the conditions tested. Maltose and sucrose were evaluated as the carbohydrate source with a constant 2,4-D (2.5 mg/L) concentration. Root tips and mature embryos had callus formation with sucrose and maltose; however, maltose had an adverse effect on stem explants after a while. All calli in NAA and IAA containing media lost their viability finally. Overall, the responding explants to different media compositions were outlined briefly in Table 4.10.

Table 4.9 Callus formation percentages of different *Brachypodium* explants with different media composition.

CALLUS FORMATION %							
Hormone concentration (2,4-D; BAP) (mg/L)	**STEM**		**ROOT**		**EMBRYO**		**LEAF**
	2 weeks after initial culture	**5 weeks after initial culture**	**2 weeks after initial culture**	**5 weeks after initial culture**	**2 weeks after initial culture**	**5 weeks after initial culture**	**2-4 weeks after initial culture**
(1; 0)	20%	0%	0%	0%	100%	**100%**	0%
(1; 0,5)	50%	0%	0%	0%	100%	0%	0%
(3; 0)	80%	**80%**	35%	0%	100%	**100%**	0%
(3; 0,5)	45%	0%	0%	0%	100%	0%	0%
(5; 0)	70%	**55%**	10%	**25%**	100%	**100%**	0%
(5; 0,5)	20%	0%	10%	0%	90%	0%	0%
Auxin (mg/L) + Carbohydrate							
2,4-D (2,5)+Maltose	80%	**80%**	40%	**20%**	100%	**100%**	0%
2,4-D (2,5)+Sucrose	70%	**70%**	45%	**45%**	100%	**100%**	0%
IAA (2,5)+Sucrose	35%	**70%**	0%	0%	100%	**30%**	0%
NAA (2,5)+Sucrose	40%	0%	0%	0%	100%	**20%**	0%

Table 4.10 Effect of plant growth regulator and carbohydrate source on callus induction

	(2,4-D; BAP) [mg/L]						**2,5 mg/L 2,4-D**	
Explant type	**(1;0)**	**(1;0,5)**	**(3;0)**	**(3;0,5)**	**(5;0)**	**(5;0,5)**	**Maltose**	**Sucrose**
Stem (mesocotyl tissue)	-	-	+	-	+	-	+	+
Root tip	-	-	-	-	+	-	+	+
Mature embryo	+	-	+	-	+	-	+	+
Leaf node	-	-	-	-	-	-	-	-

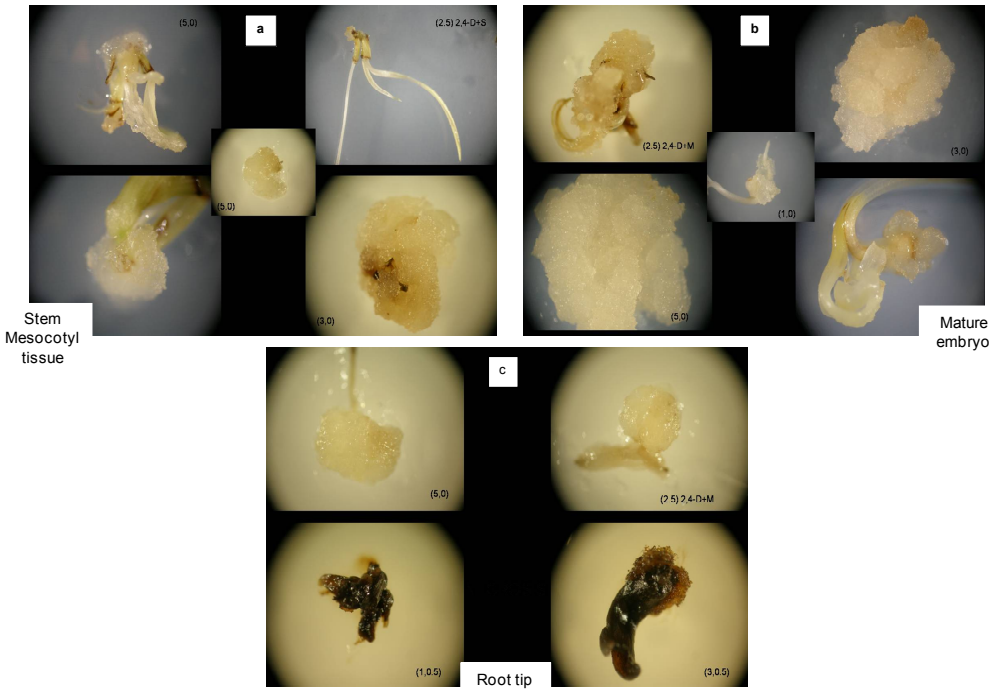


Figure 4.11 Illustration of various *Brachypodium* explants, **(a)** stem mesocotyl tissue; **(b)** mature embryo; **(c)** root tip exhibiting degrees of callus formation with different media composition (plant growth regulator type and concentration, and carbohydrate source). (Black regions representing the necrotic tissues)

These preliminary experiments were used to design conditions for the evaluation of three different genotypes (BdTR4, BdTR6 and BdTR13). These genotypes were selected according to their morphological traits (growing behavior) recorded. For callus induction frequency, we used a three factorial completely randomized design with four replications. There were three levels of the first factor (genotype), three levels of the second factor (2,4-D), and two levels of the third factor (BAP). Only mature embryos were used since embryogenic callus formation and good proliferation was observed with those explant types (Figure 4.11b). For BdTR13, the highest percentage of callus formation was 90% with supplementation of 1 mg/L 2,4-D to the medium and decreased at higher concentrations (Figure 4.12a; 4.13a). Using maltose as a carbohydrate source caused an increase in callus formation percentages with respect to sucrose and the difference was significant (Figure 4.13b). For BdTR6, application of 3 mg/L 2,4-D gave the highest rate of callus formation at a rate of almost 100% (Figure 4.12b; 4.13c). A slight difference was observed between 1 and 5 mg/L 2,4-D (significant difference between 3 and 5 mg/L 2,4-D at $p \leq 0.002$) and also between

sucrose and maltose additions (Figure 4.13d). For BdTR4, 5 mg/L 2,4-D gave the highest callus formation with 100% and it was decreased by lowering the hormone concentration (Figure 4.12c; 4.13e). 1 and 5 mg/L 2,4-D treatments were significantly different at $p \leq 0.002$. Maltose and sucrose supplementation was not significantly different (Figure 4.13f). Bd21 as the control line used in our studies performed callus formation at an average rate (79%) with respect to Turkish genotypes (Figure 4.13g; 4.13a). In BdTR4, though callus formation percentages were high, embryogenic callus formation started late. However, regeneration was quick and the efficiency was high. In BdTR6, although profuse callus formation was observed, necrosis started so quickly that those tissues had to be removed and viable tissues were subcultured. In BdTR13, callus initiation was high but the proliferation was late. Embryogenic callus formation efficiency in Bd21 was not as high as in BdTR4 but similar to BdTR13. As a source of variation, genotype and genotype-hormone treatment interaction were found to be highly significant whereas there was no genotypic effect on carbohydrate treatment (Figure 4.17a, b and Table 4.11).

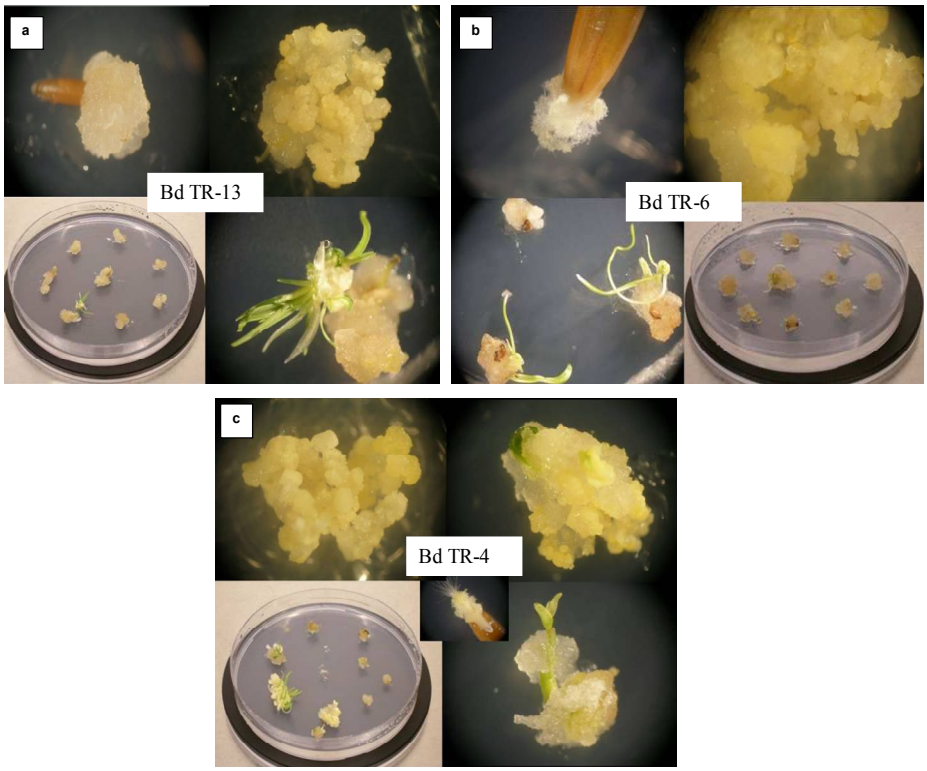


Figure 4.12 Illustration of callus formation and regeneration from mature embryos of selected genotypes, **(a)** BdTR13, **(b)** BdTR6; **(c)** BdTR4 under optimized conditions. Embryogenic callus formation could be distinguished with its yellowish color and non-translucent form.

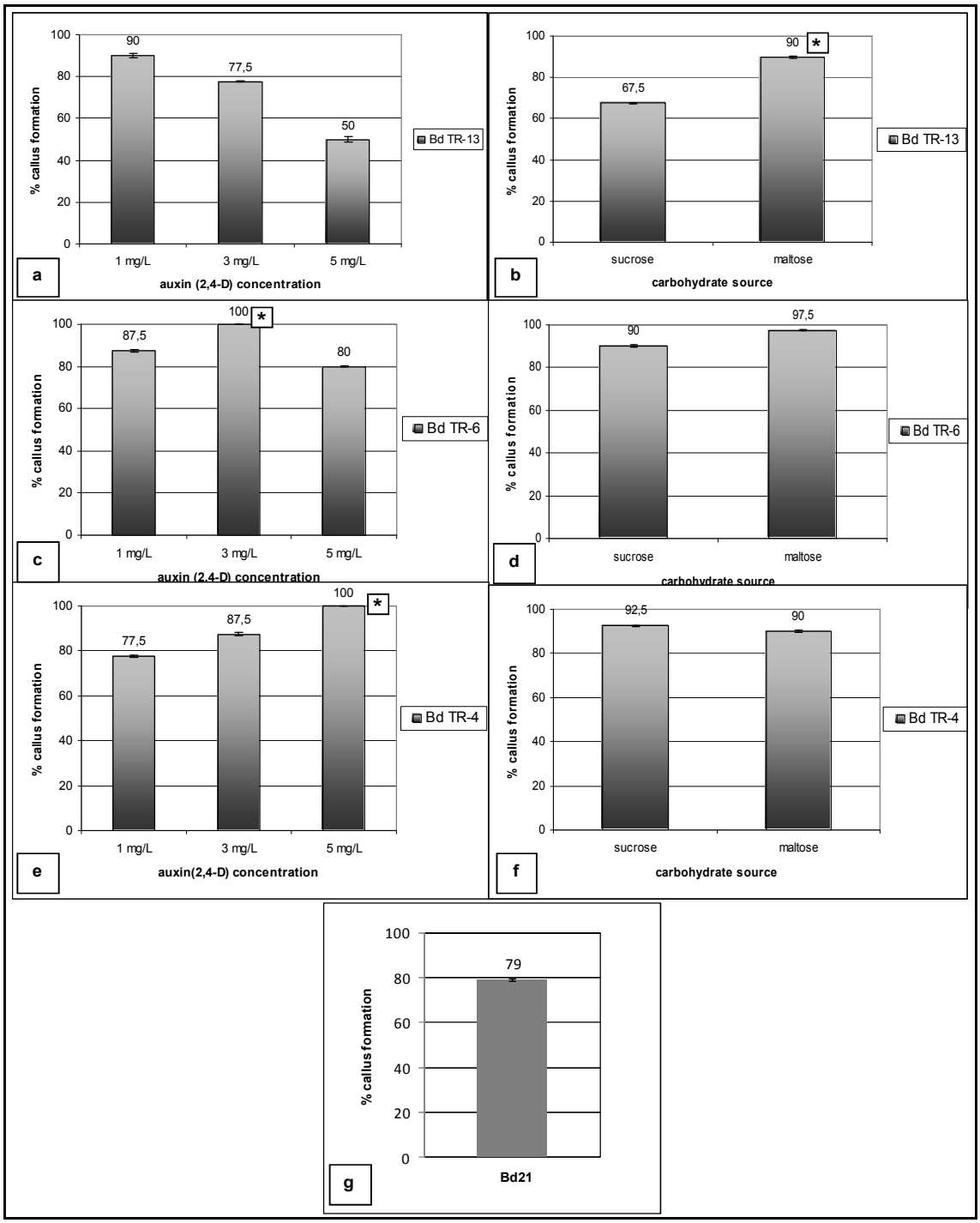


Figure 4.13 Callus formation percentages of three different *Brachypodium* genotypes; **(a, b)** BdTR13, **(c, d)** BdTR6, **(e, f)** BdTR4 and **(g)** Bd21 line. Mature embryos were supplemented with; **(a, c, e)** varied concentrations of auxin (2, 4-D) hormone and **(b, d, f)** two different carbohydrate sources. Each value represents the mean ± SE of 4 replicates (20 embryos/replicate). * Significant at $p \leq 0.05$ using Student's t-test analysis (Refer to Appendix F-1).

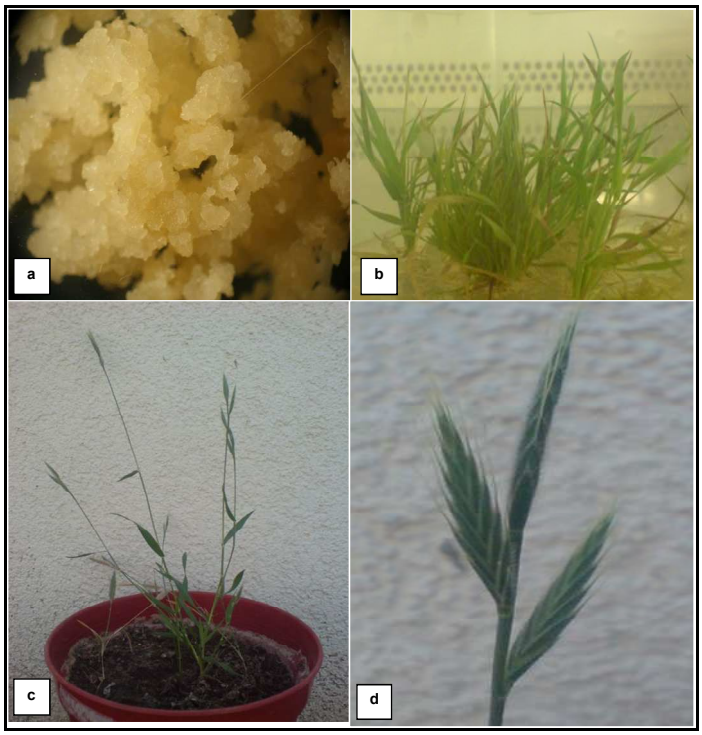


Figure 4.14 Growth cycle of *Brachypodium* Bd21 line. **(a)** Embryogenic callus formation, **(b)** plantlets regenerated in magenta boxes, **(c)** plants grown in soil pots, **(d)** seed head formation illustrated.

Different genotypes of *B. distachyon* exhibited different responses to tissue culture conditions, thus, optimization should be performed when working with new genotypes. The time required for embryogenic callus formation was observed to be varying from 1.5 to 2 months among the genotypes. During callus initiation, two different types of calli were observed as has been noted previously (Draper et al. 2001; Vogel et al. 2006a). One form of callus was translucent and watery and regenerated poorly and the other one was hard, yellowish and exhibited many lobe-like formations that could be separated into pieces easily and indicated the embryogenic callus formation having high regeneration capacity (Figure 4.12a-c).

4.3.2 Regeneration

After callus formation, the calli were transferred to regeneration medium in the petri plates (Figure 4.12a-c). Before the roots were fully developed, regenerated plantlets were subcultured to regeneration media in magenta boxes (Figure 4.15a-d). During this transfer, extra attention should be exerted in order not to give damage to root parts. For BdTR13, regeneration capacity of the calli, previously cultured with 1 mg/L and 3 mg/L 2,4-D, was similar and regeneration capacity of calli previously cultured with 5 mg/L 2,4-D was lower than the others (Figure 4.16a). There was a slight difference between sucrose and maltose additions (Figure 4.16b). On the other hand, with respect to time, regeneration process was slow. For BdTR6, highest regeneration capacity was observed in calli that had been grown in 3 mg/L 2,4-D (Figure 4.16c). Sucrose supplementation of the media seemed to increase the regeneration capacity; however, the difference was insignificant (Figure 4.16d). In BdTR4, most of the plantlets regenerated from 1 mg/L auxin hormone supplemented calli and the percentage decreased in 3 mg/L and 5 mg/L 2,4-D treatments (Figure 4.16e). There was no significant difference in regeneration percentages of the calli grown in sucrose or maltose (Figure 4.16f; 4.14b). On the other hand, regeneration capacity of Bd21 was slightly lower with respect to BdTR4 and BdTR13. We had also some difficulties during the germination of Bd21 line at both *in vivo* and *in vitro* conditions and during its growth at greenhouse conditions with respect to our genotypes (Figure 4.14c, d). This might have caused due to its vernalization requirement (3 weeks). Overall, the highest regeneration capacity was achieved with BdTR4 (grown with 1 mg/L 2,4-D), whereas BdTR6 exhibited the lowest regeneration percentages. The results obtained from BdTR6 were not surprising since there was a necrosis problem with this genotype. Hence, most of the calli were lost due to phenolic compound synthesis of the necrotic tissues. Regeneration capacity of BdTR13 was higher than BdTR6 at all applications. Regeneration capacity of BdTR4 was high and the timing was fast. In addition, this genotype also exhibited higher rates of seed production, fast growth and short life-cycle compared to the other genotypes during the greenhouse experiments (Table 4.1). As a consequence, there was no effect of previous hormone and carbohydrate supplementation for callus induction on callus regeneration. Hence, differences in regeneration capacities were only due to the genotypic effect (Figure 4.17c, d and Table 4.12). Beyond the normal regeneration process, as exhibited in the study of Draper et al. (2001), Christiansen et al. (2005) and Vogel et al. (2006a),

albino shoot formation was also detected in our study at a rate of 0.80% in BdTR4 and 6.25% in BdTR13 (Figure 4.15d). This response might be due to the prolonged periods of callus culture (Altpeter et al. 1996; Zhang et al., 2000).

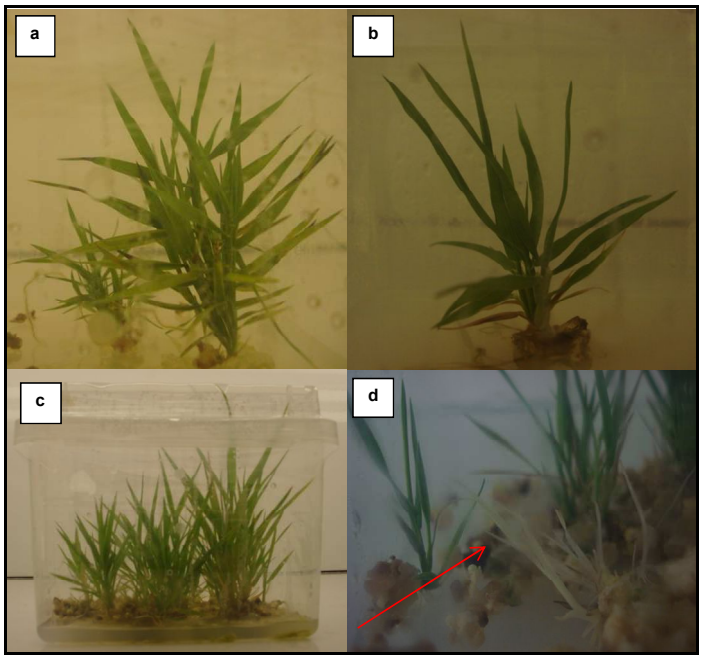


Figure 4.15 Regenerated *Brachypodium* plantlets under tissue culture conditions. Illustration of **(a)** BdTR13, **(b)** BdTR6, **(c)** BdTR4 genotypes with green shoot formation, and **(d)** albino shoot formation (indicated by the arrow) in magenta boxes.

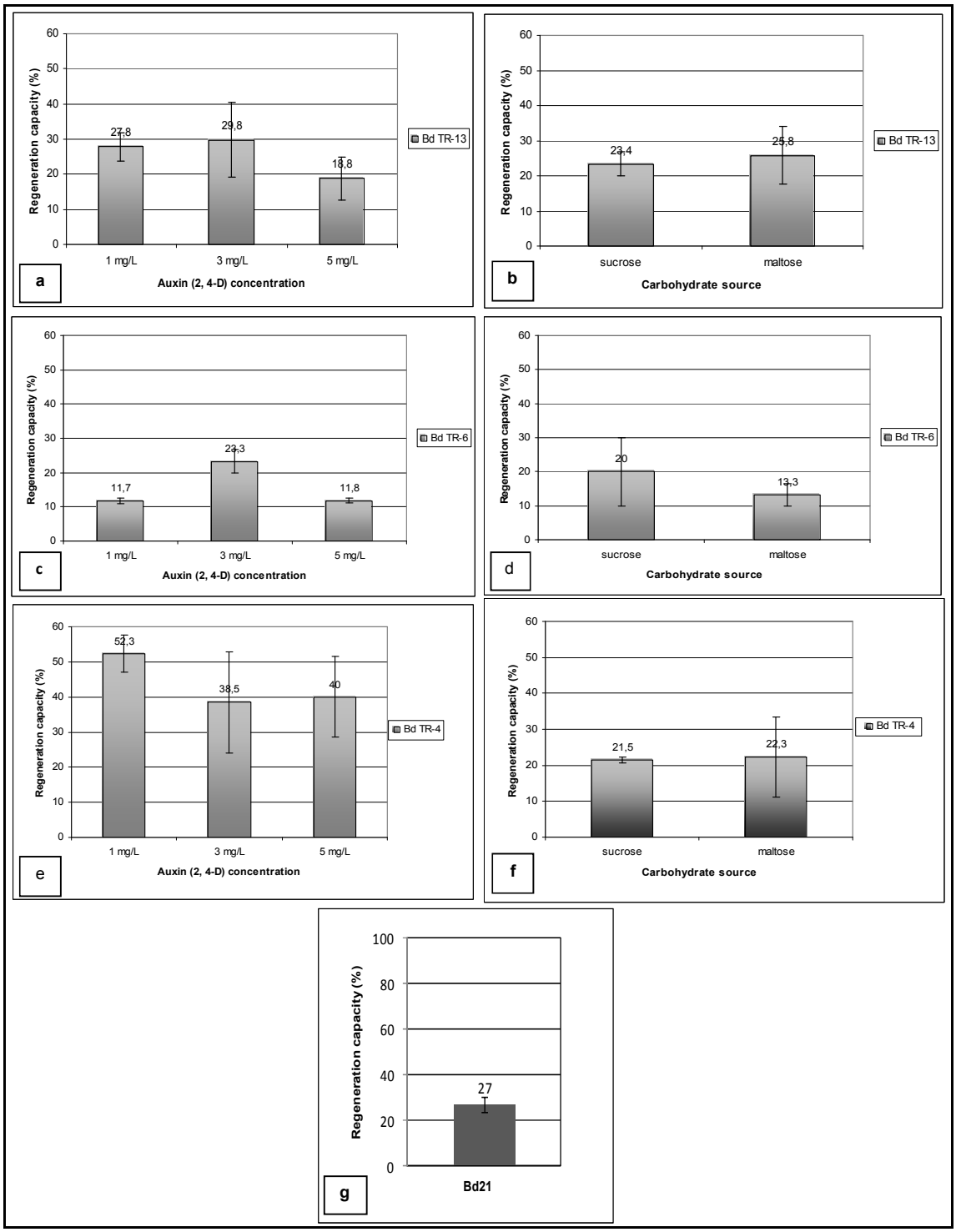


Figure 4.16 Regeneration percentages of three different *Brachypodium* genotypes; **(a, b)** BdTR13, **(c, d)** BdTR6, **(e, f)** BdTR4 and **(g)** Bd21 line. Regenerants were achieved from the calli that were previously supplemented with; **(a, c, e)** varied concentrations of auxin (2, 4-D) hormone and **(b, d, f)** two different carbohydrate sources. Each value represents the mean ± SE of 4 replicates. [Regeneration capacity % = (#of Regenerants / # of embryo with callus) x100]. (Refer to Appendix F-2)

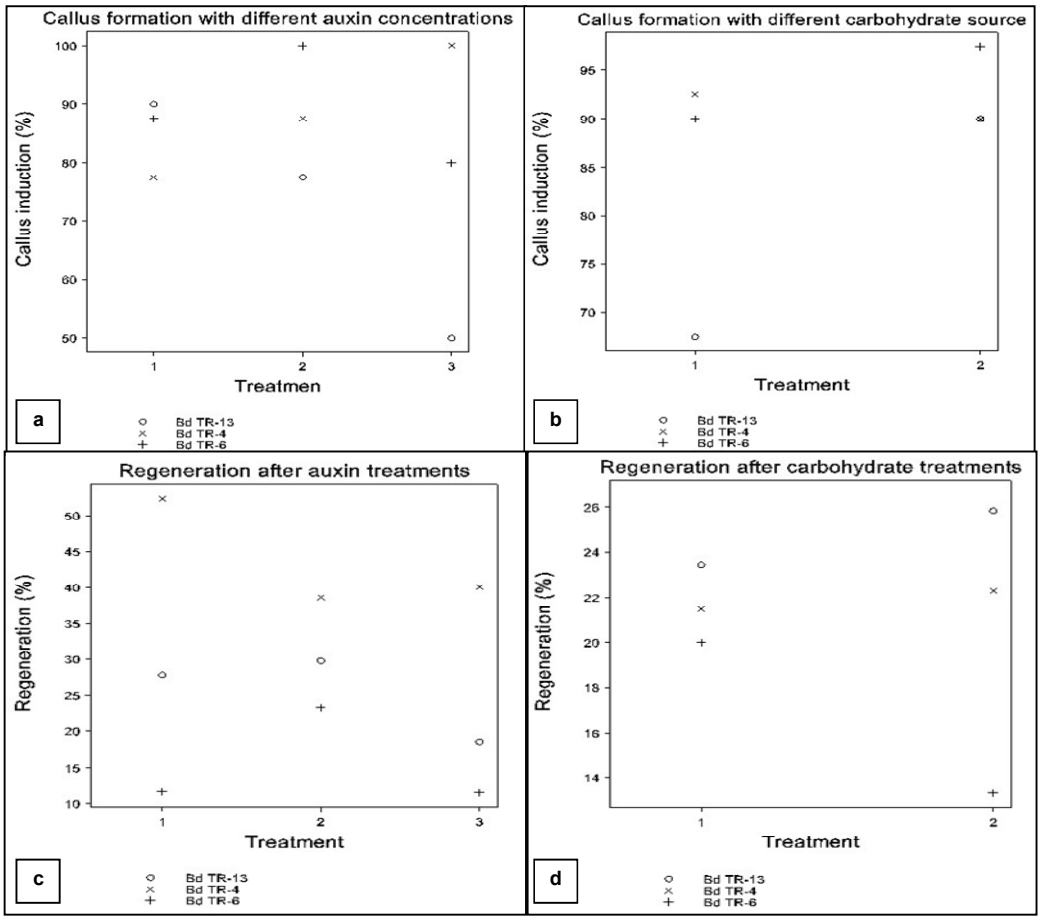


Figure 4.17 Effect of genotype, treatment and their interaction on **(a, b)** callus formation of mature embryos supplemented with varied 2,4-D concentrations or carbohydrate sources and **(c, d)** regeneration of those *Brachypodium* calli. **(a, c)** Treatment 1: 1 mg l^{-1} 2,4-D; Treatment 2: 3 mg l^{-1} 2,4-D; Treatment 3: 5 mg l^{-1} 2,4-D and **(b, d)** Treatment 1: sucrose; Treatment 2: maltose.

Table 4.11 Statistical analysis of the data represented in Figure 4.17 (a, b)

	Hormone concentration effect		Carbohydrate source effect	
Source of variation	d.f.	M.S.	d.f.	M.S.
Genotype	2	1058.3 **	2	516.67 *
Treatment	2	433.3 NS	1	504.17 *
Genotype.treatment	4	1079.2 **	2	316.67 NS
Error	24	179.2	15	96.39

*** Significant at $p \leq 0.001$

** Significant at $p \leq 0.01$

* Significant at $p \leq 0.05$

NS Not significant

Table 4.12 Statistical analysis of the data represented in Figure 4.17 (c, d)

	Hormone concentration effect		Carbohydrate source effect	
Source of variation	d.f.	M.S.	d.f.	M.S.
Genotype	2	1830 ***	2	98.2 NS
Treatment	2	156.2 NS	1	5.9 NS
Genotype.treatment	4	130.7 NS	2	35.2 NS
Error	24	160.5	10	96.39

*** Significant at $p \leq 0.001$

** Significant at $p \leq 0.01$

* Significant at $p \leq 0.05$

NS Not significant

4.4 Microprojectile bombardment

Calli derived from mature embryos of *B. distachyon* (BdTR4, BdTR6 and BdTR13) were bombarded with gold particles of 1.0 and 1.6 μm under six different bombardment pressures-sample plate distances (psi-cm) of 650-6, 650–9, 900–6, 900–9, 1100–6 and 1100–9. Transient GUS expression was observed both in *Brachypodium* calli (Figure 4.18a, b; 4.19b) and in control explants, common bean (*Phaseolus vulgaris* L.) cotyledons (Figure 4.18c; 4.19a) at all pressure-distance combinations used. Transient GUS expression on cotyledons was actually expected to be much higher due to explant type since cotyledons has smooth and flat surface and cover precisely the bombardment area. However, it could be observed in our results that GUS expression of calli was as high as that of cotyledons in various pressure and distance combinations like 1100-6 or higher as in 1100-9.

The statistical analysis of the transient GUS expression results in calli showed that there was no significant difference between pressure-distance combinations for 1.0 μm gold particles but highest efficiency was observed at 1100-9 (psi-cm) (Figure 4.18a). For 1.6 μm gold particles, pressure-distance of 1100-6 (psi-cm) had significantly increased the transient GUS expression (Figure 4.18b). Analysis of the results revealed that gold particle size had no significant effect but pressure-distance values affected the efficiency of transient gene expression significantly (Table 4.13). The interaction between gold particle size and pressure-distance parameters showed an insignificant effect, however, might have caused variation in regeneration due to varied levels of damage caused to the tissues. The high standard deviation between the replicates could be observed widely and could be explained as shot to shot variation that occurred during loading of gold suspension onto the macrocarriers (Özdemir et al., 2005). During this loading step; although many precautions has taken to spread the gold suspension homogenously, it can not be achieved fully. Because these steps are performed manually and gold suspension tends to precipitate quickly.

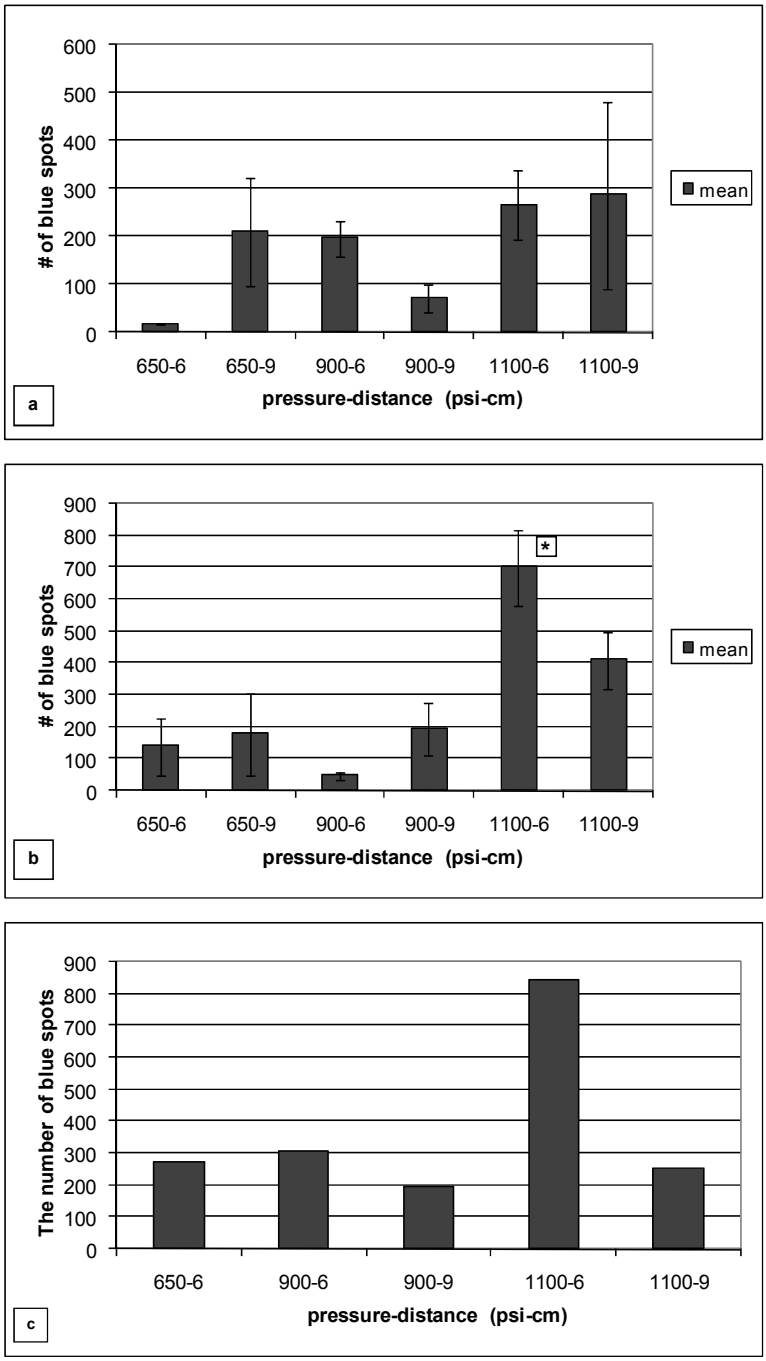


Figure 4.18 Transient GUS expression (number of blue spots) observed on *Brachypodium distachyon* calli bombarded with **(a)** 1.0 μm diameter and **(b)** 1.6 μm diameter gold particles at different pressures and distances, and **(c)** the bean cotyledons as control explants. Each data representing the mean ± SE of 3 replicates (10 calli/replicate). *Significant at $p \leq 0.05$ using Student's t-test analysis (Refer to Appendix F-3, 4).

Table 4.13 Effect of gold particle size (treatment 1) and bombardment pressure-distance (treatment 2) and their interaction on transient GUS expression.

	Hormone concentration effect	
Source of variation	d.f.	M.S.
Treatment 1	1	69382 ^{NS}
Treatment 2	5	162221 *
Treatment1.Treatment2	5	59312 ^{NS}
Error	22	28149

*** Significant at $p \leq 0.001$
** Significant at $p \leq 0.01$
* Significant at $p \leq 0.05$
^{NS} Not significant

According to the results of the regenerated hygromycin resistant plants (Figure 4.19c, e), 40% regenerated after bombarded at a pressure-distance of 650-9 (psi-cm) with 1.0 μm gold particles. Though bombardment of tissues at a pressure-distance of 1100-6 (psi-cm) and with 1.6 μm gold particles resulted in transient transformation efficiency higher than 40%, the hygromycin resistant plants mostly formed albino shoots. Albino shoot formation after transformation was observed at a higher rate with respect to non-bombarded tissues (Figure 4.19f). The increase in albino shoot formation might have not been only due to prolonged tissue culture conditions but also because of bombardment damage and selection pressure applied on the tissues. 68.2% of the putative transgenic plants were regenerated after bombarded with 1.0 μm gold particles. BdTR4 had accounted for 84.8% of all hygromycin resistant *B. distachyon* plants regenerated. Average transient transformation efficiency within all three genotypes was 28.7% with BdTR4 and BdTR6 displaying respectively the highest and lowest efficiencies. Transient transformation efficiency of control line Bd21 was 12.3%.

All the calli were carried through selection and regeneration process to calculate the transient transformation efficiency (number of plantlets/number of bombarded calli). T_0 plants were recovered in soil and seeds collected (Figure 4.19g, h). However, lower regeneration capacity and transformation efficiency of BdTR6 with respect to other genotypes, T_1 plants could not be recovered. Regenerated transformed plants from line Bd21 produced spikelets but seed formation could not be completed. From the selected hygromycin resistant lines of BdTR4 and BdTR13, T_1 seeds have been collected until now.

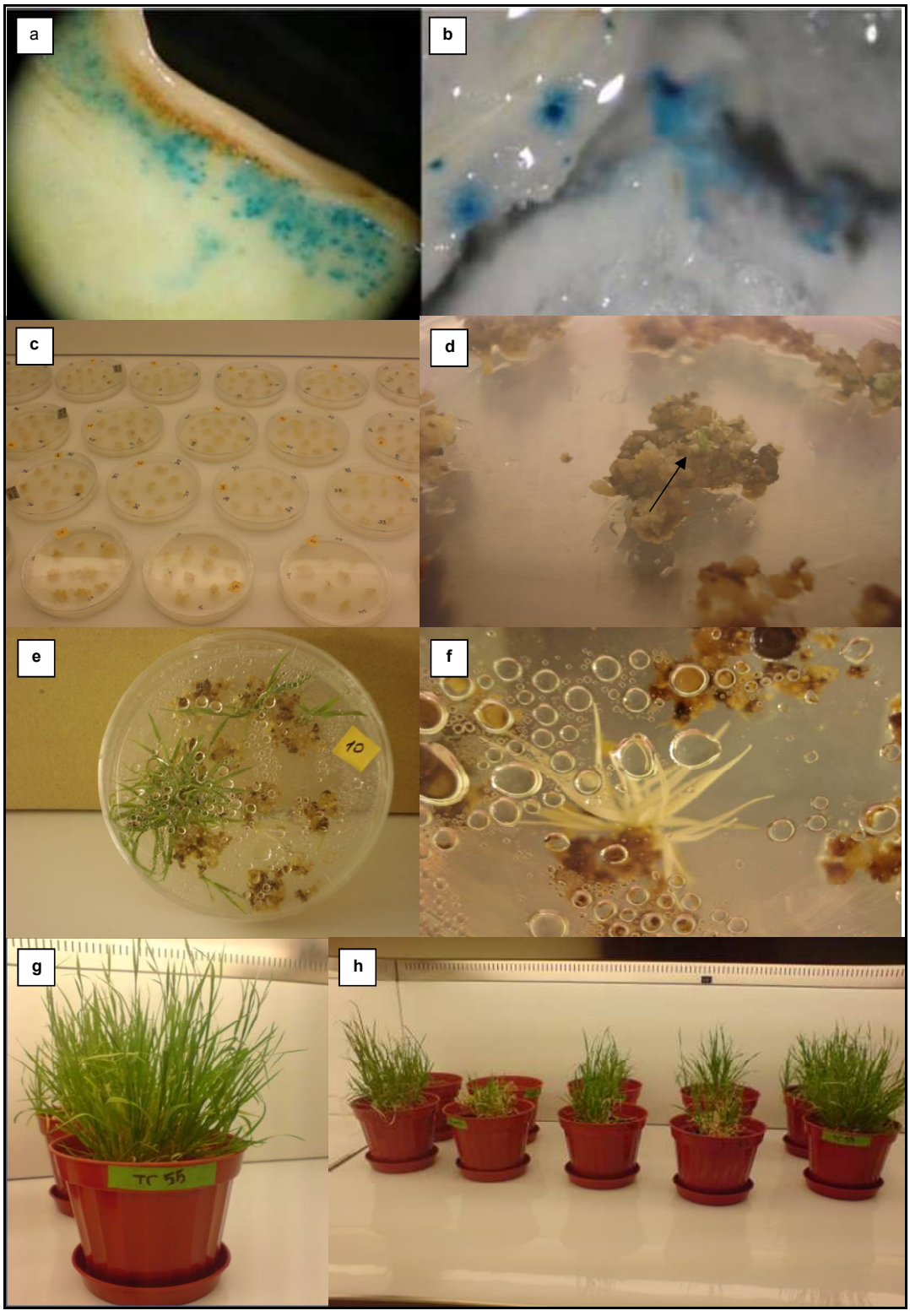


Figure 4.19 Transient GUS expression observed in **(a)** common bean cotyledons and **(b)** *Brachypodium distachyon* calli that exhibited GUS-positive blue spots, stained 48 h following bombardment. **(c, d)** Antibiotic selection of transformed *Brachypodium* calli and arrow indicating the side of shoot proliferation; **(e)** regeneration of green shoots from the transformed tissues; **(f)** albino shoot formation after selection and **(g, h)** regeneration of putative transgenic lines.

PCR was performed for hygromycin resistant T_0 plants using genomic DNA extracted from leaves and primers designed to amplify a portion of the CaMV35S promoter. Five transformed lines for each genotype (BdTR4, BdTR6 and BdTR13) produced a fragment band of the expected size of 195 bp (Figure 4.20).

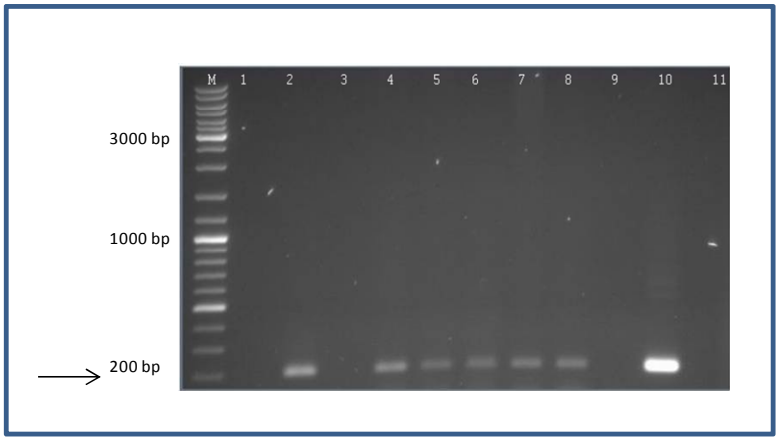


Figure 4.20 PCR amplification of CaMV35S promoter region in five different transformed lines of *Brachypodium distachyon*, visualized with ethidium-bromide stained 1% agarose gel. Arrow indicates the expected PCR product. Lane M: Molecular marker-Gene Ruler^{TM} DNA ladder mix; 1: wild-type; 2: positive control; 3: negative control; Lanes 4-8: hygromycin resistant plants; 9: negative control; 10: positive control; 11: wild-type.

4.5 *Agrobacterium*-mediated transformation

4.5.1 Transformation conditions

The Turkish *Brachypodium* genotypes (BdTR4, BdTR6 and BdTR13) and the control line Bd21 were also used in our *Agrobacterium*-mediated transformation studies to further analyze their potential as model species. The calli were infected with the virulent strains of *Agrobacterium*; LBA4404, EHA105, and AGL1. These strains were chosen according to their transformation efficiency and frequent use in monocot systems. The first two strains have not been yet used in any of the *Brachypodium* transformation studies before.

At the first step of transformation, *vir* (virulence) genes were induced by adding acetosyringone to the growth medium of bacteria. Acetosyringone as phenolic compound has importance at induction of virulence. YEB-MES medium with acetosyringone supplementation was used for the growth of EHA105 and LBA4404 strains whereas AGL1 was grown in MGL medium. AGL1 could not be grown in YEB-MES medium. The bacterial cells were harvested when OD (Abs: 600nm) was between 0.6-0.8. When bacteria were over-grown, transient gene expression efficiency decreased especially with EHA105 strain. Inoculation of explants with bacteria was carried out from 15 min up to 1 hour. However; with increased inoculation time, bacterial elimination was impossible with the use antibiotic concentrations during growth of the transformed tissues. Antibiotic concentration was increased for bacterial elimination, but it exerted a negative effect on the viability of the tissues. Although transformation efficiency could be increased with higher inoculation times, the optimum time was set as 15-20 min and at most 30 min. Time condition of cocultivation was also similarly set. In most of the studies, the cocultivation time was signed as 3 or 4 days, but in our study we found out the optimum time as 2 days and at most 3 days. After this time, it was again hard to eliminate bacterial contamination. After cocultivation step, washing of the explants with antibiotic was not sufficient so that we used two different antibiotics as Cefotaxime or Augmentin. During regeneration and selection of the transformed calli,

both antibiotics were tested separately in the growth medium for bacterial elimination. But, Augmentin caused severe damage on the tissues and the experiments were carried out using Cefotaxime at an optimum concentration of 250 mg/L. Prolonged exposure of the tissues to antibiotic for bacterial elimination was found to cause a decrease in cell viability (Wojtania et al., 2005). Also in our study, the LBA4404 infected explants did not need to be exposed to antibiotic prolonged times due to ease of bacterial elimination from these tissues that viability of these tissues was higher with respect to the tissues transformed with other strains. These preliminary conditions not only affect the transient GUS expression but also the transformation efficiency of the infected tissues.

4.5.2 Transient GUS expression efficiency and effect of wounding type

Wounding of the tissues is another important step for *Agrobacterium*-mediated transformation to increase transformation efficiency. For that reason, two kinds of wounding were applied. Macro-wounding was applied by giving damage to the tissues by a blade and micro-wounding was performed using microprojectile-mediated wounding of the tissues. The tissues were bombarded with pressure and distances of 650 psi-9 cm with gold particles of 1.0 μm in size and 1100 psi -6 cm using 1.6 μm sized gold particles.

Genotype, bacterial strain and wounding type were compared to observe their effect on transient gene expression efficiency (Table 4.14). Transient GUS expression was observed on callus tissues of all native *Brachypodium* genotypes and Bd21 line after infection with all strains of *Agrobacterium* used (Figure 4.21a, b, c). The transformed explants that were not exposed to histochemical GUS staining were selected with either kanamycin or PPT (Figure 4.21d, e). In macro-wounding, transient GUS expression was highest in BdTR4 (infection with AGL1) with GUS foci/Callus# of 6.8. Percentage of GUS gene expressing calli among total number of calli used was 93% in BdTR4 (EHA105) with the highest rate. Transient GUS expression was lowest in BdTR6 (EHA105) in both terms. Micro-wounding of the tissues by microprojectile bombardment increased the transient transgene expression of the infected tissues. Using gold particles in the size of 1.0 μm (650 psi-9 cm), transient GUS expression was increased up to 8.9 in BdTR4 (EHA105), 7.6 in BdTR6 (LBA4404), 5.1 in BdTR13

(AGL1) and 8.6 in Bd21 (LBA4404). By bombardment of tissues with 1.6 µm sized gold particles at 1100 psi-6 cm, the highest transient GUS expressions were achieved with BdTR6 (LBA4404) as 10.4, BdTR13 (AGL1) as 8.3 and 7.7 in Bd21 (AGL1). Bombardment of the callus tissues several times (2 or 3 times) had a negative effect on the transgene expression that no GUS expression was observed in almost all of these tissues. The transgene expression of the genotype and bacterial strain combinations were grouped according to their wounding type with the standard errors in Appendix F-5.

Genotypes (Bd TR4, 6, 13 and Bd21), *Agrobacterium* strains (LBA4404, EHA105, AGL1) and wounding type (macrowounding-blade; microwounding-bombardment with 1.0 um gold at 650psi-9cm and 1.6 um gold at 1100psi-6cm) used for transformation of the calli were evaluated for their individual and .interaction effect on the transgene expression efficiency. All parameters were found to have significant effect on transient transformation efficiency under *Agrobacterium*-mediated transformation (Table 4.15, 4.16).

Table 4.14 Transient GUS expressions of different *Brachypodium* genotypes recorded using different *Agrobacterium* strains and wounding type. Each data representing the 2 replicates (30 calli/replicate).

Genotype	Bacterial strain	Macro-wounding with blade		Micro-wounding by microprojectile bombardment			
				1.0 µm size gold		1.6 µm size gold	
		GUSfoci/Callus#	*GUS+Callus %*	*GUSfoci/Callus#*	*GUS+Callus %*	*GUSfoci/Callus#*	*GUS+Callus %*
BdTR4	*LBA4404*	4,4	83	3,0	60	4,6	43
	EHA105	4,7	93	8,9	85	3,3	53
	AGL1	6,8	67	5,4	80	6,2	68
BdTR6	*LBA4404*	3,2	53	7,6	35	10,4	97
	EHA105	2,3	20	4,8	30	4,4	70
	AGL1	4,3	40	3,1	45	5,4	45
BdTR13	*LBA4404*	4,4	73	4,2	30	4,6	40
	EHA105	2,8	60	0,0	0	0,0	0
	AGL1	2,9	47	5,1	38	8,3	28
Bd21	*LBA4404*	4,1	53	8,6	82	4,5	82
	EHA105	3,3	60	6,9	53	6,5	50
	AGL1	6,3	40	8,0	47	7,7	56

GUS foci/Callus#: the average number of GUS-positive foci per callus

GUS+ Callus%: percentage of GUS gene expressing calli among total number of calli used in the experiment

Table 4.15 Statistical analysis of the data represented in Table 4.14

Variable		t-Test		
Genotype	**Strain**	***Blade/1.0***	***Blade/1.6***	***1.0/1.6***
BdTR4	*LBA4404*	**0,049***	0,642	0,092
	EHA105	**0,029***	**0,010****	**0,020***
	AGL1	0,193	0,642	0,259
BdTR6	*LBA4404*	**0,029***	**0,002****	**0,045***
	EHA105	**0,005***	**0,030***	1
	AGL1	0,116	0,227	0,073
BdTR13	*LBA4404*	0,293	0,293	0,106
	EHA105	**0,023***	**0,023***	
	AGL1	**0,045***	**0,008****	**0,028***
Bd21	*LBA4404*	**0,001*****	0,259	**0,010****
	EHA105	**0,035***	**0,040***	0,106
	AGL1	**0,027***	0,075	0,504

*** Significant at $p \leq 0.001$

** Significant at $p \leq 0.01$

* Significant at $p \leq 0.05$

NS Not significant

Table 4.16 Wounding type, genotype, bacterial strain and their interaction effect on transient GUS expression of the calli of *Brachypodium distachyon* transformed with *Agrobacterium.*

Source of variation	d.f.	M.S
genotype	3	21,11*
strain	2	20,85*
wounding	2	14,97*
genotype.strain	6	14,79*
genotype.wounding	6	7,74*
strain.wounding	4	5,20*
genotype.strain.wounding	12	7,41*

* Significant at $p \leq 0.001$

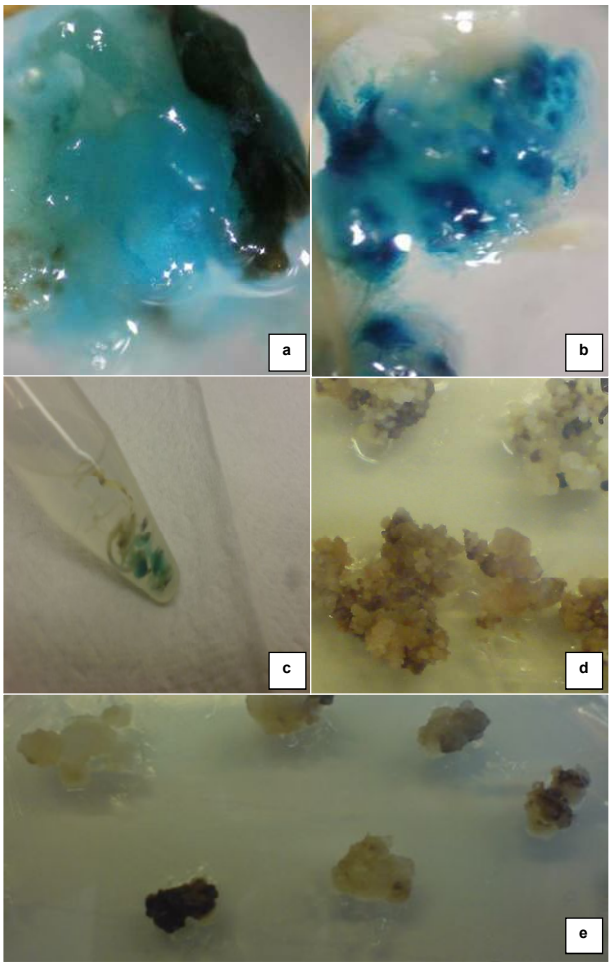


Figure 4.21 Transient GUS expression observed on callus tissues of *Brachypodium* after infection with *Agrobacterium* (a) BdTR6-LBA4404, (b) BdTR4-AGL1, (c) BdTR13-EHA105 using macro-wounding and selection of the tissues after transformation of **(d)** BdTR13- LBA4404 with 50 mg/L Kanamycin antibiotic, **(e)** BdTR6-AGL1 with 3 mg/L PPT herbicide.

Identification of genotypes with a high level of gene delivery efficiency that possess the genetic potential of regeneration under tissue culture conditions is crucial for the development of transgenic genotypes with biotic and abiotic stress, improved nutrient use efficiency, improved quality as food and feed, and higher biomass for energy purposes. This study, in addition to the establishment of new *Brachypodium* germplasm collection, has identified diploid and polyploid inbred lines with a high level of gene delivery efficiency that had also good responses at tissue culture conditions. They could aid further the genetic and genomics studies in *Brachypodium* and other temperate grass species.

CHAPTER 5

5 DISCUSSION

Researches on *Brachypodium distachyon* has been accelerated from the time it was proposed as model species in 2001 by Draper et al. Its close phylogenetic relation to important cereals and bioenergy crops make it a valuable model species for the grass family with respect to other model plants. Germplasm collections are important for the genetic, genomic and plant breeding studies for plant improvement. For both cultivated and model plants, gene pool availability is required for distinction of new or existing traits and the study of these traits in model species can provide the information to be used in other plants. For instance, it was found that some disease of *Triticeae* and rice could also infect *Brachypodium* species (Draper et al., 2001). There are two germplasm collections for *Brachypodium* that are found in Wales and USA. These collections contain diploid types with haploid chromosome number of five and some of these diploid accessions are native to Turkey (Garvin et al., 2008). Since Turkey is a rich source for *Brachypodium* diversity, a new collection that represents its different geographical regions should have been introduced to the scientific researches.

We collected 1101 *Brachypodium* individuals from diverse regions of Turkey including Marmara, Aegean, Mediterranean, South and Central Anatolian. We created 146 lines and 116 of them (diploid lines) are freely available for the scientific community (refer to Appendix D for all inbred lines). These lines were placed under 13 groups according their morphological characters. According to mean nuclear content, 116 of the lines were classified as diploid and the other 30 lines were polyploid. Thus, diploidy was common among the Turkish *Brachypodium* populations. As reported in the previous reports (Draper et al. 2001; Hasterok et al. 2004, 2006; Jenkins et al. 2003, 2005), chromosome number of 10 with same karyotypes was observed. The phenotypic

variation among the collection was diverse both in qualitative and quantitative traits. *Brachypodium* plant height was assessed to be 20 cm at maturity and 80 to 200 seeds yield per plant was observed (Draper et al., 2001). We had the average height of 35 cm and the highest yield was 793 seeds per plant in tetraploid BdTR4 and 214 seeds per plant for diploid BdTR13. The time to seed production was as low as 7 weeks in tetraploid BdTR4 and 8 weeks in diploid Bd TR1. However, this time was determined as 11-18 weeks depending on the vernalization requirements (Draper et al., 2001) and the inbred lines created from those diploid *Brachypodium* needed 3-6 weeks vernalization. Although the vernalization requirement was eliminated for some of the lines by controlling the growth conditions, they could not reach the flowering stage until 4-6 weeks.

Macro- and microelement analysis has not been performed before in *Brachypodium distachyon*. Genotypic effect on the variation of these nutrients was significant. The results evaluated as concentration and content were slightly different. We actually did the data analysis to represent the nutrient amount both in concentration (as % for macroelements and mg/kg for microelements) and content (µg/seed) to achieve more precise results to eliminate the effect of small seed size on the concentration. The significant correlations between N and S, Mn, Zn; S and Mn, Zn; Mg and P, Cu; Cu and Mn showed more distinctly the genotypic variation. In improving crop yield and quality, the consumption and storage of these elements are important. For instance, the Zn composition in some of the genotypes of *Brachypodium* (39 mg/kg for BdTR3 and BdTR13) were found to be higher than the Zn composition in some of wheat cultivars or its wild relatives for which the data was varying from 11to 154 mg/kg (Çakmak et al., 1999; Khan et al., 2006). Besides, selection of crops towards high yield decreases their micronutrient availability such as the low concentrations of Zn and Fe in cultivated modern wheat cultivars and the inadequate uptake of these nutrients in daily human diet from the commercial wheat cultivars causes negative impact on human health (Çakmak et al., 2009). The studies on nutrient availability of crops revealed that there was positive correlation between N, Fe and Zn concentrations such as in wheat (Morgounov et al., 2007) and triticale (Feil and Fossati 1995). We also found a positive correlation between N and Fe; and Zn and Fe concentrations in *Brachypodium* seeds, but the positive correlation between N and Zn concentrations was significant as in the study of Çakmak et al. (2009) where also co-localization of these elements with protein were

shown. The role of grain protein as a sink for Zn and Fe was indicated. In our study, the largest variation in grain concentration was achieved with Ca (2.3-fold) as macronutrient and Fe displayed the highest variation (4.56-fold) in terms grain micronutrient concentration. The association between S and Fe was found to be moderate whereas S and Zn strong and highly significant correlation. The differences in environments were found to have significant effect on the variation of macro- and micronutrients (Gomez-Becerra et al., 2009). The variation in macro- and microelement concentration that was found in different genotypes of *Brachypodium distachyon* could be a potential genetic resource but the effect of environmental interaction on the stability of these nutrients might be further assessed.

In tissue culture experiments, different explant sources of *Brachypodium* were used. Mature embryos, stem and root parts were the responding tissues to callus initiation. At the end of six weeks, it was observed that only 2,4-D as auxin type could be efficiently used rather than IAA or NAA, and the tissues supplied with cytokinin entered necrosis. Due to the high rate of embryogenic callus formation from mature embryos, these explants were selected as target tissues. The Turkish genotypes; BdTR4, BdTR6 and BdTR13 were chosen for tissue culture and transformation studies since they were all superior to others in terms of seed yield (seed#/plant) and two of them (BdTR4 and BdTR6) had shorter life cycles with respect to others. Besides, both polyploid (BdTR4 and BdTR6) and diploid (BdTR4) types of *B. distachyon* were compared. Highest efficiencies of callus formation were achieved at 2,4-D concentrations of 1 mg/L for BdTR13, 3 mg/L for BdTR6 and 5 mg/L for BdTR4. It was found that the hormone concentrations were required to be optimized for each genotype to obtain efficient results. Maltose and sucrose additions as carbohydrate source to the medium did not differ callus formation percentages in BdTR6 (100%) and BdTR4 (100%) but had an effect in BdTR13. However, these carbohydrate source additions were performed at a constant concentration of 2.5 mg/L 2,4-D and the optimum concentration was found to be 1 mg/L for BdTR13. This concentration of 2.5 mg/L could have been a little high but the efficiency of callus yield was increased with maltose addition and the callus formation percentage with sucrose plus 1 mg/L 2,4-D and maltose plus 2.5 mg/L 2,4-D were both at 90%. We used this constant 2,4-D concentration with variable carbohydrate source since callus formation percentages were same at all concentrations in our preliminary results and that results only represented callus initiation percentages,

and also 2,4-D concentration was set to 2.5 mg/L in previous studies (Bablak et al., 1995; Draper et al., 2001; Vogel et al., 2006a). We used the diploid Bd21 line as control in our experiments since this inbred line was used for transformation studies previously and the tissue culture conditions were optimized (Vogel et al., 2006a). Besides, it is being used as the model line in genetic and genomic researches. Bd21 mature seeds showed 79% callus formation when 3 mg/L 2,4-D and MS medium was used. We had lower callus formation percentages when they were propagated with LS medium and 2.5 mg/L 2-4-D as described in Vogel et al. (2006a). They had lower percentages of embryogenic callus formation also with the 19 genotypes used. In addition, in the study, Bd 21 was one of the genotypes performing lowest percentage of embryogenic callus (2.9%). Later that, Bd21 was indicated to be less responsive than Bd21-3 since embryogenic callus formation from immature embryos of Bd21 and Bd21-3 was 68% and 94% respectively and MS medium was used in the study rather than LS medium (Vain et al., 2008). Although we used mature embryos of *Brachypodium*, our callus formation results with diploid line BdTR13 (90%) and Bd21 (79%) was close to or higher than the previous studies. This might have been due to the fact that they had cultured the tissues at 28 °C whereas we used temperature conditions of 25 °C. The other reason could be the genotypic effect for BdTR13 and use of 3 mg/L concentration of 2,4-D for the callus induction medium of Bd21. In none of the tissue culture studies in *Brachypodium*, 2,4-D concentration different than 2.5 mg/L was used which was optimized in the study of Bablak et al. (1995). Though the genotypes used in the later studies were different than those genotypes, the concentrations were not optimized.

Although immature embryos have higher regeneration efficiencies, mature embryos have the advantages that they are available throughout the year, easily collectable, having consistent physiology and they speed up the procedure carried out in transformation studies. Besides, it was shown that with eliminating the removal step of embryo from seed, callus could be obtained by placing the whole seed in the callus induction media. Then the calli were subcultured by removing from the seeds when they could be handled after 2 weeks. This step decreases the time spent for explant preparation in tissue culture studies and make it easier since the embryos are so small to be removed without microscope.

We obtained higher callus production frequencies in tetraploid lines than diploid lines and supported with the results of Christiansen et al. (2005). They obtained callus formation at 67% in diploid and 91% in polyploid lines, whereas our results were consistent with tetraploid lines at a percentage of 100% and 90% for the diploid line. We had observed two kinds of callus formations as described in results section and the hard and yellowish ones were embryogenic calli as noted previously (Draper et al. 2001; Vogel et al. 2006a). Overall, it was found that genotype and genotype-hormone interaction effect were highly significant on callus formation percentages and there was no significant effect of carbohydrate on callus formation efficiencies.

Regeneration percentages of these calli were highest in polyploid line BdTR4 with 52.3% which had also the fastest growth and shortest life cycle in greenhouse conditions. The other lines had 22.3% (BdTR6) and 29.8% (BdTR13) regeneration at most whereas the control line Bd21 exhibited 27% regeneration. Our diploid line (BdTR13) had similar or even slightly higher regeneration when compared to Bd21. Although BdTR6 was polyploid and regeneration with higher percentages were expected, the necrosis problem, as mentioned in results section, with this line lower the efficiency of regeneration. Since elimination of every necrotic tissue decreases the amount of cultured tissues or the proliferated callus amount, it was obvious to achieve these results with BdTR6. Statistical analysis showed that there was no effect of previous treatments during callus formation stage but the genotypic effect was significant. In addition, albino shoot formation was achieved at a rate of 0.80% in BdTR4 and 6.25% in BdTR13 other than green shoot formation which was mentioned in other studies (Draper et al., 2001; Christiansen et al., 2005; Vogel et al., 2006a). However, in the study of Draper et al. (2001), the albino shoot formation percentage was 7% (in LS medium) and increased up to 45% (in N6 medium) with immature embryo derived calli and to 53% with mature embryo derived calli (Vogel et al., 2006a) which were higher than the albino shoots we observed from mature embryo derived calli. The higher percentage albino shoot formation of BdTR13 with respect to BdTR4 could be due to the incubation time of BdTR13 in callus culture. The prolonged tissue culture time affects the overall regeneration capacity (Altpeter et al., 1996; Zhang et al., 2000). The embryogenic callus formation achieved with varying time up to 2 months depending on the genotype used. BdTR13 had the latest proliferation of embryogenic callus where BdTR4 had the fastest timing. We did not get any albino shoot formation

with the line BdTR6. Albinism prevents to achieve higher transformation efficiencies. We have obtained similar or higher regeneration percentages when compared with the regeneration percentages of mature embryos (28.8%) in the study of Vogel et al. (2006a). In that study, they could not observe any regeneration from Bd21 and regeneration from immature embryos were 37.3%.

The transformation of *Brachypodium* calli was done by using both microprojectile bombardment and *Agrobacterium*-mediated transformation method. From six different bombardment pressure and distance combinations, 1100-9 (psi-cm) using 1.0 µm sized gold particles and 1100-6 (cm) using 1.6 µm sized gold particles resulted in highest transient GUS efficiencies. Although bombardment of tissues with 1100-6 (psi-cm) with 1.6 µm gold particles had significant transient GUS expression and gave transient transformation efficiency higher than 40%, the hygromycin resistant plants mostly formed albino shoots (higher than non-transformed tissues). This high rate of albinism might have been due to selection pressure and bombardment damage exerted on the tissues since the tissues bombarded at other pressure-distance combinations did not regenerate albino shoots at this rate. Average transient transformation efficiency within all three genotypes was 28.7%. Overall 1.0µm sized gold particles and 650-9 (psi-cm) pressure-distance combinations and line BdTR4 supplied the highest percentage of hygromycin resistant green shoot formation. The bombardment of highly regenerated tissues with low particle velocity and smaller sized gold particles could be the reason of this result. The polyploid types resulted in higher transformation efficiencies of 14% with GUS gene in Christiansen et al. (2005) compared to diploid types. The genotypes used in this study including Bd21 were not used before in any of the transformation studies via microprojectile bombardment. We had obtained high transient transformation efficiencies and PCR confirmation of the transgene expression in putative T_0 transgenic lines were displayed by using the designed primers for the amplification of a portion of CaMV35S promoter region. T_0 and T_1 plants were obtained from Turkish genotypes but not from Bd21. We had some difficulties with this genotype (non-transformed lines) during its growth in greenhouse conditions. It was affected from environmental stress easily and it needed 3 weeks of vernalization.

Olsen et al. (2006) using the regeneration and transformation protocol of Christiansen et al. (2005) introduced two flowering genes from ryegrass and *Arabidopsis*. The

expression of this functional alien gene in *Brachypodium distachyon* showed the potential use of this plant as a model species. However, there is still limited amount of transformation studies that has been done with *B. distachyon*. There are three transformation studies with microprojectile bombardment (Draper et al., 2001; Christiansen et al., 2005; Olsen et al., 2006) and four transformation studies by *Agrobacterium* (Vogel et al., 2006a; Pacurar et al., 2007; Vogel and Hill, 2007; Vain et al., 2008). So, further tissue culture and transformation studies using new genotypes and improvements in transformation efficiencies are required with this model plant.

Agrobacterium-mediated transformation of *B. distachyon* in previous studies showed transformation frequency of 80% (BDR018 line-AGL1 strain) in the study of Pacurar et al. (2007); 0.4-15% (diploid and polyploid lines-AGL1) in the study of Vogel et al. (2006a) where Bd21 had the efficiency of 4.2%; 10-41% (Bd21-3 line-AGL1) in the study of Vogel and Hill (2007); and 17% (Bd21 line-GFP gene) in the study of Vain et al. (2008).

For the first time in our study, the bacterial strains LBA4404 and EHA105 for transformation were used and the wounding type effect in transient transgene expression of this species was compared. We could not yet have completed the regeneration studies that all the results were exhibiting the transient GUS expression. With the optimized protocol for each genotype and bacterial strain used, wounding effect was assessed. It was found that all factors individually and together affected the transient transgene expression efficiency significantly. The highest transient GUS expression was with 10.4 (GUS foci/callus#) for the line BdTR6 that was wounded by 1.0 µm sized gold particles and infected with the strain LBA4404. The other three highest expressions were 8.9, 8.3 and 8.0 with "BDTR4-EHA105-1.0 µm gold", "BdTR13-AGL1-1.6 µm gold" and "Bd21-AGL1-1.0 µm gold" combinations respectively. All genotypes responded efficiently to *Agrobacterium*-mediated transformation with the all strains used. But it was found out that bacterial elimination was easier and faster in the tissues that were infected with LBA4404. Since antibiotic usage for bacterial elimination causes the viability of the tissues to decrease, LBA4404 strain had this advantage over the others. Although best wounding type was changing for every genotype-bacterial strain combinations, microprojectile-mediated type of wounding was found to be superior over macro-wounding with blade cutting for the improvement of transient gene

expression. Although the type of wounding affected the transformation efficiencies in various species differently, in many studies it was recorded that it enhanced the transformation frequencies like in the study of Zuker et al. (1999); Lucas et al. (2000) and Droste et al. (2000).

CHAPTER 6

6 CONCLUSION

A new *Brachypodium* germplasm collection was established from 146 lines (116 of them diploid lines) representing different geographic regions of Turkey. It is freely available for the scientific community. The phenotypic variation among the collection was diverse both in qualitative and quantitative traits. The lines were placed under 13 phenotypic groups based on their morphological traits. Diploid and polyploid lines with high yield, short life cycle and without any vernalization requirement were characterized. Diploidy was found to be common among the Turkish *Brachypodium* populations. Macro- and microelement analysis has not been performed before in *Brachypodium distachyon*. Genotypic effect on the variation of these nutrients was significant.

Mature embryos, stem and root parts were the responding tissues to callus initiation. Due to the high rate of embryogenic callus formation from mature embryos, these explants were selected as target tissues. 2,4-D as auxin type rather than IAA or NAA and MS medium rather than LS increased the callus formation percentages. It was found that the hormone concentrations were required to be optimized for each genotype to obtain efficient results. Higher callus production frequencies were obtained from tetraploid lines than diploid lines. They were consistent with tetraploid lines at a percentage of 100% and 90% for the diploid line. The control line Bd21 showed 79% callus formation. Overall, it was found that genotype and genotype-hormone interaction effect were highly significant on callus formation percentages and there was no significant effect of carbohydrate on callus formation efficiencies.

Regeneration percentages of these calli were highest in polyploid line BdTR4 with 52.3% which had also the fastest growth and shortest life cycle in greenhouse conditions. The other lines had 22.3% (BdTR6) and 29.8% (BdTR13) regeneration at most whereas the control line Bd21 exhibited 27% regeneration. There was no effect of previous treatments during callus formation stage but the genotypic effect was significant.

1100-9 (psi-cm) using 1.0 µm sized gold particles and 1100-6 (cm) using 1.6 µm sized gold particles resulted in highest transient GUS efficiencies. 1.0µm sized gold particles and 650-9 (psi-cm) pressure-distance combinations and line BdTR4 supplied the highest percentage of hygromycin resistant green shoot formation. Average transient transformation efficiency within all three genotypes was 28.7%.

The highest transient GUS expression was with 10.4 (GUS foci/callus#) for the line BdTR6 that was wounded by 1.0 µm sized gold particles and infected with the strain LBA4404. The other three highest expressions were 8.9, 8.3 and 8.0 with "BDTR4-EHA105-1.0 µm gold", "BdTR13-AGL1-1.6 µm gold" and "Bd21-AGL1-1.0 µm gold" combinations respectively. All genotypes responded efficiently to *Agrobacterium*-mediated transformation with the all strains used. It was found that genotype, bacterial strain and wounding effect all affected the transient transgene efficiency significantly. Although best wounding type was changing for every genotype-bacterial strain combinations, microprojectile-mediated type of wounding was found to be superior over macro-wounding with blade cutting in the improvement of transient gene expression.

The control line Bd21 were not used before in any of the transformation studies *via* microprojectile bombardment. For the first time; all Turkish *Brachypodium* genotypes were described, the effect of wounding types in transgene expression of this species was compared, and *Agrobacterium* strains LBA4404 & EHA105 were used with this species for transformation.

The diploid line BdTR13 and the polyploid line BdTR4 could be used as model lines and could aid further the genetic and genomics studies in *Brachypodium* and other temperate grass species.

REFERENCES

Alibert B, Lucas o, Le Gall V, Kallerhoff J and Alibert G (1999) Pectolytic enzyme treatment of sunflower explants prior to wounding and co-cultivation with *Agrobacterium tumefaciens* enhances efficiency of transient *β*-glucuronidase expression. Phsiol Plant 106: 232-237.

Allwood JW, Ellis DI, Heald JK, Goodacre R and Mur LAJ (2006) Metabolomic approaches reveal that phosphatidic and phosphatidyl glycerol phospholipids are major discriminatory non-polar metabolites in responses by *Brachypodium distachyon* to challenge by *Magnaporthe grisea*. The Plant Journal 46: 351-368.

Altpeter F, Vasil V, Srivastava V, Stoger E and Vasil IK (1996) Accelerated production of transgenic wheat (*Triticum aestivum* L.) plants. Plant Cell Rep 16:12-17.

Altpeter F, Baisakh N, Beachy R et al. (2005) Particle bombardment and the genetic enhancement of crops: myths and realities. Molecular Breeding 15: 305-327.

Anamthawat-Jonsson K, Bödvarsdottir S, Bragason B, Gudmundsson J, Martin PK and Koebner RMD (1997) Wide hybridization between wheat (*Triticum* L.) and lymegrass (*Leymus* Hochst.). Euphytica 93: 293-300.

Aragon-Alcaide L, Miller T, Schwarzacher T, Reader S and Moore G (1996) A cereal centromeric sequence. Chromosoma 105: 261-268.

Armaleo D, Ye G-N, Klein T, Shark K, Sanford J and Johnston S (1990) Biolistic nuclear transformation of *Saccharomyces cerevistae* and other fungi. Cur Genet 17: 97-103.

Arumuganathan K and Earl ED (1991) Estimation of nuclear DNA contents of plants by flow cytometry. Plant Mol Biol Rep 9: 229-241.

Bablak P, Draper J, Davey MR and Lynch PT (1995) Plant regeneration and micropropagation of *Brachypodium distachyon*. Plant Cell Tiss Org Cult 42: 97-107.

Bennett MD LI (2004) Plant DNA C-values database (release 3.0, Dec. 2004). <http://www.rbgkew.org.uk/cval/homepage.html>. [Accessed 20 May, 2007].

Bennett MD and Leitch IJ (2005) Nuclear DNA amounts in angiosperms: progress, problems and prospects. Ann Bot (Lond) 95: 45-90.

Bevan MW (2006) Establishing a BAC-based physical map of *Brachypodium distachyon* as an aid to physical mapping in bread wheat. In: Plant & Animal Genomes XIV Conference, San Diego, CA.

Bevan M, McKenzie N, Trick M, et al. (2007) Developing a genetic map of *Brachypodium distachyon* Bd21. In: Plant & Animal Genomes XV Conference, San Diego, CA.

Bhalla PL, Ottenhof HH and Singh MB (2006) Wheat transformation- an update of recent progress. Euphytica 149: 3535-366.

Bindley D, Scelonge C, Martich J, Burrus M, Sims L and Huffman G (1992) Microprojectile bombardment of plant tissues increases transformation frequency by *Agrobacterium tumefaciens*. Plant Molecular Biology 18: 301-313.

Borlaug NE (1998) Feeding a world of 10 billion people: the miracle ahead. Plant Tiss Cult Biotech 3: 119-127.

Bossolini E, Wicker T, Knobel PA and Keller B (2007) Comparison of orthologous loci from small grass genomes *Brachypodium* and rice: implications for wheat genomics and grass genome annotation. The Plant Journal 49: 704-717.

Boynton JE, Gillham NW, Harris EH, Hosler JP, Johnson AM and Jones AM et al. (1988) Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. Science 240: 1534-1538.

Budak H, Shearman RC, Parmaksiz I, Gaussoin RE, Riordan TP and Dweikat I (2004) Molecular characterization of buffalograss germplasm using sequence related amplified polymorphism markers. Theor Appl Genet 108: 328-334.

Caswell K, Leung N and Chibbar RN (2000) An efficient method for *in vitro* regeneration from immature inflorescence explants of Canadian wheat cultivars. Plant Cell Tiss Org Cult 60: 69-73.

Catalan P, Shi Y, Armstrong L, Draper J and Stace CA (1995) Molecular phylogeny of the grass genus *Brachypodium* P-Beauv based on RFLP and RAPD analysis. Bot. J. Linn. Soc. 117: 263-280.

Catalan P and Olmstead R (2000) Phylogenetic reconstruction of the genus *Brachypodium* Beauv. (Poaceae) from combined sequences of chloroplast gene and nuclear ITS. Plant System Evol 220: 1-19.

Cauderon Y (1978) Use of Agropyron species for wheat improvement. In: Procc. Broadening the Genetic Base of Crops. Zeven AC and Harten AMv (eds.) Pudoc, Wageningen, Netherlands, pp 175-186.

Chan MT, Chang HH, Ho SL, Tong WF and Yu SM (1993) *Agrobacterium*-mediated production of transgenic rice plants expressing a chimeric *α*-amylase promoter/*β*-glucuronidase gene. Plant Molecular Biology 22: 491-506.

Charles M, Choisne N, Samain S, Boudet N and Chalhoub B (2006) *Brachypodium* species as intermediate models for wheat genomics. In: Plant & Animal Genomes XIV Conference, San Diego, CA.

Cheng M, Lowe BA, Spencer TM, Ye X and Armstrong CL (2004) Factors influencing *Agrobacterium*-mediated transformation of monocotyledonous species. In Vitro Cell Dev Biol-Plant 40:31–45.

Christiansen P, Didion T, Andersen C, Folling M and Nielsen K (2005) A rapid and efficient transformation protocol for the grass *Brachypodium distachyon*. Plant Cell Reports 23: 751-758.

Çakmak I, Tolay I, Özdemir A, Özkan H, Öztürk L and Kling CI (1999) Differences in zinc efficiency among and within diploid, tetraploid and hexaploid wheats. Annals of Botany 84: 163-171.

Çakmak I, Wolfgang HP and McClafferty B (2009) Biofortification of durum wheat with zinc and iron. Cereal Chemistry (submitted).

Çürük S, Çetiner S, Elman C, Xia X, Wang A, Yeheskel A, Zilberstein L, Perl-Treves R, Watad AA and Gaba V (2005) Transformation of recalcitrant melon (*Cucumis melo* L.) cultivars is facilitated by wounding with carborundum. Eng Life Sci 5 (2): 169-177.

Dale PJ, Marks MS, Brown MM, Woolston CJ, Gunn H, Boulton MI, Mullineaux PM, Lewis DM, Kemp JM, Chen DF, Gilmour DM and Flavell RB (1989) Agroinfection of

wheat: Inoculation of *in vitro* grown seedlings and embryos. Plant Science 63: 237-245.

Delporte F, Mostade O and Jacquemin JM (2001) Plant regeneration through callus initiation from thin mature embryo fragments of wheat. Plant Cell Tiss Org Cult 67:73-80.

Dewey DR (1984) The genomic system of classification as a guide to intergeneric hybridization with the perennial *Triticeae*. In: Gene manipulation in Plant Improvement. Gustafson JP (ed.) Plenum Publishing Corp., New York, pp 209-279.

Doyle JJ and Doyle JL (1987) A rapid DNA isolation procedure from small quantities of fresh leaf tissues. Phytochem Bull 19: 11-15.

Draper J, Mur LAJ, Jenkins G, et al. (2001) *Brachypodium distachyon*. A new model system for functional genomics in grasses. Plant Physiology 127: 1539-1555.

Droste A, Pasquali G and Bodanese-Zanettini MH (2000) Integrated bombardment and *Agrobacterium* transformation system: an alternative method for soybean transformation. Plant Mol Biol Rep 18: 51-59.

Engvild KC (2005) Mutagenesis of the model grass *Brachypodium distachyon* with sodium azide. Risoe-R-1510 (EN) Report.

Eker S, Öztürk L, Yazıcı A, Erenoglu B, Romheld V and Çakmak I (2006) Foliar-applied glyphosate substantially reduced uptake and transport of iron and manganese in sunflower (*Helianthus annuus L.*) plants. J Agr Food Chem 54 (26): 10019-25.

Faris JD, Zhang Z, Fellers JP and Gill BS (2008) Micro-colinearity between rice, *Brachypodium*, and *Triticum monococcum* at the wheat domestication locus *Q*. Functional & Integrative Genomics 8: 149-164.

Feil B and Fossati D (1995) Mineral composition of triticale grains as related to grain yield and grain protein. Crop Science 35: 1426-1431.

Filiz E, Özdemir BS, Tuna M and Budak H (2009) Diploid *Brachypodium distachyon* of Turkey: Molecular and Morphologic Analysis. In The Proceedings of the 5th International Symposium on the Molecular Breeding of Forage and Turf. Edited by Yamada T and Spangenberg G, pp 83-89. ISBN: 978-0-387-79143-2.

Fofana IBF, Sangare A, Collier R, Taylor C and Fauquet CM (2004) A geminivirus-induced gene silencing system for gene function validation in cassava. Plant Molecular Biology 56: 613-24.

Foote TN, Griffiths S, Allouis S and Moore G (2004) Construction and analysis of a BAC library in the grass *Brachypodium sylvaticum*: its use as a tool to bridge the gap between rice and wheat in elucidating gene content. Functional & integrative genomics 4: 26-33.

Fromm M, Morrish F, Armstrong C, Williams R, Thomas J and Klein T (1990) Inheritance and expression of chimeric genes in the progeny of transgenic maize. Bio/Technology 8: 833-839.

Fu XD, Duc LT, Fontana S, Bong BB, Tinjuangjun P, Sudhakar D, Twyman RM, Christou P and Kohli A (2000) Linear transgene constructs lacking vector backbone sequences generate low-copy-number transgenic plants with simple integration patterns. Transgenic Research 9: 11-19.

Garvin D (2006) *Brachypodium distachyon*. A new model plant for structural and functional analysis of grass genomes. In: Model plants and crop improvement. Koebner R and Varshney R (eds.) CRC Press, Boca Raton, FL, pp 109-123.

Garvin DF, Gu YQ, Hasterok R, Hazen SP, Jenkins G, Mockler TC, Mur LAJ and Vogel JP (2008) Development of Genetic and Genomic Research Resources for *Brachypodium distachyon*, a New Model System for Grass Crop Research. Crop Science 48: S-69-S-84.

Godelle B, Cartier D, Marie D, Brown SC and Siljak-Yakovlev S (1993) Heterochromatin study demonstrating the non-linearity of fluorometry useful for calculating genomic base composition. Cytometry 14: 618-626.

Gomez-Becerra HF, Yazici A, Öztürk L, Budak H, Peleg Z, Morgounov A, Fahima T, Saranga Y and Çakmak I.(2009) Genetic variation and environmental stability of grain mineral nutrient concentrations in *Triticum dicoccoides* under five environments. Euphytica. DOI 10.1007/s10681-009-9987-3.

Gordon-Kamm W, Spencer T, Mangano M et al. (1990) Transformation of maize cells and regeneration of fertile plants. Plant Cell 2: 603-618.

Grayburn WS and Vick BA (1995) Transformation of sunflower (*Helianthhus annuus* L.) following wounding with glass beads. Plant Cell Reports 14: 285-289.

Griffiths S, Sharp R, Foote TN, Bertin I, Wanous M, Reader S, Colas I and Moore G (2006) Molecular characterization of Ph1 as a major chromosome pairing locus in polyploid wheat. Nature 439: 749-752.

Gu YQ, Huo N, Lazo GR, et al. (2007) Towards *Brachypodium* genomics: analysis of 60,000 BAC End Sequences and sequence comparison with cereal crops. In: Plant & Animal Genomes XV Conference, San Diego, CA.

Hamilton CM, Frary A, Lewis C and Tankskey SD (1995) Stable transfer of intact high molecular weight DNA into plant chromosomes. Proc. Natl. Acad. Sci. USA. 93: 9975-9979.

Hasterok R, Draper J and Jenkins G (2004) Laying the cytotaxonomic foundations of a new model grass, *Brachypodium distachyon* (L.) Beauv. Chromosome Research 12: 397-403.

Hasterok R, Marasek A, Donnison IS, et al. (2006) Alignment of the genomes of *Brachypodium distachyon* and temperate cereals and grasses using bacterial artificial chromosome landing with fluorescence *in situ* hybridization. Genetics 173: 349-362.

Hoekema A, Hirsh PR, Hooykaas PJ and Schilperoort RA (1983) A binary plant vector strategy based on separation of *vir* and T region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature 303: 179-180.

Hoffmann K, Verbeek M, Romano A, Dullemans AM, van den Heuvel JFJM and van der Wilk F (2001) Mechanical transmission of poleroviruses. J. Virol. Methods 91: 197-201.

Hoque ME, Mansfield JW (2004) Effect of genotype and explant age on callus induction and subsequent plant regeneration from root derived callus of indica rice genotypes. Plant Cell Tiss Org Cult 78 (3): 217-223.

Huo N, Gu Y, Lazo G, Vogel J, Coleman-Derr D, Luo M, Thilmony R, Garvin D and Anderson O (2006) Construction and characterization of two BAC libraries from *Brachypodium distachyon*, a new model for grass genomics. Genome 49: 1099-1108.

Huo N, Lazo GR, Vogel JP, You FM, Ma Y, Hayden DM, Coleman-Derr D, Hill TA, Dvorak J, Anderson OD, Luo MC and Gu YQ (2008) The nuclear genome of *Brachypodium distachyon*: analysis of BAC end sequences. Functional & Integrative Genomics 8: 135-147.

Huo N, Vogel JP, Lazo GR, You FM, Ma Y, MacMahon S, Dvorak J, Anderson OD, Luo MC and Gu YQ (2009) Structural characterization of *Brachypodium* genome and its syntenic relationship with rice and wheat. Plant Molecular Biology 70: 47-61.

International Rice Genome Sequencing Project (2005) The map-based sequence of the rice genome. Nature 436: 793-800.

Jefferson RA (1987) Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol Biol Rep 5: 387-405.

Jenkins G, Hasterok R and Draper J (2003) Building the molecular cytogenetic infrastructure of a new model grass. In Application of novel cytogenetic and molecular techniques in genetics and breeding of the grasses. Edited by Z. Zwierzykowski et al. Poznan, Poland: Polish Academy of Sciences pp 77-84.

Jenkins G, Mur LAJ, Bablak P, Hasterok R and Draper J (2005) Prospects for functional genomics in a new model grass. In Plant functional genomics. Edited by Leister D. Binghamton: Haworth Press pp 305-325.

Johnston SA, Anziano PQ, Shark K, Sanford JC and Butow RA (1988) Mitochondrial transformation in yeast by bombardment with microprojectiles. Science 240: 1538-1541.

Johri MM and Mitra D (2001) Action of plant hormones. Current Science 80:199-205.

Jones HD, Doherty A and Wu H (2005) Review of methodologies and a protocol for the *Agrobacterium*-mediated transformation of wheat. Plant Methods 1 (5): 1-9.

Kalendar R and Schulman AH (2006) Retrotransposons and their use as molecular markers in *Brachypodium*. In: Plant & Animal Genomes XIV Conference, San Diego, CA.

Kellogg EA (2001) Evolutionary History of the Grasses. Plant Physiology 125: 1198-1205.

Khan M and Stace C (1999) Breeding relationships in the genus *Brachypodium* (Poaceae: Pooideae). Nord J Bot 19: 257–269.

Khan H, Hassan ZU and Maitlo AA (2006) Yield and micronutrient content of bread wheat (*Triticum aestivum* L.) under multinutrient fertilizer - Hal -Tonic. Int J Agri Biol 8 (3): 366-370.

Klee H (2000) A guide to *Agrobacterium* binary Ti vectors. Trends in Plant Science 5(10): 446-451.

Klein TM, Wolf ED, Wu R and Sanford JC (1987). High-velocity microprojectiles for delivering nucleic acids into living cells. Nature 327: 70-73.

Lazar MD, Baenziger P and Schaeffer GW (1984) Combining abilities and heritability of callus formation and plantlet regeneration in wheat (*Triticum aestivum* L.) anther cultures. Theor Appl Genet 68: 131-134.

Lazo GR, Stein PA and Ludwig RA (1991) A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. Bio/Technology 9: 963–96.

Li L, Li R, Fei S and Qu R (2005) *Agrobacterium*-mediated transformation of common bermudagrass (*Cynodon dactylon*). Plant Cell Tissue and Organ Culture 83: 223-229.

Linsmaier EM and Skoog F (1965) Organic growth factor requirements of tobacco tissue cultures. Plant Physiol 18: 100−127.

Lucas O, Kallerhoff J and Alibert G (2000) Production of stable transgenic sunflowers (*Helianthus annuus* L.) from wounded immature embryos by particle bombardment and co-cultivation with *Agrobacterium* tumefaciens. Molecular Breeding 6: 479-487.

Luo H, Hu Q, Nelson K, Longo C et al. (2004) *Agrobacterium tumefaciens*-mediated creeping bentgrass (*Agrostis stolonifera* L.) transformation using phosphinothricin selection results in a high frequency of single-copy transgene integration. Plant Cell Rep 22: 645-652.

Luo M-C, Ma Y, Huo N et al. (2007) Construction Of Physical Map For *Brachypodium distachyon*. In: Plant & Animal Genomes XV Conference, San Diego, CA.

Martin A and Chapman V (1977) A hybrid between *Hordeum chilense* and *Triticum aestivum*. Cereal Research Communication 4: 365-368.

Martin A and Sanchez-Monge-Laguna E (1980) A hybrid between *Hordeum chilense* and *Triticum turgidum*. Cer Res Comm 8: 349-353.

Mayfield S and Kindle K (1990) Stable nuclear transformation of *Chlamydomonas reinhardtii* by using a *C. reinhardtii* gene as the selectable marker. Proc. Natl. Acad. Sci. 87: 2087-2091.

McCabe D and Christou P (1993) Direct DNA transfer using electrical discharge particle acceleration (ACCELL™ technology). Plant Cell Tissue Organ Culture 33: 227-236.

McCormac AC, Fowler MR, Chen DF and Elliott MC (2001) Efficient co-transformation of *Nicotiana tabacum* by two independent T-DNAs, the effect of T-DNA size and implications for genetic separation. Transgenic Research 10: 143-155.

Mockler TC, Givan S, Sullivan C and Shen R (2007) Bioinformatics and genomics resources for *Brachypodium distachyon*. In: Plant & Animal Genomes XV Conference, San Diego, CA.

Mooney PA and Goodwin PB (1991) Adherence of *Agrobacterium tumefaciens* to the cells of immature wheat embryos. Plant Cell Tissue Organ Culture 25:199-208.

Morgonuov A, Gómez-Becerra HF, Abugalieva A, Dzhunusova M, Yessimbekova M, Muminjanov H, Zelenskiy Y, Ozturk L and Cakmak I (2007) Iron and zinc grain density in common wheat grown in Central Asia. Euphytica 155: 193-203.

Murashige T and Skoog F (1962) A revised medium for rapid growth bioassays with tobacco tissue cultures. Physiol Plant 15: 473-497.

Nadolska-Orczyk A, Orczyk W and Przetakiewiez A (2000) *Agrobacterium-mediated* transformation of cereals- from technique development to its application. ACTA Physiologiae Plantarum 22: 77-88.

Norelli J, Mills J and Aldwinckle H (1996) Leaf wounding increases efficiency of *Agrobacterium*-mediated transformation of apple. Horticultural Science 31: 1026-1027.

Olsen P, Lenk I, Jensen CS et al. (2006) Analysis of two heterologous flowering genes in *Brachypodium distachyon* demonstrates its potential as a grass model plant. Plant Science 170: 1020-1025.

Opanowicz M, Vain P, Draper J, Parker D and Doonan JH (2008) *Brachypodium distachyon*: making hay with a wild grass. Trends in Plant Science 13(4): 172-177.

Özdemir BS, Önde S and Özgen M (2005) Marker gene transfer to winter durum wheat (*Triticum durum* Desf.) by microprojectile bombardment method. In: Proceedings of Balkan Scientific Conference of Biology: Molecular Biology Section. Edited by Gruev B, Nikolova M and Donev A, Plovdiv University Press, Plovdiv, Bulgaria, pp 71-78.

Özdemir BS and Budak H (2008) Genetic transformation in diploid Turkish *Brachypodium distachyon* based on a well-established tissue culture system. In vitro Cellular and Developmental Biology 44: 41-41.

Özdemir BS, Hernandez P, Filiz E and Budak H (2008) *Brachypodium* Genomics. Int J of Plant Genomics vol. 2008, Article ID 536104, 7 pages. doi:10.1155/2008/536104.

Özgen M, Türet M, Altınok S and Sancak C (1998) Efficient callus induction and plant regeneration from mature embryo culture of winter wheat (*Triticum aestivum* L.) genotypes. Plant Cell Reports 18:331-335.

Pacurar DI, Thordal-Christensen H, Nielsen KK and Lenk I (2007) A high-throughput *Agrobacterium*-mediated transformation system for the grass model species *Brachypodium distachyon* L. Transgenic Research 17: 965-975.

Paterson AH, Bowers JE and Chapman BA (2004) Ancient polyploidization predating divergence of the cereals, and its consequences for comparative genomics. PNAS 101: 9903-9908.

Pereira A (2000) A transgenic perspective on plant functional genomics. Transgenic Res 9(4-5): 245-260.

Popelka JC and Altpeter F (2003) *Agrobacterium tumefaciens*-mediated genetic transformation of rye (*Secale cereale* L.). Molecular Breeding 11: 203-211.

Powell W and Caligari PDS (1987) The in vitro genetics of barley (*Hordeum vulgare* L.): detection and analysis of reciprocal differences for culture response to 2,4 dichlorophenoxyacetic acid. Heredity 59: 293-299.

Rasco-Gaunt S, Riley A, Barcelo P and Lazzeri PA (1999) Analysis of particle bombardment parameters to optimise DNA delivery into wheat tissues. Plant Cell Rep 19: 118-127.

Repellin A, Baga M, Jauhar PP and Chibbar RN (2001) Genetic enrichment of cereal crops via alien gene transfer: new challenges. Plant Cell Tiss Org Cult 64: 159-183.

Robertson IH (1981) Chromosome numbers in *Brachypodium* Beauv. (Gramineae). Genetica 56: 55-60.

Routledge APM, Shelley G, Smith JV, Talbot NJ, Draper J and Mur LAJ (2004) *Magnaporthe grisea* interactions with the model grass *Brachypodium distachyon* closely resemble those with rice (*Oryza sativa*). Molecular Plant Pathology 5: 253-265.

Saharan V, Yadav RC, Yadav RN and Chapagain BP (2004) High frequency plant regeneration from desiccated calli of indica rice (*Oryza Sativa L.*). Afr J Biotechnol 3(5): 256-259.

Sambrook J, Manniatis T and Fritsch EF (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press. 3rd edition.

Sanford, JC, Klein TM and Wolf ED (1987) Delivery of substances into cells and tissues using a particle bombardment process. Particulate Science and Technology 6: 559-563.

Sanford JC (1990) Biolistic plant transformation. Physiologia plantarum 79: 206-209.

Sharma HC and Gill BS (1983) Current status of wide hybridization in wheat. Euphytica 32: 17-31.

Sheng OJ and Citovsky V (1996) *Agrobacterium*-plant cell DNA transport: have virulence proteins, will travel. Plant Cell 8: 1699-1710.

Shrawat AK and Lörz H (2006) *Agrobacterium*-mediated transformation of cereals: a promising approach crossing barriers. Plant Biotechnology Journal 4: 575-603.

Slater A, Scott N and Fowler M (2003) Plant Biotechnology: The genetic manipulation of plants. Oxford University Press, Inc., New York.

Stanton BG (2003) *Agrobacterium* mediated plant transformation: the biology behind of the "gene jockeying" tool. Microbiology and Molecular Biology Reviews 67(1): 16-37.

Stebbins GL (1971) The morphological, physiological, and cytogenetic significance of polyploidy. In: Chromosomal evolution in higher plants. Barrington FRS and Willis AJ (eds.) Addison-Wesley, Reading, Massachusetts.

Sunagawa M and Magae Y (2002) Transformation of the edible mushroom *Pleurotus ostreatus* by particle bombardment. FEMS Microbiology Letters 211: 143-146.

Taylor NJ and Fauquet CM (2002) Microparticle bombardment as a tool in plant science and agricultural biotechnology. DNA Cell Biol. 21: 963-977.

The Arabidopsis Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408: 796-815.

Trick HN and Finer JJ (1997) Sonication-assisted *Agrobacterium*-mediated transformation. Transgenic Research 6: 329-336.

Trigiano RN and Dennis JG (2005) Plant development and biotechnology. CRC Press, USA.

Unver T and Budak H (2009) Conserved microRNAs and their targets in model grass species Brachypodium distachyon. Planta DOI 10.1007/s00425-009-0974-7

Vain P, Worland B, Thole V, McKenzie N, Alves SC, Opanowicz M, Fish LJ, Bevan MW and Snape JW (2008) *Agrobacterium*-mediated transformation of the temperate grass *Brachypodium distachyon* (genotype Bd21) for T-DNA insertional mutagenesis. Plant Biotechnology Journal 6(3): 236-245.

Veluthamdi K, Gupta AK and Sharma A (2003) The current status of plant transformation technologies. Current Science 84(3): 368-380.

Vogel JP, Garvin DF, Leong OM and Hayden DM (2006a) *Agrobacterium*-mediated transformation and inbred line development in the model grass *Brachypodium distachyon*. Plant Cell Tissue and Organ Culture 84: 199-211.

Vogel JP, Gu YQ, Twigg P, et al. (2006b) EST sequencing and phylogenetic analysis of the model grass *Brachypodium distachyon*". Theoretical and Applied Genetics 113: 186-195.

Vogel J and Hill T (2007) High-efficiency *Agrobacterium*-mediated transformation of *Brachypodium distachyon* inbred line Bd21-3. Plant Cell Reports 27: 471-478.

Watson L and Dallwitz M (2005) The grass genera of the world: descriptions, illustrations, identification, and information retrieval; including synonyms, morphology, anatomy, physiology, phytochemistry, cytology, classification, pathogens, world and local distribution, and references. Version: 28th November 2005. <http://delta-intkey.com>. [Accessed 17 May, 2007], 1992 onwards.

Westhoff P, Jeske H, Jurgens G, Kloppstech K and Link G (1998) Molecular development from gene to plant. Oxford University Press, Inc., New York.

Wojtania A, Pulawska J and Gabryszewska E (2005) Identification and elimination of bacterial contaminants from *Pelargonium* tissue cultures. Journal of Fruit and Ornamental Plant Research 13: 101-108.

Woolston CJ, Barker R, Gunn H, Boulton MI and Mullineaux PM (1988) Agroinfection and nucleotide sequence of cloned wheat dwarf virus DNA. Plant Molecular Biology 11: 35-43.

Xue RG, Xie HF and Zhang B (2006) A multi-needle-assisted transformation of soybean cotyledonary node cells. Biotechnology Letters 28: 1551-1557.

Yang NS and Christou P (1994) Particle bombardment technology for gene transfer. Oxford University Press, New York, pp 47-51.

Zale JM, Borchardt-Wier H, Kidwell KK and Steber CM (2004) Callus induction and plant regeneration from mature embryos of a diverse set of wheat genotypes. Plant Cell Tiss Org Cult 76: 277-281.

Zamani I, Gouli-Vavdinoud E, Kovacs G, Xynias I, Roupakias D and Barnabas B (2003) Effect of parental genotypes and colchicin treatment on the androgenic response of wheat F hybrids. Plant Breeding 122: 314-317.

Zhang L, Rybczynski JJ, Langenberg WG, Mitra A and French R (2000) An efficient wheat transformation procedure: transformed calli with long-term morphogenic potential for plant regeneration. Plant Cell Reports 19:241−250.

Zhang S, Gu J, Yang NS, Kao C, Gardner TA, Eble JN and Cheng L (2002) Relative promoter strengths in four human prostate cancer cell lines evaluated by particle bombardment-mediated gene transfer. Prostate 51: 286-292.

Zhao T, Palotta M, Langridge P, Prasad M, Graner A, Schulze-Lefert P and Koprek T (2006) Mapped Ds/T-DNA launch pads for functional genomics in barley. Plant Journal 47:811–826.

Zuker A, Ahroni A, Tzfira T, Ben-Meir H and Vainstein A (1999) Wounding by bombardment yields highly efficient *Agrobacterium*-mediated transformation of carnation (*Dianthus caryophyllus* L.). Molecular Breeding 5: 367-375.

Zupan JR and Zambryski PC (1995) Transfer of T-DNA from *Agrobacterium* to the plant cell. Plant Physiology 107: 1041-1047.

Zupan J, Muth TR, Draper O and Zambryski (2000) The transfer of DNA from *Agrobacterium tumefaciens* into plants: a feast of fundamental insights. Plant Journal 23: 11-28.

APPENDIX A

Chemicals, growth media, plant growth regulators, antibiotics and enzymes

Absolute ethanol	Riedel de Haen	32221
Acetic acid	Merck	100056
Acetosyringone	Phytotechlab	A104
Agarose	PRONA	8016
Ascorbic acid	Sigma	A2174
BAP (6-Benzylaminopurine)	Sigma	B3408
Boric acid	Sigma	B6768
Calcium chloride ($CaCl_2$)	Sigma	C5080
Carbenicillin	Phytotechlab	C346
Cefotaxime	Phytotechlab	C380
Cetyl Trimethyl Ammonium Bromide (CTAB)	Phytotechlab	H276
Chloroform	Merck	102.445
2,4-D (2,4-Dichlorophenoxyacetic acid)	Sigma	D7299
Dimethylformamide (DMF)	Sigma	D4254
dNTP Mix	Fermentas	R0193
Ethidium bromide	Applichem	A1151
Ethylenediaminetetraaceticacid (EDTA)	Calbiochem	324503
Formaldehyde Solution	Fluka	47630
GeneRuler DNA Ladder Mix	Fermentas	SM0333
GeneRuler 100 bp DNA Ladder	Fermentas	SM0243

L-Glutamic acid	Sigma	G8415
Glycerol	Sigma	J8773
Hydrochloric acid	Merck	100314
Hydrogen peroxide (H_2O_2 solution %30)	Merck	107209
Hygromycin	Duchefa	H0125
IAA (Indole-3-acetic acid)	Sigma	I5148
Isoamylalcohol	Sigma	W205702
Isopropanol	Merck	1.09634
Kanamycin	Phytotechlab	K378
LB Agar	Sigma	L2897
LB Broth	Sigma	L302214
D-Mannitol	Phytotechlab	M562
Magnesium sulphate ($MgSO_4$)	Phytotechlab	M150
Maltose	Duchefa	M0811
2- mercapto ethanol	Aldrich	M370-1
MES	Phytotechlab	M825
Murashige and Skoog (MS) medium	Duchefa	M0222
NAA (1-Naphthaleneacetic acid)	Sigma	N0640
NaH_2PO_4	Riedel de Haen	04269
Na_2HPO_4	Merck	1.065.731.000
Nitric acid (HNO_3%65)	Merck	
Nutrient broth	Phytotechlab	N611
Phenol	Applichem	A1153
Plant agar	Duchefa	P1001
Potassium dihydrogen phosphate	Riedel de Haen	4243

Potassium ferricyanide	Sigma	244023
Potassium ferrocyanide	Sigma	P3289
PPT (DL-Phosphinothricin)	Duchefa	P0159
Proteinase K	Fermentas	EO0491
Rifampin	SanofiAventis	
RNase	Fermentas	EN0531
Sodium acetate	Sigma	S2289
Sodium chloride (NaCl)	Sigma	S7653
Sodium hydroxide	Merck	1.064.625.000
Spermidine	Duchefa	S1369
Streptomycin	I.E. Ulugay	
Sucrose	Duchefa	S0809
Taq DNA polymerase (recombinant)	Fermentas	EP0401
Trisbase	Sigma	T1503
TritonX-100	Sigma	58532
Tryptone	Sigma	27293
X-gluc (5,bromo-4,chloro-3,indolyl β-D-glucorinic acid)	Duchefa	X1405
Xho I restriction enzyme	Fermentas	ER0691
Yeast extract	Phytotechlab	Y892

APPENDIX B

Molecular biology and biolistic particle delivery system kits

DNeasy Plant Mini Kit	Qiagen	69106
Genopure Plasmid Midi Kit	Roche	3143414001
QIAprep Spin Miniprep kit	Qiagen	27106
Wizard Genomic DNA Purification Kit	Promega	A1120

Microprojectile bombardment kit (BioRad):

1.0 and 1.6 um Gold Microcarriers

Macrocarriers, for PDS-100/He and Hepta Systems (65-2335)

Stopping Screens for PDS-100/He and Hepta Systems (165-2336)

Macrocarrier Holders, PDS-100/He and Hepta Systems (165-2322)

650 psi Rupture Disks (165-2327)

900 psi Rupture Disks (165-2328)

1,100 psi Rupture Disks (165-2329)

APPENDIX C

Equipments

Autoclave:	Hirayama, Hiclave HV-110, JAPAN
	Nüve 0T 032, TÜRKİYE
Balance:	Sartorius, BP221S, GERMANY
	Schimadzu, Libror EB-3200 HU, JAPAN
Biolistic device:	PDS-1000/He Particle Delivery System, Bio-Rad, USA
Camera:	Olympus C-7070, JAPAN
Centrifuge:	Microfuge 18 Centrifuge Beckman Coulter, USA
	Kendro Lab. Prod., Heraeus Multifuge 3S-R, GERMANY
	Kendro Lab. Prod. Sorvall RC5C Plus, USA
	Eppendorf, 5415D, GERMANY
	Eppendorf, 5415R, GERMANY
Deepfreeze:	-20 °C Bosch, TÜRKİYE
	-80 °C Thermo electron corporation, USA
Distilled Water:	Millipore, Elix-S, FRANCE
	Millipore, MilliQ Academic, FRANCE
Electrophoresis:	Labnet Gel XL Ultra V-2, USA
	Biogen Inc., USA
	Biorad Inc., USA
Gel Documentation:	Biorad Universal Hood II F1-F2 Fuses Type T2A, USA
	Biorad, UV-Transilluminator 2000, USA
Ice Machine:	Scotsman Inc., AF20, USA

Heating block:	HDV Life Sciences, AUSTRIA
	Thermostat Bio TDB-100, LATVIA
ICP machine:	ICP-OES, Vista-Pro Axial, Varian Pty Ltd, AUSTRALIA
Incubator:	Innova 4330, USA
	Memmert, Modell 300, GERMANY
	Memmert, Modell 600, GERMANY
Laminar Flow:	Holten LaminAir Model 1.8 82034000, DENMARK
Magnetic Stirrer:	VELP Scientifica, ITALY
Microliter Pipette:	Gilson, Pipetman, FRANCE
	Eppendorf, GERMANY
Microscope:	Olympus SZ61, JAPAN
	Olympus LG-PS2, JAPAN
Microwave digestion:	CEM-MARS Xpress system, USA
Microwave Oven:	Bosch, TÜRKİYE
N measure:	Leco Truspec CN, USA
Oven:	Memmert D06062 Modell 600, GERMANY
pH meter:	WTW, pH540 GLP MultiCal, GERMANY
Power Supply:	Biorad, PowerPac 300, USA
Refrigerator:	+4 C Bosch, TÜRKİYE
Shaker:	Forma Scientific, Orbital Shaker 4520, USA
	GFL, Shaker 3011, USA
	New Brunswick Sci., Innova™ 4330, USA
	NewBrunswick Scientific Excells E24, USA
Spectrophotometer:	Amersham Biosciences Ultraspec 2100 pro, USA
	Nanodrop, ND-1000, USA

Sterilizer:	Steri 350, Simon Keller Ltd., SWITZERLAND
Thermocycler:	Eppendorf, Mastercycler Gradient, GERMANY
	Biorad Gradient Cycler DNA Engine, USA
Tissue Lyser:	Qiagen Retsch, USA
Vacuum:	Heto, MasterJet Sue 300Q, DENMARK
Vortex mixer:	VELP Scientifica $2X^3$, ITALY
Water bath:	Memmert, GERMANY

APPENDIX D

Morphological characterization of Turkish *Brachypodium distachyon* inbred lines

Genotypes	Latitude	Longitude	Elevation (m)	†Average Height (cm)	Leaf Color**	Leaf Hairiness*	Plant Stature	†Average Seed size (width/length) (cm)	***Seed Production	†Average Biomass (g)	††Seed yield	Germination Percentage (%)	DNA Contents (pg/2C)
BdTR1A	41°25'17.86"N	27°28'36.81"E	124	30.0 - 41.0	2	2	Fairly erect	0,123 / 0,644	8 weeks after	0.89	64	94	0.69
BdTR1B	41° 5'7.15"N	26°55'53.29"E	141	29.5 - 40.5	2	2	Erect	0,123 / 0,665	8 weeks after	0.88	63	94	0.69
BdTR1C	39°44'17.39"N	28° 2'24.71"E	363	28.7 - 39.8	2	2	Fairly erect	0,120 / 0,661	8 weeks after	0.89	63	95	0.69
BdTR1D	38°45'50.79"N	28°35'6.42"E	612	29.0 - 39.5	2	2	Fairly erect	0,118 / 0,622	8 weeks after	0.9	65	95	0.69
BdTR1E	38°25'0.42"N	28° 1'52.75"E	986	28.2 - 41.0	2	2	Fairly erect	0,124 / 0,666	8 weeks after	0.9	63	95	0.69
BdTR1F	41°25'17.86"N	27°28'36.81"E	124	29.5 - 40.8	2	2	Fairly erect	0,124 / 0,629	8 weeks after	0.9	64	95	0.69
BdTR1G	41° 5'7.15"N	26°55'53.29"E	141	29.0 - 39.9	2	2	Fairly erect	0,121 / 0,616	9 weeks after	0.87	64	95	0.69

BdTR1H	39°44'17.39"N	28° 2'24.71"E	363	28.9 - 40.0	2	2	Fairly erect	0,124 / 0,636	8 weeks after	0.89	64	95	0.69
BdTR1I	38° 5'35.03"N	28°34'59.02"E	841	29.1 - 41.0	2	2	Fairly erect	0,121 / 0,606	8 weeks after	0.89	64	95	0.69
BdTR1J	37°25'38.24"N	28°35'6.75"E	513	29.9 - 40.7	2	2	Fairly erect	0,114 / 0,654	8 weeks after	0.89	63	95	0.69
BdTR1K	39°45'34.18"N	29°40'40.96"E	1007	28.9 - 40.5	2	2	Erect	0,127 / 0,652	8 weeks after	0.89	62	95	0.695
BdTR1M	39° 5'12.20"N	30°15'8.93"E	1076	29.0 - 40.0	2	2	Fairly erect	0,121 / 0,673	8 weeks after	0.89	63	95	0.695
BdTR1N	38° 5'59.53"N	30°14'44.11"E	1034	29.1 - 39.9	2	2	Fairly erect	0,119 / 0,641	8 weeks after	0.89	63	95	0.696
BdTR2A	39°45'10.62"N	30°47'19.07"E	932	29.6 - 40.1	2	2	Fairly erect	0,112 / 0,593	10 weeks after	0.79	183	100	0.696
BdTR2B	40° 4'55.55"N	31°19'52.01"E	667	30.0 - 41.0	2	2	Fairly erect	0,112 / 0,585	10 weeks after	0.78	183	100	0.696
BdTR2C	39° 5'8.89"N	31°53'29.40"E	864	29.7 - 41.8	2	2	Fairly erect	0,103 / 0,591	10 weeks after	0.78	183	100	0.696
BdTR2D	38°25'46.78"N	31°18'33.71"E	1301	29.2 - 40.2	2	2	Erect	0,114 / 0,586	10 weeks after	79.8	186	100	0.696
BdTR2E	38°25'40.97"N	32°24'16.47"E	1012	28.5 - 39.0	2	2	Fairly erect	0,122 / 0,611	10 weeks after	0.8	184	100	0.696
BdTR2F	37°46'41.64"N	31°53'5.68"E	1288	28.0 - 40.8	2	2	Fairly erect	0,108 / 0,589	9 weeks after	0.78	184	100	0.696
BdTR2G	40°23'37.13"N	32°59'7.32"E	1596	30.9 - 40.0	2	2	Fairly erect	0,115 / 0,609	10 weeks after	0.78	184	100	0.696

BdTR2H	39°45'23.35"N	32°25'56.46"E	787	30.0 - 41.7	2	2	Erect	0,119 / 0,611	10 weeks after	0.78	184	100	0.696
BdTR2I	39°45'16.02"N	33°32'16.37"E	872	29.4 - 40.1	2	2	Fairly erect	0,119 / 0,617	10 weeks after	0.79	184	100	0.696
BdTR2J	39°24'46.28"N	32°59'17.24"E	1192	29.0 - 39.2	2	2	Fairly erect	0,112 / 0,586	10 weeks after	0.79	184	100	0.696
BdTR2K	38°45'28.75"N	34° 4'18.34"E	1142	29.9 - 42.2	2	2	Fairly erect	0,111 / 0,597	10 weeks after	0.8	184	100	0.696
BdTR2M	38° 5'52.78"N	34° 5'40.38"E	1406	30.0 - 41.2	2	2	Fairly erect	0,113 / 0,572	10 weeks after	0.8	184	100	0.696
BdTR2N	37°46'4.28"N	33°31'10.58"E	1013	30.0 - 42.4	2	2	Erect	0,117 / 0,610	10 weeks after	0.8	184	100	0.696
BdTR2O	39°45'23.35"N	32°25'56.46"E	787	31.1 - 39.5	2	2	Fairly erect	0,119 / 0,580	10 weeks after	0.8	184	100	0.696
BdTR2P	39°45'16.02"N	33°32'16.37"E	872	30.0 - 39.0	2	2	Fairly erect	0,110 / 0,607	10 weeks after	0.9	184	100	0.696
BdTR2R	39°24'46.28"N	32°59'17.24"E	1192	30.7 - 41.1	2	2	Fairly erect	0,115 / 0,611	10 weeks after	0.79	184	100	0.696
BdTR2S	38°45'28.75"N	34° 4'18.34"E	1142	29.9 - 40.0	2	2	Fairly erect	0,116 / 0,561	11 weeks after	0.8	184	100	0.696
BdTR3A	37°46'4.28"N	33°31'10.58"E	1013	32.0 - 40.0	2	1	Erect	0,114/ 0,692	12 weeks after	2	38	100	0.68
BdTR3B	37° 6'31.87"N	34° 4'17.06"E	2385	29.8 - 39.2	2	1	Erect	0,108/ 0,697	11 weeks after	2	36	100	0.68
BdTR3C	36°46'58.92"N	32°57'46.71"E	1957	29.0 - 38.5	2	1	Erect	0,114/ 0,681	12 weeks after	2	36	100	0.68

BdTR3D	40°24'19.91"N	34°38'10.10"E	1088	29.8 - 37.9	2	1	Erect	0,112/ 0,696	12 weeks after	2.1	38	100	0.68
BdTR3E	39°44'53.45"N	34°39'1.15"E	1035	30.2 - 38.0	2	1	Erect	0,113/ 0,690	12 weeks after	2.1	38	100	0.68
BdTR3F	39° 5'31.64"N	35°11'18.62"E	1249	30.0 - 40.2	2	1	Erect	0,100/ 0,603	12 weeks after	2.1	36	100	0.68
BdTR3G	38°24'16.86"N	35° 9'34.32"E	1086	30.0 - 39.8	2	1	Fairly erect	0,106/ 0,650	11 weeks after	2	34	100	0.68
BdTR3H	37°46'43.23"N	35°12'9.55"E	3196	29.7 - 38.5	2	1	Erect	0,100/ 0,638	11 weeks after	2	34	100	0.68
BdTR3I	39°44'51.33"N	36°48'56.85"E	1574	29.5 - 38.5	2	1	Erect	0,100/ 0,669	11 weeks after	2	34	100	0.68
BdTR3J	38°45'25.33"N	36°16'56.90"E	1849	30.2 - 38.0	2	1	Erect	0,094/ 0,600	12 weeks after	2	34	100	0.68
BdTR3K	38°25'12.10"N	37°23'26.99"E	1709	30.0 - 38.0	2	1	Erect	0,094/ 0,615	12 weeks after	2	34	100	0.68
BdTR3M	37°45'58.01"N	37°53'23.56"E	614	28.0 - 39.2	2	1	Erect	0,104/ 0,633	12 weeks after	2	34	100	0.68
BdTR3N	37°26'4.39"N	36°49'9.55"E	787	29.9 - 38.2	2	1	Erect	0,108/ 0,684	11 weeks after	1.98	34	100	0.68
BdTR3O	37° 7'2.52"N	39° 1'49.56"E	448	30.0 - 38.4	2	1	Erect	0,102/ 0,654	11 weeks after	2	34	100	0.68
BdTR3P	37°45'41.57"N	39°34'56.72"E	1156	29.6 - 38.0	2	1	Erect	0,102/ 0,650	12 weeks after	2	36	100	0.69
BdTR3R	37°26'13.88"N	40° 7'50.51"E	710	29.5 - 39.0	2	1	Erect	0,104/ 0,650	12 weeks after	2	36	100	0.69

BdTR3S	38° 5'43.50"N	40°39'30.84"E	688	29.0 - 39.1	2	1	Erect	0,105/ 0,660	12 weeks after	2.1	36	100	0.69
BdTR3T	37°27'31.68"N	41°14'39.03"E	975	29.7 - 39.0	2	1	Erect	0,108/ 0.656	11 weeks after	2	36	100	0.69
BdTR4A	37°27'31.68"N	41°14'39.03"E	975	39.0 - 50.2	2	2	Erect	0,128 / 0,698	7 weeks after	0.94	801	65	1.36
BdTR4B	37°47'51.69"N	41°46'25.09"E	612	38.5 - 49.0	2	2	Erect	0,123 / 0,663	7 weeks after	0.95	801	65	1.36
BdTR4C	38° 5'48.94"N	42°19'18.79"E	1713	38.4 - 49.2	2	2	Erect	0,128 / 0,669	7 weeks after	0.95	798	65	1.36
BdTR4D	38° 5'43.50"N	40°39'30.84"E	688	38.7 - 50.0	2	2	Erect	0,128 / 0,688	7 weeks after	0.99	798	65	1.36
BdTR4E	37°27'31.68"N	41°14'39.03"E	975	38.5 - 50.1	2	2	Erect	0,122 / 0,666	7 weeks after	0.94	790	65	1.36
BdTR4F	37°47'51.69"N	41°46'25.09"E	612	39.0 - 49.5	2	2	Fairly erect	0,127 / 0,665	7 weeks after	0.95	790	65	1.36
BdTR4G	38° 5'48.94"N	42°19'18.79"E	1713	38.9 - 49.0	2	2	Erect	0,128 / 0.690	7 weeks after	0.95	791	65	1.36
BdTR4H	38° 5'48.94"N	42°19'18.79"E	1713	38.0 - 49.0	2	2	Erect	0,131 / 0,693	7 weeks after	0.98	791	65	1.36
BdTR4I	37°28'28.23"N	43°26'45.08"E	1957	38.3 - 48.5	2	2	Fairly erect	0,130 / 0,690	7 weeks after	0.96	792	65	1.36
BdTR4J	36°45'59.24"N	44°32'6.90"E	886	38.0 - 51.0	2	2	Erect	0,128 / 0,672	7 weeks after	0.95	792	65	1.36
BdTR4K	38° 5'48.94"N	42°19'18.79"E	1713	38.1 - 49.3	2	2	Erect	0,125 / 0.680	7 weeks after	0.98	792	65	1.36

BdTR5A	36°45'59.24"N	44°32'6.90"E	886	29.3 - 37.0	3	3	Fairly erect	0,109 / 0,701	14 weeks after	1.7	24	100	0.69
BdTR5B	37°46'43.23"N	35°12'9.55"E	3196	29.2 - 37.0	3	3	Fairly erect	0,104 / 0,670	14 weeks after	1.5	24	100	0.69
BdTR5C	39°44'51.33"N	36°48'56.85"E	1574	29.3 - 37.5	3	3	Fairly erect	0,109 / 0,673	14 weeks after	1.5	24	100	0.69
BdTR5D	38°25'40.97"N	32°24'16.47"E	1012	29.0 - 37.2	3	3	Erect	0,107 / 0,691	14 weeks after	1.6	24	100	0.69
BdTR5E	37°46'41.64"N	31°53'5.68"E	1288	29.0 - 37.1	3	3	Fairly erect	0,109 / 0,683	14 weeks after	1.6	24	100	0.69
BdTR5F	40°23'37.13"N	32°59'7.32"E	1596	30.0 - 39.0	3	3	Fairly erect	0,107 / 0,700	14 weeks after	1.7	24	100	0.69
BdTR5G	39°45'23.35"N	32°25'56.46"E	787	30.0 - 37.8	3	3	Erect	0,104 / 0,692	14 weeks after	1.5	24	100	0.69
BdTR5H	39°45'16.02"N	33°32'16.37"E	872	29.2- 36.9	3	3	Fairly erect	0,107 / 0,685	14 weeks after	1.5	24	100	0.69
BdTR5I	40°23'37.13"N	32°59'7.32"E	1596	28.3 - 37.0	3	3	Fairly erect	0,112 / 0,684	14 weeks after	1.5	24	100	0.69
BdTR5J	40°23'37.13"N	32°59'7.32"E	1596	28.9 - 37.2	3	3	Erect	0,114 / 0,688	14 weeks after	1.6	24	100	0.69
BdTR5K	40°23'37.13"N	32°59'7.32"E	1596	29.1 - 36.8	3	3	Fairly erect	0,110 / 0,699	14 weeks after	1.5	24	100	0.69
BdTR5M	40°23'37.13"N	32°59'7.32"E	1596	28.9 - 37.0	3	3	Fairly erect	0,102 / 0,679	14 weeks after	1.7	24	100	0.69
BdTR5N	40°23'37.13"N	32°59'7.32"E	1596	28.9 - 36.8	3	3	Fairly erect	0,122 / 0,683	14 weeks after	1.7	24	100	0.69

BdTR5O	39°45'16.02"N	33°32'16.37"E	872	29.0 - 38.0	3	3	Fairly erect	0,102 / 0,683	14 weeks after	1.7	24	100	0.69
BdTR6A	38° 5'48.94"N	42°19'18.79"E	1713	36.0 - 51.2	3	5	Erect	0,144 / 0,793	10 weeks after	0.5	440	100	1.34
BdTR6B	37°28'28.23"N	43°26'45.08"E	1957	35.7 - 50.8	3	5	Fairly erect	0,141 / 0,799	10 weeks after	0.6	390	100	1.34
BdTR6C	38°25'40.97"N	32°24'16.47"E	1012	35.2 - 51.0	3	5	Erect	0,143 / 0,788	11 weeks after	0.5	379	100	1.34
BdTR6D	37°46'41.64"N	31°53'5.68"E	1288	35.3 - 51.4	3	5	Erect	0,144 / 0,782	11 weeks after	0.5	398	100	1.34
BdTR6E	40°23'37.13"N	32°59'7.32"E	1596	35.0 - 51.2	3	5	Erect	0,144 / 0,794	11 weeks after	0.5	438	100	1.34
BdTR6F	39°45'23.35"N	32°25'56.46"E	787	34.7 - 50.8	3	5	Erect	0,139 / 0,780	11 weeks after	0.5	437	100	1.34
BdTR6G	39°45'16.02"N	33°32'16.37"E	872	34.9 - 51.0	3	5	Erect	0,138/ 0,763	10 weeks after	0.5	437	100	1.34
BdTR6H	39°24'46.28"N	32°59'17.24"E	1192	35.1 - 52.0	3	5	Fairly erect	0,143 / 0,776	10 weeks after	0.5	437	100	1.34
BdTR6I	38°45'28.75"N	34° 4'18.34"E	1142	34.3 - 50.7	3	5	Erect	0,142 / 0,780	10 weeks after	0.5	437	100	1.34
BdTR6J	38° 5'52.78"N	34° 5'40.38"E	1406	35.0 - 51.1	3	5	Erect	0,140 / 0,781	10 weeks after	0.5	437	100	1.34
BdTR6K	37°46'4.28"N	33°31'10.58"E	1013	34.0 - 50.6	3	5	Fairly erect	0,140 / 0,800	10 weeks after	0.5	437	100	1.34
BdTR6M	37° 6'31.87"N	34° 4'17.06"E	2385	35.6 - 50.3	3	5	Erect	0,138 / 0,785	10 weeks after	0.5	437	100	1.34

BdTR6N	36°46'58.92"N	32°57'46.71"E	1957	34.8 - 52.6	3	5	Erect	0,142 / 0,790	10 weeks after	0.5	437	100	1.34
BdTR7A	39°44'53.45"N	34°39'1.15"E	1035	26.2 - 35.9	1	2	Fairly erect	0,113 / 0,638	22 weeks after	15.77	54	100	0.69
BdTR7B	39° 5'31.64"N	35°11'18.62"E	1249	25.8 - 36.1	1	2	Erect	0,112 / 0,630	22 weeks after	15.87	54	100	0.69
BdTR8A	39°45'16.02"N	33°32'16.37"E	872	24.8 - 35.3	1	2	Erect	0,124 / 0,638	22 weeks after	8.8	68	100	1.27
BdTR8C	39°24'46.28"N	32°59'17.24"E	1192	25.0 - 35.0	1	2	Erect	0,125 / 0,652	22 weeks after	8.5	69	100	1.27
BdTR8F	37°46'4.28"N	33°31'10.58"E	1013	25.0 - 34.2	1	2	Erect	0,125 / 0,640	22 weeks after	8.5	69	100	1.27
BdTR8I	37° 6'31.87"N	34° 4'17.06"E	2385	24.5 - 35.2	1	2	Fairly erect	0,126 / 0,650	22 weeks after	8.9	69	100	1.27
BdTR8M	39° 5'31.64"N	35°11'18.62"E	1249	24.9 - 34.8	1	2	Erect	0,128 / 0,641	22 weeks after	8.9	68	100	1.27
BdTR8N	38°24'16.86"N	35° 9'34.32"E	1086	25.2 - 35.0	1	2	Erect	0,129 / 0,650	22 weeks after	8.7	68	100	1.27
BdTR9A	39°44'51.33"N	36°48'56.85"E	1574	29.4 - 45.2	1	3	Branchy	0,122/ 0,599	9 weeks after	2.67	26	88	0.69
BdTR9B	39°44'17.39"N	28° 2'24.71"E	363	29.7 - 45.0	1	3	Branchy	0,128/ 0,618	9 weeks after	2.59	31	88	0.69
BdTR9C	38°45'50.79"N	28°35'6.42"E	612	29.9 - 44.8	1	3	Branchy	0,130/ 0,602	9 weeks after	2.7	27	86	0.69
BdTR9D	38°25'0.42"N	28° 1'52.75"E	986	30.2 - 45.2	1	3	Branchy	0,126/ 0,621	9 weeks after	2.8	19	86	0.69

BdTR9E	38° 5'35.03"N	28°34'59.02"E	841	30.0 - 46.0	1	3	Branchy	0,133/ 0,622	9 weeks after	2.8	21	86	0.69
BdTR9F	37°25'38.24"N	28°35'6.75"E	513	28.9 - 45.0	1	3	Branchy	0,122/ 0,622	9 weeks after	2.8	23	86	0.69
BdTR9G	39°45'34.18"N	29°40'40.96"E	1007	30.4 - 44.9	1	3	Branchy	0,122/ 0,610	9 weeks after	2.78	24	86	0.69
BdTR9H	39° 5'12.20"N	30°15'8.93"E	1076	30.8 - 45.9	1	3	Branchy	0,118/ 0,618	9 weeks after	2.75	23	86	0.69
BdTR9I	38° 5'59.53"N	30°14'44.11"E	1034	29.9 - 44.5	1	3	Branchy	0,119/ 0,611	9 weeks after	2.68	23	86	0.69
BdTR9J	37° 7'38.06"N	30°13'59.22"E	1073	30.3 - 44.7	1	3	Branchy	0,122/ 0,625	9 weeks after	2.9	23	86	0.69
BdTR9K	39°45'10.62"N	30°47'19.07"E	932	30.2 - 45.3	1	3	Branchy	0,125/ 0,625	9 weeks after	2.87	26	86	0.69
BdTR9M	40° 4'55.55"N	31°19'52.01"E	667	29.7 - 45.0	1	3	Branchy	0,119/ 0,619	9 weeks after	2.67	26	86	0.69
BdTR10A	38°25'46.78"N	31°18'33.71"E	1301	30.2 - 41.0	1	3	Erect	0,121 / 0,633	20 weeks after	2.33	18	92	0.7
BdTR10B	38°25'40.97"N	32°24'16.47"E	1012	29.0 - 39.0	1	3	Erect	0,126 / 0,626	20 weeks after	2.34	18	92	0.7
BdTR10C	37°46'41.64"N	31°53'5.68"E	1288	29.8 - 40.0	1	3	Erect	0,121 / 0,613	20 weeks after	2.35	19	92	0.7
BdTR10D	40°23'37.13"N	32°59'7.32"E	1596	30.3 - 39.8	1	3	Fairly erect	0,122 / 0,624	20 weeks after	2.34	19	92	0.7
BdTR10E	37° 7'2.52"N	39° 1'49.56"E	448	29.5 - 41.0	1	3	Erect	0,128 / 0,620	20 weeks after	2.34	20	92	0.7

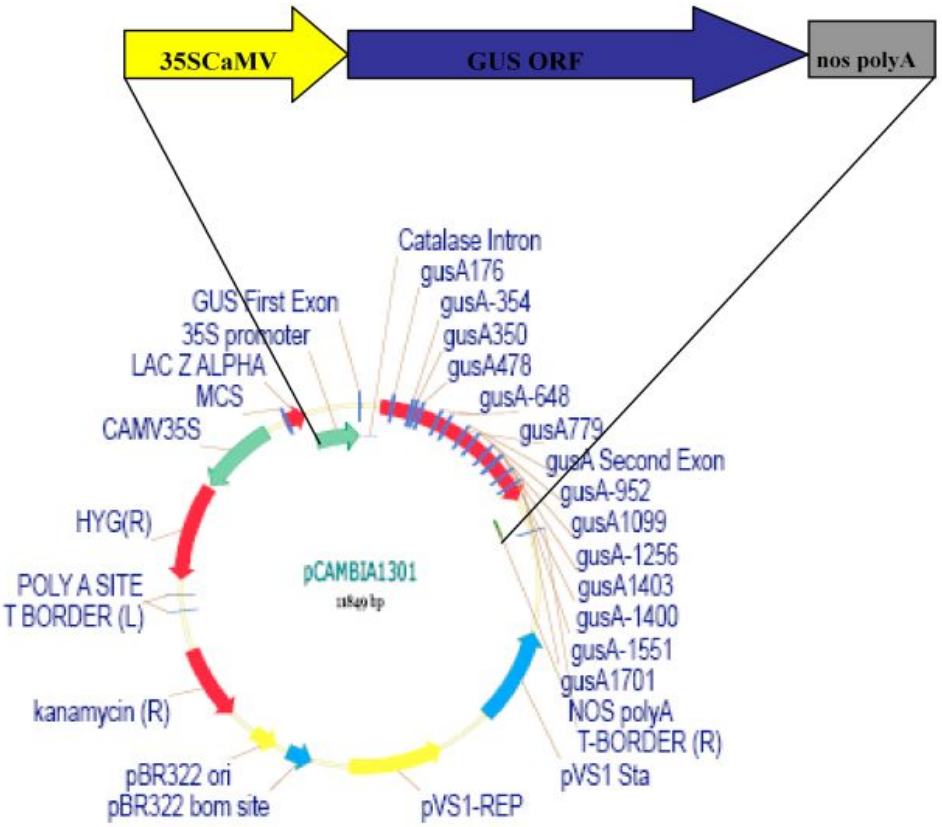
BdTR10F	37°45'41.57"N	39°34'56.72"E	1156	30.5 - 41.7	1	3	Fairly erect	0,123 / 0,620	20 weeks after	2.37	21	92	0.7
BdTR10G	37°26'13.88"N	40° 7'50.51"E	710	30.2 - 40.3	1	3	Erect	0,122 / 0,613	20 weeks after	2.18	21	92	0.7
BdTR10H	38° 5'43.50"N	40°39'30.84"E	688	29.7 - 40.0	1	3	Erect	0,133 / 0,621	20 weeks after	2.15	21	92	0.7
BdTR10I	37°27'31.68"N	41°14'39.03"E	975	29.2 - 40.1	1	3	Fairly erect	0,124 / 0,610	20 weeks after	2.18	21	92	0.7
BdTR10J	37°47'51.69"N	41°46'25.09"E	612	30.0 - 40.8	1	3	Erect	0,125 / 0,634	20 weeks after	2.15	23	92	0.7
BdTR10K	38° 5'48.94"N	42°19'18.79"E	1713	30.2 - 40.4	1	3	Erect	0,118 / 0,630	20 weeks after	2.14	23	92	0.7
BdTR10M	41°25'17.86"N	27°28'36.81"E	124	29.8 - 39.6	1	3	Fairly erect	0,123 / 0,619	20 weeks after	2.15	24	92	0.7
BdTR10N	41° 5'7.15"N	26°55'53.29"E	141	30.3 - 39.9	1	3	Erect	0,125 / 0,622	20 weeks after	2.17	24	92	0.7
BdTR10O	39°44'17.39"N	28° 2'24.71"E	363	29.6 - 40.9	1	3	Erect	0,123 / 0,620	20 weeks after	2.16	27	92	0.7
BdTR11A	38°25'0.42"N	28° 1'52.75"E	986	30.0 - 39.5	1	3	Fairly erect	0,120 / 0,618	17 weeks after	2.1	112	95	0.69
BdTR11B	41°25'17.86"N	27°28'36.81"E	124	28.3 - 39.0	1	3	Erect	0,125 / 0,613	17 weeks after	2	112	95	0.69
BdTR11C	41° 5'7.15"N	26°55'53.29"E	141	28.1 - 38.9	1	3	Erect	0,119 / 0,621	17 weeks after	2.2	118	95	0.69
BdTR11D	37°27'31.68"N	41°14'39.03"E	975	29.6 - 39.1	1	3	Erect	0,123 / 0,610	18 weeks after	2.1	119	95	0.69

BdTR11E	37°47'51.69"N	41°46'25.09"E	612	27.6 - 38.4	1	3	Erect	0,124 / 0,627	18 weeks after	2.1	118	95	0.69
BdTR11F	38° 5'48.94"N	42°19'18.79"E	1713	29.0 - 37.6	1	3	Fairly erect	0,122 / 0,612	18 weeks after	2.3	118	95	0.69
BdTR11G	41°25'17.86"N	27°28'36.81"E	124	28.4 - 37.7	1	3	Erect	0,127 / 0,621	18 weeks after	2	119	95	0.69
BdTR11H	41° 5'7.15"N	26°55'53.29"E	141	29.0 - 39.0	1	3	Erect	0,123 / 0,618	18 weeks after	2	119	95	0.69
BdTR11I	39°44'17.39"N	28° 2'24.71"E	363	28.5 - 38.0	1	3	Erect	0,126 / 0,615	18 weeks after	2	120	95	0.69
BdTR12A	36°46'58.92"N	32°57'46.71"E	1957	25.1 - 32.0	1	3	Erect	0,114 / 0,568	20 weeks after	4.71	19	67	0.69
BdTR12B	40°24'19.91"N	34°38'10.10"E	1088	25.0 - 32.4	1	3	Fairly erect	0,111 / 0,588	20 weeks after	4.72	16	67	0.68
BdTR12C	39°44'53.45"N	34°39'1.15"E	1035	24.9 - 32.2	1	3	Erect	0,113 / 0,580	20 weeks after	4.7	16	67	0.69
BdTR13A	39°45'23.35"N	32°25'56.46"E	787	30.2 - 41.9	3	4	Branchy	0,102 / 0,654	19 weeks after	0.5	200	95	0.7
BdTR13B	39°45'16.02"N	33°32'16.37"E	872	31.2 - 42.0	3	4	Branchy	0,100 / 0,652	19 weeks after	0.6	218	95	0.7
BdTR13C	39°24'46.28"N	32°59'17.24"E	1192	30.3 - 42.5	3	4	Branchy	0,100 / 0,650	19 weeks after	0.4	219	95	0.7
BdTR13D	37°46'4.28"N	33°31'10.58"E	1013	29.5 - 41.9	3	4	Branchy	0,100 / 0,672	19 weeks after	0.4	219	95	0.7
BdTR13E	37° 6'31.87"N	34° 4'17.06"E	2385	29.7 - 42.5	3	4	Branchy	0,110 / 0,649	19 weeks after	0.6	210	95	0.7

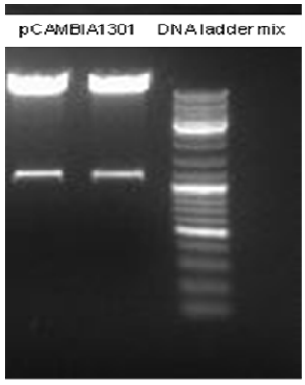
BdTR13F	39° 5'31.64"N	35°11'18.62"E	1249	30.0 - 41.4	3	4	Branchy	0,102 / 0,661	19 weeks after	0.6	211	95	0.7
BdTR13G	38°24'16.86"N	35° 9'34.32"E	1086	31.0 - 40.8	3	4	Branchy	0.100 / 0,664	19 weeks after	0.6	214	95	0.7
BdTR13H	37°27'31.68"N	41°14'39.03"E	975	31.5 - 41.3	3	4	Branchy	0,109 / 0,675	19 weeks after	0.7	216	95	0.7
BdTR13I	37°47'51.69"N	41°46'25.09"E	612	30.6 - 42.1	3	4	Branchy	0,104 / 0,650	19 weeks after	0.5	216	95	0.7
BdTR13J	38° 5'48.94"N	42°19'18.79"E	1713	31.0 - 41.5	3	4	Branchy	0,112 / 0,675	19 weeks after	0.5	218	95	0.7
BdTR13K	38° 5'48.94"N	42°19'18.79"E	1713	29.4 - 43.0	3	4	Branchy	0,110 / 0,673	19 weeks after	0.5	219	95	0.7
BdTR13M	37°28'28.23"N	43°26'45.08"E	1957	29.9 - 42.6	3	4	Branchy	0,109 / 0,670	19 weeks after	0.5	219	95	0.72
BdTR13N	36°45'59.24"N	44°32'6.90"E	886	31.0 - 41.5	3	4	Branchy	0,109 / 0,679	19 weeks after	0.5	217	95	0.71
BdTR13O	38° 5'48.94"N	42°19'18.79"E	1713	31.2 - 41.2	3	4	Branchy	0,100 / 0,673	19 weeks after	0.5	216	95	0.71

APPENDIX E

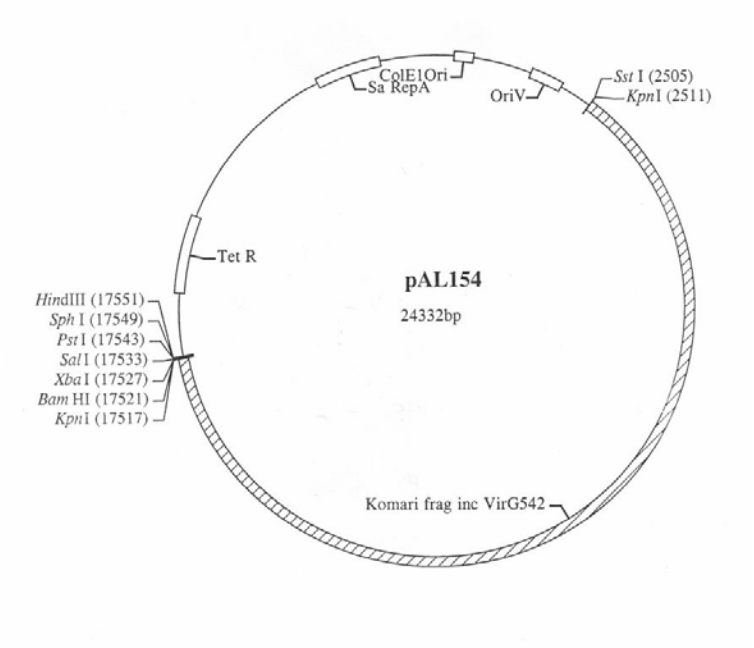
E-1 Plasmid map of pCAMBIA1301 (www.cambia.org)

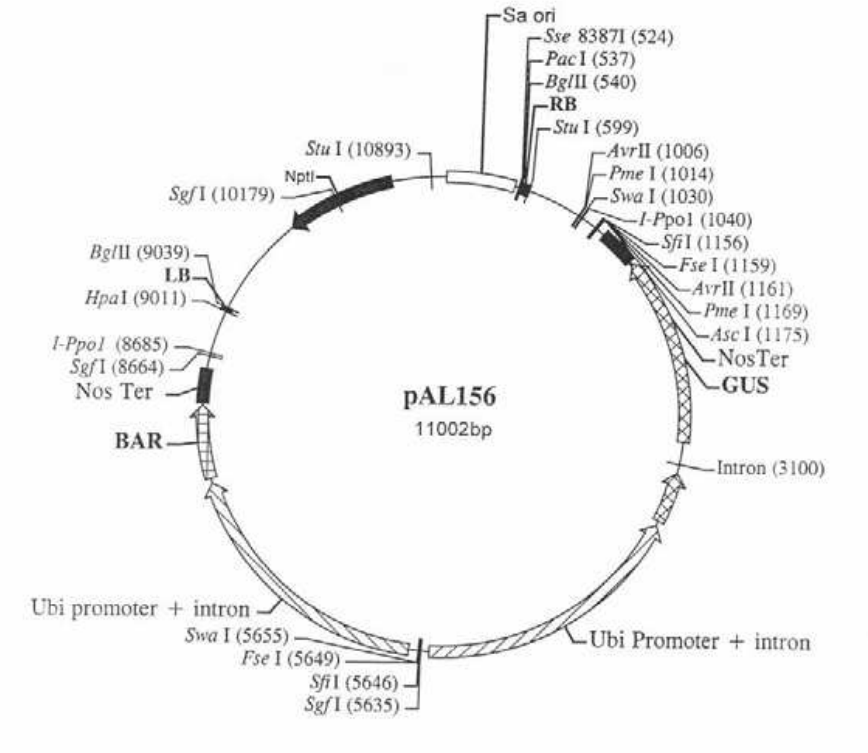


Plasmid pCAMBIA1301 digested with XhoI enzyme (Fermentas). All plasmid preparations contain the constructs at an approximate size of 1100 and 10700 bp.

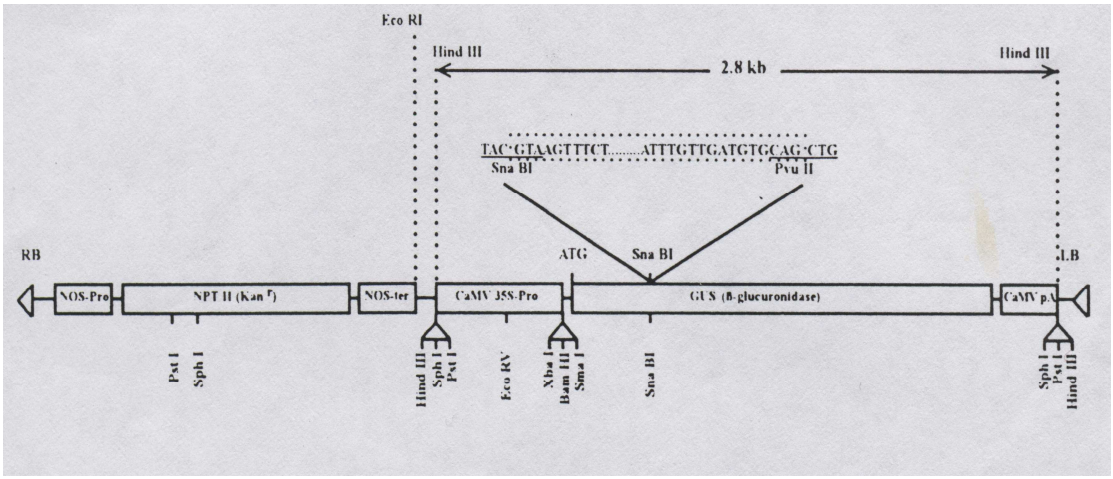


E-2 Plasmid maps of *Agrobacterium* AGL1 strain





E-3 T-DNA region of pGUSINT



35S-GUS-INT gene with following restriction sites in the surroundings;

Hind III- sphI- PstI- HincII - **35S promoter** - XbaI- BamHI- SmaI- **GUS-gene** - SstI- KpnI- **35S-3'-end** - sphI - Pst I- HindIII

APPENDIX F

F-1 Statistical analysis of the data represented in Figure 4.13 (a-f)
* : significant at p<0.05

Genotype	Variable	T-test
BdTR4	1 mg/ 3 mg	0,31
	1mg/ 5 mg	*0,02**
	3mg/ 5 mg	0,19
	sucrose/ maltose	0,62
BdTR6	1 mg/ 3 mg	0,08
	1mg/ 5 mg	0,28
	3mg/ 5 mg	*0,02**
	sucrose/ maltose	0,38
BdTR13	1 mg/ 3 mg	0,30
	1mg/ 5 mg	0,052
	3mg/ 5 mg	0,12
	sucrose/ maltose	*0,03**

F-2 Statistical analysis of the data represented in Figure 4.16 (a-f)
* : significant at p<0.05

Genotype	Variable	T-test
BdTR4	1 mg/ 3 mg	0,45
	1mg/ 5 mg	0,41
	3mg/ 5 mg	0,94
	sucrose/ maltose	0,95
BdTR6	1 mg/ 3 mg	0,07
	1mg/ 5 mg	0,91
	3mg/ 5 mg	0,07
	sucrose/ maltose	0,58
BdTR13	1 mg/ 3 mg	0,87
	1mg/ 5 mg	0,36
	3mg/ 5 mg	0,44
	sucrose/ maltose	0,81

* Significant at $p \leq 0.05$

F-3 Statistical analysis of the data represented in Figure 4.18a
* : significant at p<0.05

Variable	T-test
650-6/650-9	0,817
650-6/900-6	0,409
650-6/900-9	0,673
650-6/1100-6	*0,022**
650-6/1100-9	0,101
650-9/900-6	0,908872
650-9/900-9	0,917092
650-9/1100-6	*0,039821**
650-9/1100-9	0,219146
900-6/900-9	0,21251
900-6/1100-6	*0,029697**
900-6/1100-9	0,690163
900-9/1100-6	*0,029762**
900-9/1100-9	0,154788
1100-6/1100-9	0,126253

F-4 Statistical analysis of the data represented in Figure 4.18b
* : significant at p<0.05

Variable	T-test
650-6/650-9	0,227454
650-6/900-6	*0,038481**
650-6/900-9	0,201126
650-6/1100-6	0,0751
650-6/1100-9	0,301691
650-9/900-6	0,908872
650-9/900-9	0,338223
650-9/1100-6	0,70393
650-9/1100-9	0,757873
900-6/900-9	0,056138
900-6/1100-6	0,450131
900-6/1100-9	0,690163
900-9/1100-6	0,099414
900-9/1100-9	0,383835
1100-6/1100-9	0,929387

F-5 Transient GUS gene expression of the *Brachypodium* calli after *Agrobacterium* transformation. (Std Err: Standard Errors)

Group	Mean	Std Err
Bd TR-4, LBA4404, blade	4,4	0,2
Bd TR-4, LBA4404, 1.0 µm gold	3,0	0,1
Bd TR-4, LBA4404, 1.6 µm gold	4,6	0,3
Bd TR-4, EHA105, blade	4,7	0,1
Bd TR-4, EHA105, 1.0 µm gold	8,9	0,3
Bd TR-4, EHA105, 1.6 µm gold	3,3	0,1
Bd TR-4, AGL1, blade	6,4	0,3
Bd TR-4, AGL1, 1.0 µm gold	5,4	0,4
Bd TR-4, AGL1, 1.6 µm gold	6,2	0,2
Bd TR-6, LBA4404, blade	3,2	0,2
Bd TR-6, LBA4404, 1.0 µm gold	7,6	0,0
Bd TR-6, LBA4404, 1.6 µm gold	10,4	0,2
Bd TR-6, EHA105, blade	2,3	0,1
Bd TR-6, EHA105, 1.0 µm gold	4,4	0,1
Bd TR-6, EHA105, 1.6 µm gold	4,4	0,0
Bd TR-6, AGL1, blade	4,3	0,3
Bd TR-6, AGL1, 1.0 µm gold	3,1	0,4
Bd TR-6, AGL1, 1.6 µm gold	5,4	0,5
Bd TR-13, LBA4404, blade	4,4	0,1
Bd TR-13, LBA4404, 1.0 µm gold	4,2	0,1
Bd TR-13, LBA4404, 1.6 µm gold	4,6	0,1
Bd TR-13, EHA105, blade	2,8	0,1
Bd TR-13, EHA105, 1.0 µm gold	0,0	0,0
Bd TR-13, EHA105, 1.6 µm gold	0,0	0,0
Bd TR-13, AGL1, blade	2,9	0,3
Bd TR-13, AGL1, 1.0 µm gold	5,1	0,4
Bd TR-13, AGL1, 1.6 µm gold	8,3	0,2
Bd21, LBA4404, blade	4,1	0,1
Bd21, LBA4404, 1.0 µm gold	8,6	0,1
Bd21, LBA4404, 1.6 µm gold	4,5	0,2
Bd21, EHA105, blade	3,3	0,3
Bd21, EHA105, 1.0 µm gold	6,9	0,1
Bd21, EHA105, 1.6 µm gold	6,5	0,1
Bd21, AGL1, blade	6,3	0,2
Bd21, AGL1, 1.0 µm gold	8,0	0,2
Bd21, AGL1, 1.6 µm gold	7,7	0,3